Effects of Growth Hormone, IGF-1, or Combination Therapy on Muscle Fiber Type Composition in Diabetic Mice

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Sean R. Schumm
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This dissertation titled

Effects of Growth Hormone, IGF-1, or Combination Therapy on Muscle Fiber Type

Composition in Diabetic Mice

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ABSTRACT

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Effects of Growth Hormone, IGF-1, or Combination Therapy on Muscle Fiber Type Composition in Diabetic Mice

Director of Dissertation: Robert S. Staron

Introduction: Type 2 diabetes mellitus (T2DM) is a growing health problem. Despite inducing insulin resistance in many circumstances, growth hormone (GH) has been studied as a treatment for T2DM in both humans and mice. Many of the effects of GH are mediated via insulin-like growth factor-1 (IGF-1). Skeletal muscle is an important mediator of insulin resistance and blood glucose uptake. GH may cause a shift toward a slower fiber type profile in skeletal muscle, which may be associated with greater glucose uptake.

Overall Goal: To examine the effects of administration of GH, IGF-1, or both on the muscle fiber type profile and muscle fiber cross-sectional area in diabetic mice.

Methods: C57BL/6J mice were split into five groups as follows: Control (low-fat diet + PBS; n=10), DM (high-fat diet + PBS; n=10), GH (high-fat diet + GH; n=10), IGF-1 (high-fat diet + IGF-1; n=10), Combo (high-fat diet + GH and IGF-1; n=9). Mice were on their respective diets for 16 weeks followed by 3 weeks of hormone or PBS injections. The high-fat diet has previously been shown to induce type 2 diabetes. At the end of the injection period, the mice were sacrificed and the triceps surae muscle group (soleus, plantaris, and gastrocnemius muscles) was removed and weighed. Muscle fiber type
composition was determined using myosin ATPase histochemistry and immunohistochemistry and muscle fiber cross-sectional area was analyzed.

**Results:** Triceps surae mass was greater in the GH and Combo groups compared to the Control, DM, and IGF-1 groups. The DM group had a greater muscle CSA across muscle fiber types in all muscle groups, but no difference in muscle fiber type profile compared to the Control group. The GH, IGF-1, and Combo groups demonstrated a shift toward IIA fibers in the soleus muscle with decreased proportions of type I and/or type IIAD fibers. There were few significant changes in fiber type composition in the plantaris or gastrocnemius muscles. GH increased muscle fiber CSA across fast fiber subtypes, while IGF-1 preferentially increased CSA in slower fiber types of each respective muscle. Combo treatment yielded intermediate results.

**Conclusions:** Type 2 diabetes in mice increased muscle fiber CSA, but did not affect the fiber type profile of the triceps surae muscle group. GH administration resulted in significant muscle hypertrophy, while IGF-1 yielded a less robust hypertrophic response. GH administration to diabetic mice caused a shift toward type IIA fibers in the soleus with a concomitant decrease in type I and IIAD fibers, while IGF-1 caused a shift toward IIA fibers with a concomitant decrease in IIAD fibers. GH preferentially increased muscle fiber CSA in fast fiber types while IGF-1 increased muscle fiber CSA in the slower fiber types of each respective muscle.

Approved: ________________________________

Robert S. Staron

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I want to dedicate this dissertation to my little boy Spencer Robert Schumm who was born on March 31, 2011.
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LIST OF ABBREVIATIONS

ADP: adenosine diphosphate
ALS: acid labile subunit
ANOVA: analysis of variance
ATP: adenosine triphosphate
bGH: bovine growth hormone
CHF: congestive heart failure
CSA: cross-sectional area
DAG: diacylglycerol
DEXA: dual energy x-ray absorptometry
DGAT2: acyl-CoA:diacylglycerol acetyltransferase 2
DRG: deep, red gastrocnemius muscle
ECW: extra-cellular water
EDL: extensor digitorum longus muscle
ETC: electron transport chain
FFA: free fatty acid
GH: growth hormone
GLUT4: glucose transporter 4
GTT: glucose tolerance test
IGF-1: insulin-like growth factor 1
IGFBP: insulin-like growth factor binding protein
IRS-1: insulin receptor substrate-1

LBM: lean body mass

MAPK: mitogen activated protein kinase

mATPase: myofibrillar adenosine triphosphatase

MGF: mechano-growth factor

MHC: myosin heavy chain

mTOR: mammalian target of rapamycin

OGTT: oral glucose tolerance test

PBS: phosphate buffered saline

PCr: phosphocreatine

PGC 1α: peroxisome proliferator-activated receptor gamma coactivator 1α

PI3K: phosphatidylinositol 3 kinase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

STAT: signal transducer and activator of transcription

SWG: superficial, white gastrocnemius muscle
CHAPTER 1: INTRODUCTION

Relevance

Type 2 diabetes mellitus, which will be referred to as type 2 diabetes unless otherwise noted, is a growing public health concern both in the United States and worldwide. The incidence of type 2 diabetes in the U.S. has nearly doubled over the past 40 years from approximately 3% to approximately 6% (Taubes, 2009). This increase in the prevalence of type 2 diabetes has been mirrored by an increase in the obesity rate, which increased from 14% to 34% in the same time frame (Taubes). Obesity is closely linked to insulin resistance and thus appears to be a significant factor in the development of type 2 diabetes. Metabolic syndrome, an associated malady, is a constellation of conditions, including insulin resistance, and is estimated to affect as many as 50 million Americans (Taubes).

The pathology of type 2 diabetes often begins with insulin resistance in which skeletal muscle becomes resistant to the effects of insulin. Insulin secretion increases to compensate for the relative insulin sensitivity (see Figure 1 for disease progression overview). Early in the disease progression, fasting blood glucose concentrations are maintained but post-prandial glucose levels begin to rise (Ramlo-Halsted & Edelman, 1999; Rao, Disraeli, & McGregor, 2004). Dysfunction of the β-cells of the pancreas may also contribute to type 2 diabetes because increased insulin output from these cells cannot be maintained to overcome insulin resistance (American Diabetes Association position
statement, 2006; Rao et al.). When insulin output decreases fasting blood glucose concentrations rise, leading to a diagnosis of diabetes (see Figure 1.).

Given the impact of type 2 diabetes and insulin resistance on the health care system, many avenues for treatment of type 2 diabetes have been explored. Lifestyle factors such as dietary intake, habitual exercise, increased physical activity, and body mass are typically modified in the treatment of type 2 diabetes (Rao et al., 2004). In addition, drug therapy is used in the treatment of type 2 diabetes. The different types of oral medication for type 2 diabetes include insulin secretagogues, α-glucosidase
inhibitors, insulin sensitizers, and DPP-4 inhibitors (American Diabetes association website; Krentz & Bailey, 2005). Insulin may also be used for the treatment of type 2 diabetes in certain situations (Krentz & Bailey). In general, insulin secretagogues stimulate insulin output from the pancreas, α-glucosidase inhibitors slow the digestion of carbohydrates to decrease post-prandial hyperglycemia, insulin sensitizers decrease insulin resistance, and DPP-4 inhibitors decrease blood insulin inactivation (Krentz & Bailey) (see Table 1 for a summary of oral diabetes medications). Insulin secretagogues include sulfonylureas and meglitinides. Insulin sensitizers include biguanides and thiazolidinediones. Different types of diabetes medications with varying durations of action can be utilized (Table 1).
Table 1.

*Type 2 diabetes medications*

<table>
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<td>Sulfonylureas</td>
<td>Clorpropamide, Glipizide,</td>
<td>Increase insulin output</td>
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<td>Glyburide, Glimepiride</td>
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<td>Meglitinides</td>
<td>Repaglinide, Nateglinide</td>
<td>Increase prandial insulin output</td>
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<td>Biguanides</td>
<td>Metformin</td>
<td>Decrease hepatic glucose output, increase insulin</td>
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<td>sensitivity</td>
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<tr>
<td>Thiazolidinediones</td>
<td>Rosiglitazone, Pioglitazone</td>
<td>Decrease hepatic glucose output, increase insulin</td>
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<td></td>
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<td>Alpha-glucosidase</td>
<td>Acarbose, Meglitol</td>
<td>Decreased dietary carbohydrate breakdown</td>
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<td>Inhibitors</td>
<td></td>
<td></td>
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<tr>
<td>DPP-4 Inhibitors</td>
<td>Sitagliptin, saxagliptin</td>
<td>Prevent GLP-1 breakdown, prolong insulin circulation</td>
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<tr>
<td>Incretin Mimics</td>
<td>Exenatide</td>
<td>Increases insulin secretion</td>
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<tr>
<td>Rapid-acting Insulin</td>
<td>Lispro, Insulin Aspart,</td>
<td>Insulin replacement</td>
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<td></td>
<td>Insulin Glulisine</td>
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<td>Short-acting Insulin</td>
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Summary of information from Krentz & Bailey (2005) and American Diabetes Association website.

Growth hormone (GH) is one compound that has been investigated in the treatment of type 2 diabetes, but has not yet been accepted for widespread use due to its potential diabetogenic effects. Many years of research have indicated that GH induces insulin resistance. Zierler & Rabinowitz (1963) noted insulin resistance induced by GH using human forearm blood sampling. Subsequently, many investigations have found GH to be diabetogenic and induce insulin resistance (Barbour et al., 2004; Clemmons, Snyder, Williams, & Underwood, 1987; Christopher, Hew, Oakley, Rantzau, & Alford, 1998; Jessen et al., 2004; Kim et al., 1999; Pasarica, Zachwieja, DeJonge, Redman, &
Smith, 2007; Snyder, Underwood, & Clemmons, 1990). Because of this, the use of GH as a diabetes treatment is controversial. There are also safety concerns with the use of GH in other situations such as anti-aging therapy (Liu et al., 2007). Even though GH has shown some promise as a treatment agent, concerns regarding long-term side effects make further research warranted (Ahn et al., 2006; List et al., 2009; Nam et al., 2001). Because many of the effects of GH are mediated by insulin-like growth factor 1 (IGF-1) and it does not have the diabetogenic effects of GH, it may also have promise as a treatment option for type 2 diabetes (Butler & LeRoith, 2001; Dohm et al., 1990; Dominici et al., 2005; Laviola, Natalicchio, & Giorgino, 2007).

Skeletal muscle has been estimated to be responsible for 80% or more of blood glucose uptake and thus plays an important role in maintaining normal blood glucose concentrations (Baron, Brechtel, Wallace, & Edelman, 1988; DeFronzo, Gunnarsson, Bjorkman, Olsson, & Wahren, 1985; Phielix & Mensink, 2008; Stump, Henricksen, Wei, & Sowers, 2006; Zurlo, Larson, Bogardus, & Ravussin, 1990). Both GH and IGF-1 have been noted to influence muscle fiber type and muscle fiber cross-sectional area (CSA). The muscle fiber type composition of skeletal muscle may be related to its glucose uptake capacity. Overall, a slower muscle fiber type profile has been associated with greater glucose uptake capability (Daugaard & Richter, 2001; Gaster, Poulsen, Handberg, Schroder, & Beck-Nielsen, 2000; Gaster et al., 2001; Marette et al., 1992; Oberbach et al., 2006). There is also some evidence of a faster muscle fiber type profile in humans with type 2 diabetes, although this finding is not universal (Daugaard & Richter, 2001; Gaster et al., 2001; He, Watkins, & Kelley, 2001; Oberbach et al.). GH administration
has been shown to induce a slower muscle fiber type profile in some instances (Ayling, Moreland, Zanelli, & Schulster, 1989; Daugaard et al., 1998; Libera et al., 2004; Schuenke et al., 2008). It is widely believed that the effects of GH on skeletal muscle are mediated via IGF-1, although the effects of IGF-1 on muscle fiber type have been equivocal (Lynch, Cuffe, Plant, & Gregorevic, 2001; Roy et al., 1996; Song et al., 2005). If GH or IGF-1 did indeed cause a shift to a slower fiber type profile, it may partially explain the favorable effects of GH on glucose uptake in type 2 diabetes. Therefore, further investigation of the effects of GH and IGF-1 on muscle characteristics in diabetes could prove valuable in understanding the glucose-lowering effects of GH in type 2 diabetes.

Muscle Fiber Type

Myosin Heavy Chain

Myosin heavy chain (MHC) proteins are a part of the larger hexameric myosin molecule. Myosin is comprised of two MHC’s, which are approximately 200 kDa each, and two pairs of myosin light chains that are approximately 16-24 kDa each. An enzyme called myofibrillar adenosine triphosphatase (mATPase) is contained in the head of each MHC. It is responsible for cleaving a phosphate bond from adenosine triphosphate (ATP) to release the energy utilized to fuel muscle contraction. It has been established that four MHC isoforms exist in substantial amounts in mammalian skeletal muscle; MHC Iβ, MHC Ila, MHC IId, and MHC Iib (Pette & Staron, 2001). Muscle fiber type is determined by the MHC content with MHC Iβ = type I, MHC Ila = type IIA, MHC IId = type IID, and MHC Iib = IIB. Muscle fiber type has also been correlated with
contraction velocity with a continuum of slow to fast in the following order: I, IIA, IID, and IIB.

Different MHC isoforms have different mATPase pH stability profiles. These pH stability differences can be used to determine the MHC profile of individual fibers and thus determine muscle fiber type. Antibodies specific to different MHC isoforms can also be used to identify muscle fiber type. It should be noted that specific MHC isoforms will subsequently be identified with a Roman numerals and a lower case letter (i.e. Iia), while overall muscle fiber type will be identified with a Roman numeral and an upper case letter (i.e. IIA).

Muscle Fiber Type Plasticity and Hypertrophy

Muscle fiber type, MHC gene expression, and MHC protein expression have been shown to be adaptable to different stimuli including muscle loading, activity or exercise, hormone exposure, and changes in innervation or neural stimulation patterns (Baldwin & Haddad, 2001; Castro, Apple, Staron, Campos, & Dudley, 1999; Hodgson et al., 2005; Pette, 2001; Pette & Staron, 2001; Staron et al., 1991). Thyroid hormones are thought to have a profound effect on muscle fiber phenotype with hypothyroidism causing a fast-to-slow fiber type transformation and hyperthyroidism causing a slow-to-fast shift (Pette & Staron, 2001). The effects of other hormones such as testosterone, GH, and IGF-1 on muscle fiber type composition are not as clear (Aroniadou-Anderjaska, Lemon, & Gilloteaux, 1996; Daugaard, Lausten, Hansen, & Richter 1998; Daugaard et al., 1999; Libera et al., 2004; Everitt, Terry, Phillips, Kerry, & Shorey, 1996; Florini & Ewton, 1989; Hikida, Knapp, Chen, Gozdanovic, & Kopchick, 1995; Lynch, Cuffe, Plant, &
The cellular signaling events related to fiber type and MHC isoform expression have been associated with both Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) and calcineurin (Delling et al., 2000; Geng, et al., 2010; Handschin et al., 2007; Mortensen, Frandsen, Schjerling, Nishimura, & Grunnet, 2006; Naya et al., 2000; Parsons, Wilkins, Bueno, & Molkentin, 2004; Torgan & Daniels, 2001; Vescovo, Ravara, Gobbo, Angelini, & Libera, 2005).

Increases in expression of both PGC-1α and calcineurin have been correlated with increases in expression of MHC Iβ or increases in type I fibers both in vivo and in muscle cell cultures (Chin et al., 1998; Delling et al., 2000; Meißner, Gros, Scheibe, Scholz, & Kubis, 2001; Mortensen et al., 2006; Naya et al., 2000; Parsons et al., 2004; Serrano et al., 2001; Vescovo et al., 2005). PGC-1α functions as a nuclear transcription coactivator involved in many metabolic processes including those associated with mitochondrial biogenesis and insulin secretion (Russell, 2005). Calcineurin is a serine and threonine phosphatase with activity controlled by intracellular calcium levels (Crabtree, 2001). It has been implicated in a number of cellular processes including muscle and neuron development (Crabtree). There is also evidence that PGC-1α influences some fast MHC expression. Adding PGC-1α to rat skeletal muscle cell cultures increased MHC Iβ mRNA content and decreased MHC IId and IIb mRNA (Mortensen et al.). Handschin et al. found an increase in the percentage of type IID and IIB fibers and a decrease in type I and IIA fibers in PGC-1α knockout mice compared to control mice. Interestingly, PGC-1α knockout mice still demonstrate IIB to IIA fiber type transitions with exercise (Geng
et al.). When taken together, the available evidence suggests that PGC-1α promotes the expression of MHC Iβ and IIA and suppresses MHC IID and IIB expression, although it may not be the only determining factor in MHC expression and fiber type shifts.

It is well known that overloading a muscle or exposure to increases in certain hormone concentrations such as testosterone, GH, and IGF-1 can induce muscle hypertrophy and increases in muscle fiber CSA (Adams & Haddad, 1996; Adams & McCue, 1998; Bhasin et al., 2003; Booth, Tseng, Fluck, & Carson, 1998; Hostler et al., 2001; Mikesky, Giddings, Matthews, & Gonyea, 1991; Schuenke et al., 2008; Semsarian et al., 1999; Sinha-Hikim, Cornford, Gaytan, Lee, & Bhasin, 2006). There may be a certain GH concentration threshold that is necessary for significant muscle hypertrophy as increasing doses or exposure have yielded a larger hypertrophic response (Hikida, Knapp, Chen, Gozdanovic, & Kopchick, 1995; List et al., 2009). The signaling events that lead to muscle hypertrophy are thought to involve the Akt/mTOR pathway with activation of satellite cells (Adams, 2006; Bodine et al., 2001; Booth et al., 1998; Chakravarthy, Abraha, Schwartz, Fiorotto, & Booth, 2000; Glass, 2003; Latres et al., 2005; Petrella, Kim, Cross, Koseck, & Bamman, 2006). Blocking the Akt/mTOR pathway has been shown to blunt the hypertrophy response, which speaks to the importance of this pathway (Bodine et al.). Gamma radiation that inactivates satellite cells has also been shown to prevent muscle hypertrophy in mice (Barton-Davis, Shoturma, & Sweeney, 1999). There is also evidence that calcineurin is involved in some aspects of hypertrophy signaling, but does not appear essential (Bodine et al., Chin et al., 1998; Naya et al., 2000; Parsons et al., 2004; Serrano et al., 2001). GH signaling
may also act on myostatin, which is a negative regulator of skeletal muscle hypertrophy. GH treatment for GH deficient adults has been shown to decrease myostatin mRNA expression (Liu et al., 2003). There is also evidence that indicates that GH signaling upregulates androgen receptor gene expression and thus also promotes hypertrophy indirectly by increasing androgen receptor content (Klover, Chen, Zhu, & Hennighausen 2009).

mATPase Histochemistry

Early use of mATPase properties to determine muscle fiber type yielded results indicating that type I fibers were acid-stable and alkali-labile while type II fibers were the opposite (Engel, 1962; Guth & Samaha, 1969; Guth & Samaha, 1970). Further refinement and analysis yielded a procedure utilizing three pre-incubation pH values and delineated a range of fiber types to include types I, IC, IIC, IIAC, IIA, IIA, IID, IID, IID, IIB (Brooke & Kaiser, 1970; Staron, 1991; Staron & Hikida, 1992; Staron, Hikida, & Hagerman, 1983; Staron & Pette, 1987). As mentioned previously, MHC isoforms have been identified as Iβ, IIa, IId, and IIB. The other fiber types are hybrids that contain more than one MHC isoform. Muscle fiber types IC, IIC, and IIAC are comprised of different proportions of Iβ and IIa MHC isoforms. Fiber types IIAD and IIDB are made up of combinations of MHC IIa + IId and IId + IIB, respectively. There may also be rarely occurring fibers that co-express more than two MHC isoforms. For a review of fiber type descriptions and adaptation see Pette & Staron (2001). Using mATPase histochemistry may not be optimal in identifying all hybrid fiber types, particularly those containing MHC IId (Linnanne, Serrano, & Rivero, 1999; Staron & Pette, 1993). It is also
noteworthy that the fiber type originally defined as IIB in humans is actually more similar to the IID in small mammals (Ennion, Pereira, Sargeant, Young, & Goldspink, 1995; Sant’ Ana Pereira, Ennion, Sargeant, Moorman, & Goldspink, 1997; Smerdu, Karsch-Mizrachi, Campione, Leinwald, & Schiaffino, 1994). This has led to inconsistency in nomenclature in the literature regarding human muscle fiber types. Literature older than approximately 10 years ago typically uses the fiber type IIB to name the fastest fiber type in humans, while more recent literature typically uses IID to refer to the same fiber type. It should also be noted that the fiber type IID and IIX are used interchangeably in the literature to identify the same fiber type.

_MHC Immunohistochemistry_

Another method for determining muscle fiber type is through the use of monoclonal anti-bodies specific to different MHC isoforms. Myosin immunohistochemistry can identify type I, IIA, IID, and IIB fibers (Sant’ et al., 1997; Schiffano et al., 1989; Schuenke et al., 2008). Because there is no antibody specific to only MHC IId, combinations of different antibodies must be compared and cross-referenced to identify IID fibers. Most hybrid fiber types can be identified using a wide range of antibodies, with the possible exception of IIDB fibers given the lack of MHC IId specific antibodies.

Single fiber analysis using SDS-PAGE is the best way to determine the presence of two or more MHC isoforms but is too laborious for use with a large numbers of muscle fibers (Staron & Pette, 1987; Staron & Pette, 1993). Given these limitations, mATPase histochemistry and myosin immunohistochemistry appear to be the most
practical methods for determining the fiber type composition in large samples. With the aforementioned limitations of each method in detecting the different hybrid fiber types, utilizing a combination of both methods seems most appropriate. Recent work has shown it is possible to delineate a range of fiber types in mice to include I, IC, IIC, IIA, IIAD, IID, IIDB, and IIB using a combination of mATPase histochemistry and myosin immunohistochemistry (Schuenke et al.).

Type 2 Diabetes Physiology

Type 2 diabetes is characterized by elevated blood glucose concentrations and insulin resistance accompanied by possible β-cell defects that affect insulin secretion (Leng, Karlsson, & Zierath 2004) (see Figure 2 for an overview). Therefore, type 2 diabetes is a disease that has both endocrine (insulin secretion) and peripheral (insulin resistance) components. Target tissues for insulin include the liver, adipose tissue, and skeletal muscle (Dominici et al., 2005). In skeletal muscle, the insulin receptor is a heterodimer composed of two alpha and two beta chains. It spans the plasma membrane with two binding domains on the cell surface and two intracellular signaling domains that have intrinsic tyrosine kinase activity (Dean & McEntyre, 2004; Dominici et al., 2005) (see Figure 3).
Pathophysiology of Type 2 Diabetes

Figure 2. Pathophysiology of type 2 diabetes. Insulin resistance can increase blood glucose by reducing glucose uptake in skeletal muscle and increasing hepatic glucose output. Defective β-cell insulin secretion plus peripheral insulin resistant ultimately result in elevated blood glucose concentrations. Figure courtesy of Dr. John Kopchick.
Figure 3. Insulin receptor structure. The extracellular alpha subunits provide a binding site for circulating insulin. The beta subunits have intrinsic tyrosine kinase activity that is activated when insulin binds to the insulin receptor. Figure adapted from Dean & McEntyre, 2004.

Circulating insulin binds to insulin receptors, which triggers intrinsic tyrosine kinase activity located in the beta chains, which, in turn, leads to an intracellular signaling cascade that ultimately results in the migration of glucose transport 4 (GLUT4) proteins to the cell membrane (Ivy & Kuo, 1998; Moller et al., 1996; Zorzano, Palacin, & Guma, 2005). GLUT4 locating on the cell membrane creates channels that allow glucose diffusion into the cell. This GLUT4 exocytosis accounts for nearly all the increase in insulin-stimulated glucose transport (Karlsson et al., 2009) (see Figure 4 for an overview of insulin signaling). Thus, blood insulin concentrations and tissue responsiveness to insulin play an essential role in modulating glucose transport and maintaining normal blood glucose concentrations.
Figure 4. Insulin signaling cascade. Insulin binding results in a signaling cascade that involves IRS and PI3K that ultimately leads to GLUT4 translocation to the cell membrane. GLUT4 translocation allows glucose diffusion into the cell. Insulin signaling also results in lipid synthesis, protein synthesis, glycogen synthesis, and inhibition of gluconeogenesis. Insulin Signaling Pathways by C. Hooper. http://www.abcam.com/index.html?pageconfig=resource&rid=10602&pid=7. Figure courtesy of Dr. John Kopchick.

With insulin resistance there appears to be an insulin signaling defect that results in decreased recruitment of GLUT4 proteins to the cell membrane (Leng et al., 2004; Stump, Henriksen, Wei, & Sowers, 2006). The exact nature of the signaling defect associated with insulin resistance is still not fully characterized, although multiple levels of the signaling cascade may be affected (Leng et al.; Stump et al., 2006). There may be alterations in protein expression or activation involving the insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3 kinase (PI3K), PDK-1, Akt, PKC, and
GSK-3 (see Leng et al. for review). Those with a family history of type 2 diabetes have been shown to have a lower glucose disposal rate than normal control subjects during a hyperinsulinemic-euglycemic clamp procedure (Pratipanawatr et al. (2001). This decreased glucose disposal was associated with decreased tyrosine phosphorylation of IRS-1 (Pratipanawatr et al.). They also correlated glucose disposal with IRS-1 and PI3K association (Pratipanawatr et al.). Others have implicated different insulin signaling impairments in insulin resistance and type 2 diabetes. Shelley et al. (2009) examined the vastus lateralis of C57BL/6J mice that were the offspring of both obese and normal weight dams. The female offspring of obese dams had lower protein expression levels of the p110β subunit of PI3K and lower levels of Akt serine phosphorylation than the offspring of control dams (Shelley et al.). PI3K activity is impaired in insulin resistance and type 2 diabetes (Beeson et al., 2003; Cusi et al., 2000; Kim et al., 2003). Akt phosphorylation is also decreased with type 2 diabetes (Cozzone et al., 2008; Morino et al., 2005). Insulin-resistant humans have been found to have both increased IRS-1 serine phosphorylation and decreased Akt activation compared to normal controls (Morino et al.). Given the various levels of the insulin-signaling cascade that are affected with insulin resistance, it is difficult to isolate only one step that is responsible for the signaling defect.

With defects in insulin signaling blood glucose disposal is attenuated. Insulin output is typically increased to compensate for the insulin resistance. If the insulin resistance worsens, even with increasing blood insulin concentrations, glucose transport can be impaired to the point that it causes elevations in blood glucose concentrations.
Normal fasting blood glucose concentrations are 70-100 mg/dl. Diabetes is diagnosed when any one of the following three criteria are met according to the American Diabetes Association: 1) Symptoms of diabetes which include polyuria, polydipsia, and unexplained weight loss combined with a casual blood glucose of $\geq 200$ mg/dl; 2) Fasting blood glucose of $\geq 126$ mg/dl; 3) 2 hour post-load glucose of $\geq 200$ mg/dl during an oral glucose tolerance test (OGTT) (American Diabetes Association position statement, 2006). With type 2 diabetes, resting blood glucose concentrations are elevated even compared to those with impaired glucose tolerance that are not yet diabetic or obese subjects who have normal glucose tolerance (Mensink, Blaak, Baak, Wagenmakers, & Saris, 2001). Impaired fasting glucose is defined as fasting glucose concentrations that are 100-125 mg/dl and impaired glucose tolerance is defined as blood glucose of 140-199 mg/dl two hours after a glucose load in an OGTT (American Diabetes Association position statement). Skeletal muscle is believed by many to be the primary tissue responsible for insulin resistance in type 2 diabetes (Ivy & Kuo, 1998; Leng et al. 2004; Ryder, Chibal, & Zierath, 2001; Stump et al., 2006).

Recent work demonstrated the importance of skeletal muscle in glucose disposal utilizing myostatin deficient mice, which have greater than normal muscle mass (Guo et al., 2009). Myostatin is a protein implicated in regulating muscle mass and hypertrophy potential. When fed a high fat diet, the myostatin null mice showed increased carbohydrate utilization and better glucose tolerance than control mice (Guo et al.). The myostatin null mice also had lower blood glucose and insulin levels and increased sensitivity when compared to the control mice (Guo et al.). One potential limitation in
interpreting these results is that the myostatin deficient mice also have lower levels of body fat than the control mice, making it difficult to separate the effects of increased muscle mass and decreased fat mass (Guo et al.). It is also difficult to know if the improved insulin sensitivity is due to an intrinsic property of myostatin or simply the myostatin related changes in body composition.

The etiology behind the development of insulin resistance and type 2 diabetes is not universally agreed upon. Two of the most prominent ideas for the etiology of insulin resistance are related to inflammation and lipid accumulation as recently outlined by Taubes (2009). The inflammation hypothesis postulates that inflammatory signals from enlarged fat cells have a negative effect on insulin signaling (Stump et al., 2009; Taubes). The lipid overload hypothesis postulates that enlarged fat cells leak fatty acids into the bloodstream and end up as accumulated diacylglycerol (DAG) in muscle cells. This lipid accumulation subsequently interferes with lipid signaling and causes insulin resistance (Stump et al., 2009; Taubes). There are also some who believe that mitochondrial dysfunction is the primary cause of insulin resistance (Kim, Wei, & Sowers, 2008; Mogensen et al., 2007; Morino et al., 2005; Schrauwen-Hinderling et al., 2007; Toledo et al., 2007). There is compelling evidence that supports both the lipid overload and mitochondrial dysfunction hypotheses, making it difficult to isolate one primary trigger of insulin resistance in type 2 diabetes.

Many investigations have associated insulin resistance with intramuscular lipid accumulation. For example, both obesity and type 2 diabetes have been associated with increases in intramuscular lipid accumulation (He, Watkins, & Kelley, 2001; Malenfant,
et al., 2001). This muscle lipid accumulation in obese or diabetic subjects appears to be consistent across muscle fiber types (I, IIA, IID) in humans (He et al.; Malenfant et al.). In some cases, this increase in muscle lipid content has been associated with decreases in relative oxidative enzyme activity within each muscle fiber type (He et al.). What is not clear is whether lipid accumulation is the cause of insulin resistance or a consequence of it. There is some evidence that disruptions in lipid metabolism may contribute to development of insulin resistance (Levin et al., 2007). Lipid accumulation in glycolytic muscle promotes insulin resistance (Levin et al.). They utilized a transgenic mouse model that overexpressed human acyl-CoA:diacylglycerol acetyltransferase 2 (DGAT2) in glycolytic muscle, which resulted in increased triglyceride synthesis in glycolytic muscle (Levin et al.). Enhanced triglyceride deposition in glycolytic muscle resulted in impaired glucose uptake. This also caused an impairment of whole body insulin and glucose tolerance (Levin et al.). Specifically they found decreased PI3K activity (Levin et al.). Since PI3K is involved in a key step in the insulin-signaling cascade, decreased activity can lead to insulin resistance (Dominici et al., 2005). There is some thought that specific lipid intermediates such as fatty acetyl-CoA, DAG’s, or ceramides accumulate in skeletal muscle and are responsible for insulin resistance (for review see Timmers, Schrauwen, & Vogel, 2008). The direct link between the accumulation of various lipids in muscle fibers and insulin resistance has not been definitively identified, although some have proposed interactions between lipid intermediates and the insulin signaling pathways (see Figure 5). Despite this association between insulin resistance and lipid
accumulation in the muscle, it is still not completely clear why DAG’s would accumulate in the muscle tissue to begin with (Taubes, 2009).

Figure 5. Potential interactions between lipid accumulation and insulin signaling. Incomplete lipid oxidation leads to accumulation of lipid intermediates. The insulin receptor and several downstream intermediates are potential targets for the effects of lipid intermediates IR = insulin receptor. Adapted from Timmers et al. (2008).

Dietary factors can also play a role in fat accumulation in muscle and insulin and glucose homeostasis. Even relatively short-term, high-fat feeding can cause perturbations in insulin and glucose regulation in mice (Wilde et al., 2007). C57BL/6J mice were fed either a low-fat (10% of total calories) or high-fat (45% of total calories) diet. Just three days into the diet, the high-fat diet group already demonstrated significant increases in both blood glucose and insulin concentrations (Wilde et al.). These changes remained persistent at 28 days (Wilde et al.). This suggests that an increased influx of fatty acids
from a high-fat diet can result in perturbations in glucose metabolism. This is presumably due to lipid accumulation in skeletal muscle causing insulin resistance, although this was not specifically investigated (Wilde et al.) To support this notion, Sprague Dawley rats have been shown to have greater intramuscular triglyceride content with a high-fat diet compared to a high carbohydrate diet (Lee et al. (2002). Thus, if muscle lipid accumulation is a primary cause of insulin resistance, then dietary fat content may play a significant role in the development of insulin resistance. It is still somewhat unknown why a high-fat diet leads to muscle lipid accumulation or whether it is the high-fat content of the diet in isolation or the combination high-fat diet with a positive energy balance.

It should also be noted that not all lipid accumulation in skeletal muscle is associated with insulin resistance. It has been established that endurance-trained athletes have greater intramuscular triglyceride storage than non-trained individuals (Goodpaster, He, Watkins, & Kelley, 2001; Moro, Bajpevi, & Smith, 2008; Van Loon et al., 2004). Yet this increase in muscle lipid content does not result in insulin resistance (Goodpaster et al.; Moro et al.; Van Loon et al., 2004). In fact, endurance athletes may have greater overall intramuscular lipid content than overweight, sedentary subjects or subjects with type 2 diabetes (Van Loon et al.). Given this paradox regarding intramuscular lipid storage, it is difficult to attribute insulin resistance solely to muscle lipid storage. There is some thought that there are different storage forms of intramuscular lipids in endurance athletes compared to diabetics who often have an accumulation of fatty acetyl-CoA, DAG’s, or ceramides (Timmers et al., 2008).
Some have also linked mitochondrial dysfunction to insulin resistance and type 2 diabetes (Kim et al., 2008; Mogensen et al., 2007; Morino et al., 2005; Schrauwen-Hinderling et al., 2007; Toledo et al., 2007). Schrauwen-Hinderling et al. compared subjects with type 2 diabetes to BMI-matched controls and found a longer phosphocreatine (PCr) recovery time with type 2 diabetes. They also found that intramuscular lipid content was similar between the two groups (Schrauwen-Hinderling et al.). Others have demonstrated decreased mitochondrial respiration in subjects with type 2 diabetes compared to obese controls (Mogensen et al.). Ritov et al. (2005) found that electron transport chain (ETC) activity was decreased in the subsarcolemmal mitochondria of type 2 diabetic subjects compared to weight-matched obese controls and lean controls. They found a hierarchy of subsarcolemmal mitochondrial ETC activity in subjects ranging from greatest in the lean subjects and least in the type 2 diabetics with obese controls having intermediate ETC activity (Ritov et al.). They also found that the reduction of ETC activity was proportionally greater than the decrease of overall subsarcolemmal mitochondria, suggesting mitochondrial dysfunction (Ritov et al.). Interestingly they found that overall ETC activity in the muscle was not different between the obese control and type 2 diabetics, even with the differences noted in the subsarcolemmal mitochondrial fractions (Ritov et al.). Some have recorded parallel improvements in mitochondrial density and oxidative enzyme capacity and improved insulin resistance in type 2 diabetics with a 4-month exercise program (Toledo et al., 2007). There is also evidence that high concentrations of fatty acid metabolites inhibit mitochondrial function in lean, healthy humans and in mice (Abdul-Ghani et al., 2008).
Similarly, elevated plasma free fatty acid (FFA) levels from a lipid infusion induced insulin resistance and decreased ATP synthesis in healthy males (Brehm et al., 2006). These results appear to indicate that mitochondrial function may be better correlated with insulin resistance than muscle lipid accumulation.

Others have found that the link between mitochondrial dysfunction and insulin resistance is not as clear-cut (Boushel et al., 2007; De Feyter et al., 2008; Dumas, Simard, Flamment, Ducluzea, & Ritz, 2009; Morino et al., 2005; Phielex & Mensink, 2008; Toledo et al., 2008). Morino et al. found that those with insulin resistance did have decreased mitochondrial density but also had increased muscle lipid content when compared to non-insulin resistance control subjects making it difficult to attribute insulin resistance solely to mitochondrial function. Another possibility is that mitochondrial density, not necessarily mitochondrial function, is related to insulin resistance. To support this notion, there is evidence that decreased mitochondrial density accounted for decreased oxygen flux in the vastus lateralis of type 2 diabetics (Boushel et al.). Similary, type 2 diabetics, subjects with impaired fasting glucose, and normal controls and had no differences in PCr and adenosine diphosphate (ADP) recovery time constants suggesting no differences in mitochondrial function between the groups (De Feyter et al.).

Improvements in mitochondrial density or function do not necessarily lead to improvements in insulin sensitivity. In some cases, dietary intervention improved insulin sensitivity in sedentary obese subjects without any improvement in mitochondrial density or function (Toledo et al., 2008). Interestingly, dietary intervention plus exercise also
yielded improvements in insulin sensitivity accompanied by improvements in mitochondrial density and function (Toledo et al., 2008). Given the ambiguous results of the aforementioned research, many have questioned the relationship between mitochondrial dysfunction and insulin resistance (for review see Dumas et al. 2009). Other potential mechanisms in type 2 diabetes include increased inflammatory adipokines, increased renin-angiotensin-aldosterone system activity, and increased oxidative stress (Stump et al., 2009).

**GH and IGF-1 in Diabetes Treatment**

GH is a poly-peptide hormone secreted by the lateral wing of the anterior pituitary gland that is controlled via the hypothalamus (Kopchick & Chen, 1991; Velloso, 2008). GH secretion is pulsatile and is stimulated by growth hormone releasing hormone (GHRH) and inhibited by somatostatin (Velloso; Vijayakumar, Novosyadlyy, Wu, Yarkar, & LeRoith, 2010). The hormone ghrelin has also been demonstrated as a potent stimulator of GH secretion (Nass, Gaylinn, Rogel, & Thorner, 2010). GH has been implicated as an important mediator of growth and cell differentiation and proliferation. It is made up of a single peptide chain of 191 amino acids with a molecular weight of ~22 kDa (Harvey, Scanes, & Daughaday, 1995; Kopchick & Chen). Target tissues for GH include liver, skeletal muscle, and adipose tissue (Dominici et al., 2005) (see Figure 6 for an overview).

GH exists in multiple isoforms. These isoforms are due to differences in the genome, alternative mRNA splicing, and post-translational events (Baumann, 2009). The primary pituitary forms of GH are the previously mentioned 22kDa isoform and a 20kDa
isoform both of which are the result of alternative splicing of the GH1 gene transcript (Baumann). The 22kDa isoform makes up the majority of the pulsatile pituitary GH output (Baumann). Other isoforms are created due to post-translational events including N-acetylation, deamidation, and glycosylation (Baumann). A different gene encodes the placental isoform of GH. Much of the biological activity of GH has been attributed to the 22kDa isoform, although it is generally believed the 20kDa isoform has very similar effects (Baumann).

Figure 6. Growth Hormone feedback and target tissues. GH induces IGF-1 secretion in a number of target tissues. The liver is responsible for the majority of circulating IGF-1. In skeletal muscle, GH-induced IGF-1 secretion works in a local autocrine or paracrine manner. Elevated GH and IGF-1 levels provide negative feedback for GH secretion. Figure courtesy of Dr. John Kopchick.
The action of GH at the cellular level is mediated by GH binding to two GH receptor molecules to form a trimer (Lanning & Carter-Su, 2006) (see Figure 7). The binding of GH with its receptor causes tyrosine phosphorylation in the GH receptor by Janus Kinase 2 (JAK2) (Lanning & Carter-Su; Tanassijevic et al., 1993). These phosphorylation events lead to the formation of docking sites for several intracellular mediators and signaling molecules (Dominici et al., 2005; Lanning & Carter-Su). There are two important signaling pathways activated by GH. One pathway involves signal transducer and activator of transcription (STAT) proteins, particularly STAT5b (Davey et al., 2004; Herrington, Smit, Schwartz, & Carter-Su, 2000; Woelfle & Rotwein, 2004). This pathway includes the induction of IGF-1 gene expression (Davey et al.; Woelfle, Chia, & Rotwein, 2003). The other GH signaling pathway involves Ras-mitogen activated protein kinase (MAPK) pathway (Herrington & Carter-Su, 2001; Lanning & Carter-Su, 2006). This pathway has been implicated in promoting cell differentiation and proliferation (Dominici et al., 2005; Liang, Jiang, & Frank, 2000; Liang et al., 1999). GH has also been associated with increased expression of PGC-1α and calcineurin (Vescovo et al., 2005). As mentioned previously, signaling via both PGC-1α and calcineurin have been implicated in promoting expression of MHC Iβ (Adachi et al., 2006; Chin et al., 1998; Delling et al., 2000; Geng et al., 2010; Handschin et al., 2007; Kramer et al., 2006; Meißner et al., 2001; Mortensen et al., 2006; Naya et al., 2000; Parsons et al., 2004; Torgan & Daniels, 2001).
Figure 7. Growth hormone signaling. GH signaling works through STAT5 to increase transcription of target genes, including the IGF-1 gene. This triggers IGF-1 secretion in target tissues. GH signaling through the MAPK pathway promotes cell differentiation and proliferation. Figure courtesy of Dr. John Kopchick.

IGF-1 is a poly-peptide hormone produced by the liver and other target tissues. Liver production of IGF-1 is the primarily source of circulating IGF-1. In other target tissues such as skeletal muscle, GH-stimulated IGF-1 production acts in a paracrine or autocrine fashion with local effects. GH is known to stimulate IGF-1 production in the liver and in skeletal muscle (Dominici et al., 2005; Hameed et al., 2004) (see Figure 6).

It has a growth promoting effect in a number of body tissues, including skeletal muscle. IGF-1 is composed of a peptide chain of 70 amino acids with a molecular weight of ~7.6 kDa. Like GH, IGF-1 also exists in multiple isoforms. Specifically, the IGF-1 protein can be derived from multiple isoforms of prepropeptides (Barton 2006; Barton, DeMeao, & Lei, 2010; Booth 2010). Multiple splicing events at both ends of the IGF-1 gene can
result in differences in the use of exons 1 or 2 and the inclusion or exclusion of exon 5. These differences result in different E-peptide extensions in the IGF-1 propeptide (Barton 2006; Barton et al., 2010). Different IGF-1 isoforms are predominant depending on the stimulus and may have differential effects based on the circumstances (Barton 2006; Barton et al., 2010; Booth 2010).

IGF-1 also works by binding to its membrane bound receptor, which sets off a series of phosphorylation events that activates the IGF-1 signaling pathway. The IGF-1 receptor belongs to the same subfamily of tyrosine kinase receptors as the insulin receptor (Dominici et al., 2005; Vijayakumar et al., 2010). It has two identical extracellular α-subunits where IGF-1 binds and two β-subunits with intrinsic tyrosine kinase activity (Dominici et al., 2005; Laviola et al., 2007). There are also hybrid receptors in tissues such as skeletal muscle that have both insulin and IGF-1 receptors, which contain one α and β subunit from the IGF-1 receptor and one α and β subunit from the insulin receptor (Dominici et al., 2005; Laviola et al.; Soos, Whittaker, Lammers, Ullrich, & Siddle, 1990). The insulin receptor can bind IGF-1 and the IGF-1 receptor can bind insulin, but with much less affinity than its normal ligand (Dominici et al., 2005; Ezzat et al., 2008; Laviola et al.; Steele-Perkins et al., 1988). For example, IGF-1 receptors bind insulin with ~1% of the affinity of IGF-1 (Steele-Perkins et al.). The hybrid receptor can also bind both insulin and IGF-1 but with about 20 times greater affinity for IGF-1 (Soos, Field, & Siddle, 1993). Hybrid receptors may compose up to 85-90% of IGF-1 binding sites in skeletal muscle and account for the glucose uptake associated with IGF-1 (Bailyes et al., 1997; Dohm et al., 1990; Dominici et al., 2005).
Like GH, IGF-1 ultimately results in cell proliferation and differentiation through the MAPK pathway (Adams, 2002; Coolican, Samuel, Ewton, McWade, & Florini, 1997; Dominici et al., 2005; Philippou, Halapas, Maridaki, & Koutsilieris, 2007). IGF-1 also activates IRS, which in turn works through PI3K and activates Akt. This Akt activation is believed to be involved in insulin signaling as well as activating the mTOR-P70S6K pathway to promote muscle hypertrophy (see Figure 8)(Bodine et al., 2001; Dominici et al., 2005, Latres et al., 2005; Philippou et al., 2007). This pathway appears to work by activating satellite cells to promote hypertrophy and muscle repair and regeneration (Adams & Haddad, 1996; Adams & McCue, 1998; Chakravarthy et al., 2000; Dardevet, Sornet, Vary, & Grizard, 1996; Jacquemin et al., 2004; Sakuma et al., 2003; Semsarian et al., 1999). Although IGF-1 can induce muscle hypertrophy, it is not universally accepted that IGF-1 is required for muscle hypertrophy. With functional overload of the plantaris, a functional IGF-1 receptor was not necessary for Akt phosphorylation or hypertrophy (Spangenburg, LeRoith, Ward, & Bodine, 2008). IGF-1 is also believed to activate the calcium/calcineurin pathway involving GATA-1 and NFAT, which ultimately promotes expression of MHC Iβ (Bodine et al., 2001; Chin et al., 1998; Fiorotto, Schwartz, & Delaughter, 2003; McCullagh et al., 2004; Musaro, McCullagh, Naya, Olson, & Rosenthal, 1999; Naya et al., 2000; Serrano et al., 2001; Wu et al., 2000). See Figures 7 and 8 for an overview of GH and IGF-1 signaling.
Figure 8. IGF-1 signaling in skeletal muscle. IGF-1 promotes muscle hypertrophy via increases in protein synthesis and satellite cell activation. Satellite cell activation leads to differentiation and proliferation to increase the number of myonuclei. IGF-1 also increases intracellular Ca+ concentrations, resulting in signaling via calcineurin. Adapted from Philippou et al., 2007.

The role of GH and IGF-1 in treating obesity and type 2 diabetes is not clear.

There are signaling intermediates associated with GH and IGF-1 that overlap with insulin signaling in skeletal muscle (Dominici et al., 2005; Laviola, Natalicchio, & Giorgino, 2007; Ricon et al., 2007; Takano et al., 2001) (see Figure 9). It is not certain what the consequences are for this overlap in intracellular signaling in the context of already existing type 2 diabetes.
Figure 9. Overview of signaling events for GH, IGF-1, and insulin. Note that GH and insulin share some signaling intermediates including IRS proteins, PI3K, and SHC. IGF-1 and insulin also share some signaling intermediates including IRS proteins and SHC. Adapted from Dominici et al., 2005.

One of the places GH and insulin signaling overlap is at the level of the IRS proteins, IRS-PI3K association, and PI3K (Dominici et al., 2005; Ricon et al., 2007; Takano et al., 2001) (see Figure 9 for overview). PI3K is involved in both GH and insulin signaling. PI3K is made up of two subunits, with p85α considered the regulatory subunit and p110β the catalytic subunit (Dominici et al., 2005). GH signaling appears to increase overall p85α protein levels and increase the number of p85α monomers. This increase in p85α content has been linked with an inhibitory effect on insulin signaling and may lead to insulin resistance (Dominici et al. 2005; Ricon et al.). Mice with GH overexpression have been shown to have decreased insulin receptor content in the liver
compared to control mice (Dominici, Cifone, Bartke, & Turyn, 1999). IRS-1-PI3K association and PI3K activity in the liver was also maximized at baseline and did not respond to insulin (Dominici et al., 1999). Conversely, mice with decreased circulating GH or targeted GH receptor disruptions have enhanced insulin sensitivity associated with increased liver insulin receptor content, increased IRS-1 and IRS-2 content and insulin-stimulated phosphorylation, and normal PI3K activity (Dominici, Arostequi, Bartke, Kopchick, & Turyn, 2000; Dominici, Hauck, Argentino, Bartke, & Turyn, 2002). Others have found that transgenic mice that over-expressed human placental GH had higher plasma insulin levels than wild-type littermates (Barbour et al., 2004). They also demonstrated decreased insulin-stimulated GLUT4 translocation to the cell membrane, suggesting impaired insulin signaling (Barbour et al.). There was also no difference in insulin receptor or IRS-1 phosphorylation in the transgenic mice, but did find increased expression of p85α and a decrease in IRS-1 associated PI3K activity (Barbour et al.). GH excess caused by liver specific IGF-1 gene deletions also results in increased p85α content in skeletal muscle (Barbour et al., 2005). The increase in p85α appeared to be primarily be due to GH excess since a GH antagonist decreased p85α levels (Barbour et al., 2005). In white adipose tissue in mice with GH excess, p85α is also increased compared to GH insufficient mice or normal mice (Ricon et al., 2007). p85α was also increased acutely with GH administration to GH insufficient or GH sufficient mice (Ricon et al.). These data indicate that p85α plays an important role in GH induced insulin resistance. There are also multiple other common elements in the GH and insulin-signaling pathways that may contribute to GH induced insulin resistance. For example,
acute GH infusion during a hyperinsulinemic/euglycemic clamp procedure in healthy males induced insulin resistance, but did not affect IRS-1 associated PI3K activity, Akt content or activity, or GLUT4 content (Jessen et al., 2005). These results suggest that other signaling events may also be involved in GH-induced insulin resistance. There is also some suggestion that GH induces expression of suppressor of cytokine signaling (SOCS) 1 and 3, which may in turn inhibit insulin signaling (Vijayakumar et al., 2010). When taken in total, the available evidence indicates that while p85α may play a pivotal role in GH-induced insulin resistance, other intermediates in the insulin-signaling cascade may be affected as well. Given the insulin resistance often induced by GH, it is difficult to understand the cellular mechanisms of how GH administration could lead to improved blood glucose control in type 2 diabetes.

IGF-1 also shares multiple substrates with the insulin-signaling pathway (Figure 9). Despite this, IGF-1 does not seem to induce the insulin resistance that is observed with GH administration. IGF-1 itself can lower blood glucose concentrations (Guler, Zapf, & Froesch, 1987; Morrow, O’Brien, Moller, Filer, & Moses, 1994). This glucose lowering effect may be due to IGF-1 increasing insulin sensitivity (Clemmons 2002; Clemmons 2004; Hussain et al., 1995; Yakar et al., 2004). Some have postulated that IGF-1 helps offset some of the decreased insulin sensitivity associated with GH (Clemmons 2002, Clemmons 2004, Yakar et al., 2004). While both GH and IGF-1 share signaling intermediates with insulin, only GH appears to promote insulin resistance.

Interestingly, there is some evidence that GH may decrease blood glucose levels in diabetic mice (List et al., 2009). Similar results have been demonstrated in two
published human trials as well (Ahn et al. 2006; Nam et al., 2001). List et al. (2009) demonstrated that high doses of GH in C57BL/6J mice with diet-induced type 2 diabetes led to improvements in fasting glucose concentrations and glucose tolerance. They theorized that the improvements in glucose metabolism were due to increases in lean body mass (LBM) and decreases in fat mass associated with GH administration (List et al., 2009). What makes this research compelling is that independent of type 2 diabetes, GH is thought to be diabetogenic and induce insulin resistance as mentioned previously (Barbour et al., 2004; Clemmons et al., 1987; Christopher et al., 1998; Jessen et al., 2004; Kim et al., 1999; Pasarica et al., 2007; Snyder et al., 1990).

One of the potential mechanisms that may explain why GH can improve glucose disposal in type 2 diabetes is that GH may induce increases in LBM, particularly muscle mass. In theory, more muscle mass could lead to a greater potential for whole body glucose uptake since skeletal muscle is responsible a large proportion of total glucose disposal. If GH were able to preserve or even build LBM during weight loss, it would result in more favorable body composition. Given the detrimental health effects of obesity, improving body composition via GH therapy in addition to caloric restriction could theoretically optimize the health outcomes. In obese humans, multiple studies have demonstrated that 3-4 weeks of GH administration during caloric restriction resulted in attenuation of nitrogen loss compared to placebo (Clemmons et al., 1987; Snyder, Clemmons, & Underwood, 1988; Snyder, Clemmons, & Underwood, 1989; Snyder et al., 1990; Snyder, Underwood, & Clemmons, 1995). In theory, attenuation of nitrogen loss could lead to better retention of LBM while losing fat. As would be expected based on
these results, GH administration has also been shown to increase LBM or attenuate LBM loss associated with calorie restriction (Albert & Mooradian, 2004; Franco et al., 2005; Kim et al., 1999; Pasarcia et al., 2007; Tagliaferri et al., 1998; Thompson et al., 1998). Even with the favorable changes in LBM, the effects of GH on insulin sensitivity and glucose levels were variable with some trials showing no significant difference between GH and placebo and others showing elevated fasting insulin and glucose levels (Clemmons et al., 1987; Franco et al., 2005; Kim et al., 1999; Pasarica et al.; Snyder et al., 1990). These differential effects of GH on insulin sensitivity may be due to the time of administration of GH. Johannsson et al. (1997) found GH administration in males with abdominal obesity decreased glucose disposal after 6 weeks, but was similar to placebo administration after 9 months. Similarly, improved insulin sensitivity has been demonstrated in post-menopausal women with abdominal obesity given GH for 12 months compared to placebo administration (Franco et al., 2005). It appears that short-term GH administration may indeed induce insulin resistance, but longer courses of GH therapy may improve insulin sensitivity or at least not have a detrimental effect.

Muscle Fiber Type and Type 2 DM

There is also some thought that type 2 diabetes may be related to muscle fiber type composition (Daugaard & Richter, 2001). While MHC proteins are not responsible for glucose transport, some of the properties of particular fiber types are associated with differences in glucose transport. For instance, there is some evidence that suggests that there is increased GLUT4 expression in red-muscle, which typically has a higher proportion of type I muscle fibers (Daugaard & Richter; Gaster et al., 2001). In healthy
subjects, Gaster et al. (2000) found a 13-38% greater GLUT4 density in slow fibers versus fast fibers. In sedentary humans, Stuart et al. (2010) also found a 37% greater GLUT4 content in type I fibers compared to type II fibers. GLUT4 content located at the cell membrane may also be greater in slower fiber types. There is also evidence of increased GLUT4 content in the cell membranes of type I and IIA fibers compared to IIB fibers in the quadriceps, gastrocnemius, and soleus muscles of rats (Marette et al., 1992). GLUT4 content was greater in these fiber types both at rest and with insulin stimulation (Marette et al.). Glucose transport is also greater in muscles with a predominantly slower fiber makeup. There appears to be not only greater GLUT4 content but also increased glucose uptake in the deep portion of mouse gastrocnemius muscle compared to the more superficial portion (Hayasaki, Shimada, Kanbara, & Watanabe, 2001). Glucose uptake in the middle portion of the gastrocnemius muscle also appears fiber type dependent (Hayasaki et al.). The proportion of type I fibers in the mouse gastrocnemius muscle increases from superficial to deep, and thus there was greater glucose transport with a slower fiber type profile (Hayasaki et al.). Others have reported similar results with ~2 times greater glucose transport in the red gastrocnemius muscle compared to the white gastrocnemius muscle in rats (Goodyear, Hirshman, Smith, & Horton, 1991). There is also evidence of increased glucose transport both at rest and with insulin stimulation in the soleus muscle of Wistar rats, which has a slower fiber type profile, compared to the plantaris and extensor digitorum longus (EDL) muscles, which have faster fiber type profiles (Bonen, Tan, & Watson-Wright, 1981). In humans, some have found a similar relationship between increased type I muscle fibers and insulin sensitivity and glucose
uptake (Hoeg et al., 2009). Females had a greater type I percentage area in the vastus lateralis compared to males. The females also displayed 22% greater whole-body insulin sensitivity and a 29% greater leg glucose uptake than the males (Hoeg et al.). It is noteworthy that the higher insulin sensitivity could not be explained by GLUT4 content, insulin receptor expression, Akt expression, or Akt phosphorylation (Hoeg et al.). Thus, it appears that insulin sensitivity may be related to type I fiber expression, while the cellular mechanism behind the association is not completely clear. Given this tendency toward greater glucose transport in muscle with a slower fiber type profile, it seems that a shift toward a slower fiber type profile may be beneficial for the prevention or treatment of type 2 diabetes.

It is also possible that insulin sensitivity is related to the oxidative capacity of muscle. For example, increased glucose disposal has been shown in highly oxidative fibers compared to less oxidative fibers of Wistar rats (James, Jenkins, & Kraegen, 1985). Oxidative enzyme activity in different muscle fiber types follows a general trend with type I fiber being the most oxidative and IIB the least, but there is overlap between fiber types, particularly amongst the fast fiber subtypes (IIA, IID, IIB) (Pette, Peuker, & Staron, 1999). Lillioja et al. (1987) found a significant correlation between insulin action and muscle fiber type with type I fibers associated with greater insulin action and type IIB fibers associated with decreased insulin action. While the mechanisms behind the interplay between muscle fiber type and insulin sensitivity are not completely understood, the relationship between the two warrants further investigation.
While muscle fiber type may be related to insulin sensitivity, it is unclear if alterations in muscle fiber type expression play a significant role in the development of type 2 diabetes. The evidence regarding any alterations in fiber type or MHC expression relating to type 2 diabetes is ambiguous. Some evidence suggests alterations in muscle fiber type composition with type 2 diabetes while other research does not indicate a relationship between muscle fiber type and type 2 diabetes. Some have reported that obese humans or those with type 2 diabetes have a greater proportion of fast-twitch fibers and a lower percentage of slow-twitch fibers (Gaster et al., 2001; Oberbach et al., 2006). Utilizing metabolic enzyme based fiber typing, Oberbach et al. found a 16% decrease in the relative frequency of slow-oxidative fibers and a 49% increase in the relative frequency of fast glycolytic fibers in the vastus lateralis muscle of those with type 2 diabetes compared to control subjects. Conversely, other research has found no difference in muscle fiber type of the vastus lateralis muscle determined by mATPase histochemistry between humans that were lean, obese, or had type 2 diabetes (He et al., 2001). There is also evidence that blood glucose control does not directly influence muscle fiber type. No significant change in muscle fiber type composition was noted in the vastus lateralis muscle of humans after insulin treatment for poorly controlled type 2 diabetes (Cederholm, Sylven, Esbjornsson-Liljedahl, & Jansson, 2000). It should be noted that in some cases fiber type differences could be related to differences in physical activity levels, as a sedentary lifestyle may be a contributing factor to development of type 2 diabetes. Untrained or sedentary subjects typically have a faster muscle fiber type profile than active or trained subjects within the fast-twitch fiber subtypes (Staron et al.,
Muscle fiber classification methods may also influence differences detected with type 2 diabetes. Metabolic enzyme activity can vary within the same MHC-based fiber type and thus, results may vary when comparing fiber type profile when using different methods. Regardless of the role of muscle fiber type in the development of type 2 diabetes, shifts toward an overall slower fiber type profile could be beneficial in treating the disease assuming a concomitant increase in glucose uptake with a slower fiber type profile.

Potential mediators or mechanisms that are linked to both MHC expression and diabetes are calcineurin and/or PGC-1α. As mentioned previously, GH has been linked to increased expression of both calcineurin and PGC-1α (Vescovo et al., 2005). Conversely, diabetes has been linked to decreases in calcineurin and PGC-1α (Bonen 2009; Olesen, Kiilerich, & Pilegaard, 2010; Roberts-Wilson et al., 2010). More specifically, Barres et al. (2009) found that humans with type 2 diabetes have more PGC-1α methylation than non-diabetics. PGC-1α also appears to play a role in stimulating mitochondrial biogenesis and insulin production (Russell 2005). It appears increases in PGC-1α expression within normal physiological limits increase insulin sensitivity, while increases to supra-physiological levels do not have further benefit (Lira, Benton, Yan, & Bonen, 2010). PGC-1α expression also increases with chronic exercise and appears to play a role in the favorable metabolic adaptations associated with exercise (Lira et al., 2010; Russell) (see Figure 10 for an overview of PGC-1α related functions and insulin resistance). This suggests that expression or bioavailability of calcineurin or PGC-1α
may affect both insulin resistance and muscle fiber type expression and thus provide a link between the two.

**Figure 10.** Overview of PGC-1α functions related to insulin resistance and exercise. PGC-1α is involved in many processes that could favorably affect type 2 diabetes including mitochondrial biogenesis, decreased muscle uptake of free fatty acids, and promoting a type I muscle phenotype. Adapted from Russell 2005.

Like muscle fiber type composition, muscle fiber CSA also may not change with type 2 diabetes. There appear to be no significant differences in muscle fiber CSA in the vastus lateralis between obese subjects with type 2 diabetes, obese subjects without type 2 diabetes, and lean subjects without type 2 diabetes (Gaster et al. (2001). Similarly, there may not be any significant differences in muscle fiber CSA in any fiber type in the vastus lateralis muscle between poorly controlled diabetics and non-diabetic control...
subjects (Cederholm et al., 2000). Interestingly, insulin treatment of the diabetics lead to significant increases in muscle fiber CSA in type IIA, IIB, and IIC fibers (Cederholm et al.). Even with this change, the diabetic subjects did not differ significantly in muscle fiber CSA from the control subjects after insulin treatment (Cederholm et al.).

Muscle Fibers and GH or IGF-1

There have been conflicting reports regarding the effects of GH and IGF-1 on muscle fiber type and MHC isoform expression. There is evidence that suggests GH may cause shifts to a slower MHC profile or may result in no change in MHC profile (Daugaard et al., 1999; Daugaard et al., 1998, Everitt et al., 1996; Florini & Ewton, 1989; Hikida et al., 1995; Schuenke, et al., 2008). Interpretation is further complicated by the use of different animals, which makes comparisons difficult (Daugaard et al., 1999; Daugaard et al., 1998; Everitt et al.; Florini & Ewton; Hikida et al., 1995; Schuenke et al.). In many of the animals used it is difficult to separate the effects of GH from IGF-1. GH is known to induce IGF-1 gene expression and secretion in a variety of tissue including skeletal muscle (Dominici et al., 2005).

In a mouse strain with bovine GH (bGH) over-expression, Schuenke et al. (2008) found a shift toward a slower fiber type profile in the soleus, plantaris, and gastrocnemius muscles (see Table 2 for a summary of the fiber type results from Schuenke et al.). Similarly, administration of GH to GH-deficient rats resulted in an increase in expression of MHC Iβ and decreased expression of MHC IIa in the soleus muscle compared to GH deficient rats with placebo administration (Daugaard et al., 1998). This essentially “normalized” the expression of MHC in the soleus muscle similar to that of control rats.
that were not GH deficient (Daugaard et al., 1998). Interestingly, the same study found that GH administration had no effect on the MHC expression of the EDL (Daugaard et al., 1998). In another study examining the tibialis anterior muscle of rats with congestive heart failure (CHF), administration of high doses of GH attenuated the decrease in MHC IIa content and increase in MHC IIb content associated with CHF (Libera et al., 2004). Similarly, GH administration to hypophysectomized rats attenuated a shift away from type I fibers in the soleus and EDL muscles (Ayling et al., 1989). Again this seems to suggest that GH causes a fast to slow shift in MHC profile and muscle fiber type or at least maintains slow fiber type expression.
Table 2.

Summary of Muscle Fiber Type Data from Schuenke et al. (2008)

<table>
<thead>
<tr>
<th>Muscle fiber type percentages (mean values ± SD)</th>
<th>%I</th>
<th>%IC</th>
<th>%IIC</th>
<th>%IIA</th>
<th>%IID</th>
<th>%IID</th>
<th>%IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantaris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bGH</td>
<td>7±4.3*</td>
<td>1.6±1.1</td>
<td>1.2±0.8*</td>
<td>14±4.4*</td>
<td>5±1.8</td>
<td>31±4.0</td>
<td>22±5.0</td>
</tr>
<tr>
<td>NT</td>
<td>1±1.1</td>
<td>0.4±0.6</td>
<td>0.4±0.6</td>
<td>21±5.9</td>
<td>6±1.6</td>
<td>29±3.5</td>
<td>22±2.7</td>
</tr>
<tr>
<td>GHR -/-</td>
<td>1±0.9</td>
<td>0.5±0.5</td>
<td>0.0</td>
<td>17±5.0</td>
<td>8±2.0*</td>
<td>32±2.0*</td>
<td>22±3.4</td>
</tr>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bGH</td>
<td>62±5.0*</td>
<td>4.3±2.8</td>
<td>2.1±1.2*</td>
<td>22±4.2*</td>
<td>5±3.2*</td>
<td>5±6.0</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>NT</td>
<td>40±6.8</td>
<td>2.8±1.3</td>
<td>1.2±0.7</td>
<td>37±6.9</td>
<td>14±3.1</td>
<td>5±3.5</td>
<td>0.0±0.1</td>
</tr>
<tr>
<td>GHR -/-</td>
<td>39±4.8</td>
<td>2.3±1.0</td>
<td>0.8±0.4*</td>
<td>37±2.4</td>
<td>17±3.2</td>
<td>4±3.3</td>
<td>0.0</td>
</tr>
<tr>
<td>GDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bGH</td>
<td>18±4.4*</td>
<td>1.7±0.8</td>
<td>1.0±0.8</td>
<td>17±4.4</td>
<td>7±2.2</td>
<td>35±6.3</td>
<td>12±3.9</td>
</tr>
<tr>
<td>NT</td>
<td>11±2.1</td>
<td>1.6±1.0</td>
<td>1.1±1.2</td>
<td>19±3.7</td>
<td>8±2.3</td>
<td>33±5.1</td>
<td>14±3.2</td>
</tr>
<tr>
<td>GHR -/-</td>
<td>11±4.6</td>
<td>0.6±0.3*</td>
<td>0.4±0.5</td>
<td>19±6.6</td>
<td>11±3.8</td>
<td>32±5.8</td>
<td>14±2.2</td>
</tr>
<tr>
<td>GSW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bGH</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1±0.3</td>
<td>0.4±0.4</td>
<td>11±3.1*</td>
<td>22±3.7</td>
</tr>
<tr>
<td>NT</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1±0.3</td>
<td>5±2.4</td>
<td>22±3.5</td>
</tr>
<tr>
<td>GHR -/-</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2±0.6</td>
<td>6±2.8</td>
<td>25±3.4*</td>
</tr>
</tbody>
</table>

* Mean values are significantly different from NT (p<0.05)

GDR = deep red gastrocnemius; GSW = superficial white gastrocnemius; bGH = bovine GH over-expression group; NT = non-transgenic mice; GHR -/- = GH receptor deficient mice.

Other animals with increases in muscle GH exposure have found no changes in muscle fiber type or MHC expression. For example, Hikida et al. (1995) used a transgenic mouse strain with altered bGH expression to produce giant, larger than normal, and dwarf mice and compared them to non-transgenic mice. They found no significant differences in muscle fiber type profile in the soleus or gastrocnemius muscles between any of the groups (Hikida et al., 1995). There is also evidence that administration of GH to normal rats does not significantly alter muscle fiber type in the
soleus, EDL, or diaphragm muscles (Florini & Ewton, 1989). GH administration for 72 hours did not alter fiber type in the diaphragm muscle of nutrient-deprived rats and normally fed rats (Lewis, Feinberg, & Fournier, 1998; Lewis, LoRusso, & Fournier, 1997a; Lewis LoRusso, & Fournier, 1997b). GH has also been shown to have no effect on fiber type profile in the soleus muscle of rats that underwent hindlimb suspension (Roy et al., 1996). In humans with GH deficiency, the data suggest that GH treatment does not significantly alter muscle fiber type profile in the vastus lateralis muscle (Daugaard et al., 1999). It is difficult to determine exactly why there is conflicting evidence regarding the effects of GH on muscle fiber type. This discrepancy could be due to a number of factors including species, alterations in other hormones, age or gender of the animals, method of GH exposure (injection vs. gene over-expression), duration of dosing, and timing of dosing (see Table 3 for a summary of research regarding GH and muscle fiber type).
Table 3.

Summary of GH and MHC/fiber type Research

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal model</th>
<th>Hormone Administration</th>
<th>Muscle</th>
<th>Fiber Type or MHC Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roy et al. 1996</td>
<td>HS rats</td>
<td>Injection</td>
<td>Soleus</td>
<td>No Change</td>
</tr>
<tr>
<td>Schuenke et al. 2008</td>
<td>Mice</td>
<td>bGH over-expression</td>
<td>Soleus, plantaris, DRG, SWG</td>
<td>Slower</td>
</tr>
<tr>
<td>Daugaard et al. 1998</td>
<td>GHD rats</td>
<td>Injection</td>
<td>Soleus</td>
<td>Slower</td>
</tr>
<tr>
<td>Lewis et al. 1997a</td>
<td>ND rats</td>
<td>Injection</td>
<td>Diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Daugaard et al. 1999</td>
<td>GHD humans</td>
<td>Injection</td>
<td>Vastus lateralis</td>
<td>No Change</td>
</tr>
<tr>
<td>Lewis et al. 1997b</td>
<td>Rats</td>
<td>Injection</td>
<td>Diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Libera et al. 2004</td>
<td>Rats with CHF</td>
<td>Injection</td>
<td>Tibialis anterior</td>
<td>Slower</td>
</tr>
<tr>
<td>Ayling et al. 1989</td>
<td>HS rats</td>
<td>Injection</td>
<td>Soleus, EDL</td>
<td>Slower</td>
</tr>
<tr>
<td>Florini &amp; Ewton 1989</td>
<td>Rats</td>
<td>Injection</td>
<td>Soleus, EDL, diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Aroniadou et al. 1996</td>
<td>Rats</td>
<td>Injection</td>
<td>Soleus, rectus femoris</td>
<td>No Change</td>
</tr>
<tr>
<td>Hikida et al. 1995</td>
<td>Mice</td>
<td>bGH over-expression</td>
<td>Soleus, gastrocnemius</td>
<td>No Change</td>
</tr>
</tbody>
</table>

HS= hypophysectomized; GHD= GH deficient; ND= nutrient deprived; CHF= congestive heart failure

Increases in GH concentration generally appear to enhance overall muscle growth and size parameters (Daugaard et al., 1999; Daugaard et al., 1998; Everitt et al., 1996; Hikida et al., 1995; Kim et al., 2005; Libera et al., 2004; McCall et al., 1998; Schuenke et al., 2008). In most cases, GH administration or GH over-expression increases overall LBM and/or muscle weight of specifically identified muscles (Daugaard et al., 1999; Daugaard et al., 1998; Everitt et al.; Hikida et al., 1995; Kim et al., 2005; Libera et al.; McCall et al.; Schuenke et al.). More specifically it appears the GH may increase muscle fiber CSA if present in adequate concentrations (Everitt et al.; Hikida et al., 1995; Kim et al., 2005; McCall et al.; Schuenke et al.). There is some evidence that GH must be
administered or expressed above a certain threshold to induce significant increases in muscle weight or muscle fiber CSA (Hikida et al., 1995).

It should be noted that some believe that the increases in LBM associated with GH are not primarily due to actual muscle hypertrophy, but chiefly due to excess fluid retention (Ehrnborg, Ellegard, Bosaeust, Bengtsson, & Rosen, 2005; Llu et al., 2008). Depending on the body composition method used, fluid volume expansion may be interpreted as increased LBM in some cases. For example, common body composition methods such as dual energy x-ray absorptometry (DEXA) and bioimpedance may not be valid in populations with abnormal tissue fluid distribution such as those with GH deficiency or excess (Moller, 2003). There is evidence that GH-deficient humans have decreased extra-cellular water (ECW) levels that increase with GH replacement therapy (Bengtsson et al., 1993; Carrol et al., 1998; Moller, 2003). GH administration to non-GH deficient humans has also demonstrated increases in ECW (Ehrnborg et al.; Llu et al.; Meinehardt et al., 2010; Moller, 2003). GH has been shown to increase ECW significantly in recreational athletes but increases in body cell mass were not significant (Meinehardt et al.). Therefore it appeared the increase in LBM was primarily due to increased ECW (Meinehardt et al.).

However, these results should be interpreted with caution. Potential limitations of the research regarding GH and body composition are the length of GH administration and dosages provided. Providing GH for 4-8 weeks, as in some studies, may not be sufficient to induce muscle hypertrophy (Ehrnborg et al. 2005; Meinehardt et al., 2010). As noted earlier, GH has been shown to increase muscle fiber CSA and therefore not all
hypertrophy is due to ECW expansion (Everitt et al., 1996; Hikida et al., 1995; Kim et al., 2005; McCall et al., 1998; Schuenke et al., 2008).

One difficulty in interpreting the effects of GH on muscle characteristics is that it is often impossible to separate the effects of GH from IGF-1 in the animal used. Increases in GH concentration are typically accompanied by increases in IGF-1 concentrations because GH binding to GH receptors results in the expression of IGF-1 in multiple tissues, including the liver and skeletal muscle (Dominici et al., 2005). Thus, it is difficult to differentiate the independent actions of GH from IGF-1 on muscle without blocking the expression or action of IGF-1.

There is also some evidence available regarding the effects of IGF-1 administration on muscle characteristics. Because many of the outcomes of GH administration are modulated by IGF-1, it is plausible to expect the effects of IGF-1 administration on muscle to be very similar to those of GH administration. Thus, IGF-1 treatment may be predicted to promote increases in LBM and muscle fiber CSA similar to GH administration if the effects on muscle were mediated by IGF-1. Any effect on muscle fiber type would be difficult to predict given the variable effects of GH (Daugaard et al., 1999; Daugaard et al., 1998; Florini & Ewton, 1989; Hikida et al., 1995; Schuenke et al., 2008) (see Table 4 for a summary of the effects of IGF-1 on muscle fiber type or MHC content).
Summary of IGF-1 and MHC/fiber type

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal model</th>
<th>Hormone Administration</th>
<th>Muscle</th>
<th>Fiber Type or MHC Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roy et al. 1996</td>
<td>HS rats</td>
<td>Injection</td>
<td>Soleus</td>
<td>No Change</td>
</tr>
<tr>
<td>Lynch et al. 2001</td>
<td>Laminin def. mice</td>
<td>Injection</td>
<td>Soleus, EDL</td>
<td>Faster, slower respectively</td>
</tr>
<tr>
<td>Lewis et al. 1997a</td>
<td>ND rats</td>
<td>Injection</td>
<td>Diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Lewis et al. 1997b</td>
<td>Rats</td>
<td>Injection</td>
<td>Diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Lewis et al. 1998</td>
<td>Rats</td>
<td>Injection</td>
<td>Diaphragm</td>
<td>No Change</td>
</tr>
<tr>
<td>Musaro et al. 2001</td>
<td>Mice</td>
<td>IGF-1 over-expression</td>
<td>EDL</td>
<td>Faster</td>
</tr>
<tr>
<td>Song et al. 2005</td>
<td>Mice</td>
<td>IGF-1 over-expression</td>
<td>Gastrocnemius</td>
<td>Faster</td>
</tr>
</tbody>
</table>

HS= hypophysectomized; ND= nutrient deprived

Like GH, the effects of IGF-1 on muscle fiber type have been inconsistent. Some evidence suggests that IGF-1 does not alter muscle fiber type (Lewis et al., 1998; Lewis et al., 1997a; Lewis et al., 1997b). In nutrient-deprived adolescent rats that were administered IGF-1 for 72 hours, IGF-1 had no significant impact on muscle fiber type profile in the diaphragm muscle (Lewis et al., 1997a). The same group also found that IGF-1 had no effect on muscle fiber type composition in the diaphragm muscle of well-nourished adolescent rats (Lewis et al., 1998). It is questionable whether 72 hours is a sufficient time of hormone exposure to manifest in fiber type changes. Similarly, IGF-1 administration to rats undergoing hindlimb suspension does not affect fiber type profile in the soleus muscle (Roy et al., 1996).

Conversely, others have found that IGF-1 may alter fiber type composition in soleus and EDL muscles of mice (Lynch et al., 2001). The administration of IGF-1 for four weeks resulted in a slower muscle fiber type profile with an increase in the
proportion of type I fibers in the EDL (Lynch et al.). They also observed a shift to a faster muscle fiber type profile with an increase in the proportion of type IIA fibers and decrease in type I fibers in the soleus muscle of the mice given IGF-1 (Lynch et al.). The predominantly fast EDL shifted to a slightly slower fiber type profile, while the predominantly slow soleus shift toward a faster fiber type profile (Lynch et al.). Conversely, overexpression of IGF-1 may result in a faster muscle fiber type profile in the EDL compared to wild-type mice (Musaro et al., 2001). A similar trend in the gastrocnemius muscle toward increased IID and IIB fibers in mice with IGF-1 overexpression compared to wild type littermates has also been noted (Song et al., 2005). These results make it difficult to interpret whether IGF-1 exposure leads to consistent alterations in muscle fiber type profile.

With the conflicting data, it becomes difficult to extrapolate and make generalizations regarding the effects of IGF-1 on muscle fiber type. The short duration of IGF-1 administration makes the results from some of the experiments somewhat questionable regarding fiber type transformations (Lewis et al., 1997a; Lewis et al., 1997b; Lewis et al., 1998). It is questionable if only 72 hours of IGF-1 administration would have any significant effect on muscle fiber type based on MHC histochemical properties. While others administered IGF-1 for a much longer time period (4 weeks), they found different effects depending on the muscle being examined (Lynch et al., 2001). Again, this makes generalizations more difficult.

Similar to GH, IGF-1 appears to increase both muscle weight and muscle fiber CSA in certain situations (Lewis et al., 1998; Lewis et al., 1997a; Lewis et al., 1997b;
Lynch et al., 2001; Musaro et al., 2001; Roy et al., 1996). In a series of publications examining the effects of IGF-1 on diaphragm muscle properties in rats, Lewis et al. (1997b, 1998) noted that administration of IGF-1 for 72 hours attenuated decreases in muscle fiber CSA due to nutrient deprivation. The same group also found that 72 hours of IGF-1 administration increases diaphragm muscle mass in normally fed adolescent rats (Lewis et al., 1997a). Increases in muscle weight in a variety of muscles including pectoralis, soleus, EDL, and gastrocnemius muscles in transgenic mice with enhanced IGF-1 expression have been noted as well (Musaro et al.). IGF-1 also seems to increase both muscle mass and muscle fiber CSA in the soleus and EDL muscles of dystrophic mice (Lynch et al.).

In some cases, both GH and IGF-1 have been administered simultaneously. In most instances, the results were not different than simply giving GH or IGF-1 individually (Lewis et al., 1998; Lewis et al., 1997a; Lewis et al., 1997b; McCall et al., 1998; Roy et al., 1996). As with GH and IGF-1 given individually, GH and IGF-1 given simultaneously increased muscle fiber CSA in the diaphragm, but had no effect on muscle fiber type profile in both nutrient deprived and normally fed rats (Lewis et al., 1998; Lewis et al., 1997a; Lewis et al., 1997b). There also did not appear to be any additive or synergistic effects between GH and IGF-1 (Lewis et al., 1998). Similarly, a combination of GH and IGF-1 given to rats with functionally overloaded soleus muscles resulted in a significantly greater increase in soleus muscle wet weight and fiber CSA compared to functional overload alone (McCall et al.). Similar effects have been found with GH and IGF-1 in maintaining muscle mass in hindlimb suspended rats (Roy et al.,
1996). Overall, the effects of GH and IGF-1 on skeletal muscle appear to be relatively similar, although it is difficult to isolate the effects of GH from IGF-1. More recent mouse models, such as those with disrupted GH receptor genes and IGF-1 gene disruption, may help better pinpoint the individual effects of GH and IGF-1 in the future (Clark, Schuenke, Keeton, Staron, & Kopchick, 2006).

There does not currently appear to be any data regarding interaction between GH or IGF-1 exposure and type 2 diabetes in regards to muscle characteristics. While GH has been shown to increase LBM in C57BL/6J mice with diet-induced diabetes, specific characteristics of that muscle have not been examined (List et al., 2009). Novel findings in this area could help clarify a number of questions regarding the relationship between muscle fiber type and CSA and type 2 diabetes, GH, and IGF-1. It could help elucidate if muscle fiber type shifts are related to the development of diet-induced type 2 diabetes. Studying muscle fiber characteristics in the context of GH treatment of type 2 diabetes could also provide novel insight into potential mechanisms of improved glucose tolerance with GH. If increases in overall muscle mass are preferentially from increases in the percentage or CSA of type I fibers, which tend to have higher GLUT4 content and greater glucose transport capability than faster fiber types, it may explain some of the improvements in glucose tolerance with GH administration. Given that many of the effects of GH on skeletal muscle are thought to be mediated via IGF-1, examining muscle characteristics in diabetic mice given IGF-1 could help clarify some of the independent effects of GH and IGF-1. This research could also provide insight into whether muscle fiber type transformations in general could play a role in future treatment options for type
2 diabetes. Research regarding the effects on muscle fiber type and muscle fiber CSA with both GH and IGF-1 administration in type 2 diabetes would provide novel data regarding the interaction of GH and IGF-1 and type 2 diabetes on skeletal muscle. Examining muscle fiber type and muscle fiber CSA could also provide a more complete picture regarding the glucose lowering effects of GH in type 2 diabetes.

**Specific Aims**

The relationship between muscle fiber type and type 2 diabetes, GH, and IGF-1 has not been fully elucidated. Given the recent data regarding the effects of GH over-expression on muscle fiber type and the beneficial effects of GH administration on type 2 diabetes, examining muscle fiber type and muscle fiber CSA could provide a more complete picture regarding the physiological events related to the beneficial action of GH. (Ahn et al., 2001; List et al., 2009; Nam et al., 2006; Schuenke et al., 2008). Because GH over-expression may lead to a shift toward a slower muscle fiber type profile and GH improves glucose tolerance, we hypothesized that GH administration may lead to a slower muscle fiber type profile in mice with type 2 diabetes. Because some of the effects of GH are mediated by IGF-1, administering IGF-1 to diabetic mice should help elucidate the effects of GH versus IGF-1 (Dominici et al., 2005). The present research investigated the relationship between GH and/or IGF-1 treatment on muscle fiber type composition and muscle fiber CSA in diabetic mice.

The specific aims of the study were as follows:
1. To determine the effects of type 2 diabetes on skeletal muscle fiber type composition and CSA in the soleus, plantaris, deep red gastrocnemius (DRG), and superficial white gastrocnemius (SWG) muscles.

2. To determine the effects of GH administration in diabetic mice on skeletal muscle fiber type composition and CSA in the soleus, plantaris, DRG, and SWG muscles.

3. To determine the effects of IGF-1 administration in diabetic mice on skeletal muscle fiber type composition and CSA in the soleus, plantaris, DRG, and SWG muscles.

4. To determine the effects of simultaneous administration of both GH and IGF-1 in diabetic mice on skeletal muscle fiber type composition and CSA in the soleus, plantaris, DRG, SWG muscles.

The following null hypotheses were investigated:

H$_{01}$: The wet weight of the triceps surae muscle group does not differ significantly between Control, DM, GH, IGF-1, and Combo mice.

H$_{02}$: The fiber type composition of the soleus muscle does not significantly differ between Control, DM, GH, IGF-1, and Combo mice.

H$_{03}$: The fiber type composition of the plantaris muscle does not significantly differ between Control, DM, GH, IGF-1, and Combo mice.

H$_{04}$: The fiber type composition of the DRG does not significantly differ between Control, DM, GH, IGF-1, and Combo mice.
H05: The fiber type composition of the SWG does not significantly differ between Control, DM, GH, IGF-1, and Combo mice.

H06: The muscle fiber CSA does not differ, within each fiber type, for the soleus muscle between Control, DM, GH, IGF-1, and Combo mice.

H07: The muscle fiber CSA does not differ, within each fiber type, for the plantaris muscle between Control, DM, GH, IGF-1, and Combo mice.

H08: The muscle fiber CSA does not differ, within each fiber type, for the DRG between Control, DM, GH, IGF-1, and Combo mice.

H09: The muscle fiber CSA does not differ, within each fiber type, for the SWG between Control, DM, GH, IGF-1, and Combo mice.
CHAPTER 2: METHODS

Animals

The animal procurement, housing, testing, and hormone treatment described below were conducted as part of an investigation by Dr. Ed List and associates at the Konneker Research Center at Ohio University. C57BL/6J mice were housed at the Konneker Research Center and fed a high-fat diet to induce obesity and type 2 diabetes as had been previously demonstrated (Berryman et al., 2006; List et al., 2007; List et al., 2009; Kelder et al., 2007; Okada, List, Sankaran, & Kopchick, 2010; Wilde et al., 2008) (see Figure 11 for sample pictures of normal, obese, and bGH overexpression mice). The high-fat diet produces an animal model of type 2 diabetes that is characterized by high levels of adiposity and insulin resistance, which closely resembles human type 2 diabetes. With a high-fat diet, this mouse strain has demonstrated an increase in glucose concentrations from ~7 mmol/L to ~11 mmol/L and in insulin from ~100 pmol/L to ~500 pmol/L (List et al., 2009). Dr. List procured 49 male C57BL/6J mice at 21 days of age and split them into 5 groups of 9-10 mice. Groups included control (Control group; n=10), diabetic (DM group; n=10), diabetic plus GH (GH group; n=10), diabetic plus IGF-1 (IGF-1 group; n=10), and diabetic plus GH and IGF-1 (Combo group; n=9). Calculations utilizing muscle fiber type percentages and mean muscle fiber CSA data from similar research by Schuenke et al. (2008) showed statistical power of 80% or greater with a sample size of 9-10 animals per group.
Figure 11. Sample Pictures of Mice. Control = lean control; DM = high-fat diet; bGH = bovine GH overexpression transgenic mouse. While bGH mice were not used in the current investigation, it does provide some insight into the effects of increased GH exposure in mice. Pictures courtesy of Dr. Ed List and Dr. John Kopchick.

The Control group was fed standard laboratory rodent chow (ProLab RMH 3000; LabDiet, Richmond, VA, USA) that provided 60% of calories from carbohydrate, 26% from protein, and 14% from fat. The remaining groups were fed a high fat diet (D12492; Research Diets, New Brunswick, NJ, USA) that provided 20% of calories from carbohydrate, 20% from protein, and 60% from fat for 16 weeks prior to hormone treatment. The mice remained on their respective diets throughout the duration of the study. All animal handling procedures were done in accordance with local, state, and federal guidelines and were approved by the Ohio University Institutional Animal Care and Use Committee.
Study Overview

All groups were on their respective diets for 16 weeks prior to administration of GH, IGF-1, or combination treatment. For those on the high-fat diet, this time frame has previously been shown to induce type 2 diabetes (List et al., 2009). Following 16 weeks of feeding, each group received twice-daily injections of hormone treatment or phosphate buffered saline (PBS) placebo for 3 weeks (see Figure 12 for overview). During this time the groups stayed on their respective diets. Following the three weeks of injections, the mice were sacrificed and tissues were collected.

![Figure 12](image_url)

*Figure 12*. Study overview of research performed by Dr. Ed List and associates. Each group was on its respective diet for 16 weeks then received hormone or placebo injections for 3 weeks while continuing on the same diet. HF= high-fat diet; LF= low-fat diet; GTT= glucose tolerance test.
Hormone Treatment

As mentioned previously, GH, IGF-1, and combination therapy was started by Dr. List after 16 weeks on the high fat diet. Purified recombinant bovine GH was given as a gift from Monsanto (St Louis, MO, USA). GH powder was suspended in PBS and divided into single doses and stored at –80°C. Human recombinant IGF-1 solution was given as a gift from Tercica Incorporated (Brisbane, CA, USA). IGF-1 was diluted in PBS and divided into single doses and stored at –80°C. Individual doses were thawed just prior to use. GH was administered at a dose of 10 µg/g per day split into twice-daily doses (5 µg/g per dose) for three weeks. IGF-1 was administered at 8 µg/g per day split into twice-daily doses (4 µg/g per dose) for three weeks. The Combo group received GH (10 µg/g per day) combined with IGF-1 (8 µg/g per day) in twice daily doses. The Control and DM groups were injected with an equal volume of PBS on the same schedule as the hormone treated groups (i.e. twice-daily doses). This normalized any stress response associated with the injections.

Muscle Extraction and Processing

After completion of the 16-week diet period and 3-week hormone treatment period, the left triceps surae muscle group (gastrocnemius, soleus, and plantaris muscles) of each mouse was harvested as part of the tissue collection process with Dr. Ed List and associates upon sacrifice. The limb was first skinned to expose the underlying musculature. The triceps surae muscle group was then extracted and surrounding tissue removed. First the Achilles tendon was reflected to free the muscle group. The triceps
surae muscle group was then transected near the origin of the soleus muscle. The muscle sample was weighed, frozen in isopentane cooled in liquid nitrogen, and stored at -80°C. Later, the muscle samples were mounted in an OTC-tragacanth gum mixture at -22°C. The mounted muscle samples were serially sectioned in a -24°C cryostat into 12 µm thick sections and mounted on poly-L-lysine coated coverslips for mATPase histochemical and MHC immunohistochemical analyses. Three to four serial sections from at least two different muscle samples were mounted on each coverslip. Muscle sections were also arranged so that each coverslip contained samples from different treatment groups as a further control measure. The samples were air dried and then stored at -40°C until further processing.

mATPase Histochemistry

Myosin ATPase histochemistry was performed using previously published techniques (Brooke & Kaiser, 1970; Guth & Samaha, 1969; Guth & Samaha, 1970; Schuenke et al., 2008). Preincubation pH values of 4.1, 4.4, and 10.0 were utilized based on the acid and alkaline stability of the different muscle fiber types in mice established in previously published work (Brooke & Kaiser; Rowlerson, 1991; Schuenke et al.). For more detailed procedures and chemical composition of solutions see Appendix A. Briefly, coverslips that underwent alkaline preincubation were first fixed with a 10% formalin solution at 4°C and then preincubated at a pH of 10.0 for 20 minutes. Pilot work with muscle sections from other C57BL/6J mice demonstrated a better contrast in staining intensity from different fiber types with fixation in a buffered 10% formalin (3.7% formaldehyde) solution compared to the 5% formalin solution used in previously
published studies (Brooke & Kaiser, Schuenke et al.). The buffered 5% formalin solution yielded very dark staining that obscured the fibers in the plantaris and gastrocnemius muscles. The buffered 10% formalin yielded a lighter overall staining intensity that allowed better differentiation between fast fiber subtypes. When utilizing the acid (4.1) and intermediate (4.4) pH preincubations, coverslips were started with a 7-minute preincubation with no formalin fixation followed by a rinse in buffer at pH 7.8. After these preincubation steps, all procedures were identical. In summary, coverslips with consecutive sections were preincubated in their respective pH solutions (4.1, 4.4, and 10.0). After this, a series of incubations that include cobalt chloride and ammonium sulfide allow substitution reactions in fibers with remaining ATPase activity that ultimately results in a dark precipitate that yields different staining intensities in different muscle fiber types. The substitution reactions that yielded the dark precipitate staining exploited in the mATPase histochemistry procedure are summarized in Table 5. Finally, after dehydration and clearing, coverslips were mounted on glass slides with Canada Balsam with serial sections of a given muscle preincubated in all three pH values arranged successively. Microscopic views of each section under the three different preincubations were compared to determine the muscle fiber type composition.

Table 5.

<table>
<thead>
<tr>
<th>Substitution Reactions in mATPase Histochemistry</th>
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<tbody>
<tr>
<td>Reactions</td>
</tr>
<tr>
<td>$2\text{ATP} + 3\text{CaCl}_2 \rightarrow \text{Ca}_3(\text{PO}_4)_2$</td>
</tr>
<tr>
<td>$\text{Ca}_3(\text{PO}_4)_2 + 3\text{CoCl}_2 \rightarrow \text{Co}_3(\text{PO}_4)_2$</td>
</tr>
<tr>
<td>$\text{Co}_3(\text{PO}_4)_2 + 3(\text{NH}_4)_2\text{S} \rightarrow 3\text{CoS}$</td>
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</tbody>
</table>
Type I fibers maintain mATPase activity under acidic and intermediate preincubations, while mATPase activity is lost under alkaline preincubation conditions. This results in type I fibers staining dark with acid and intermediate preincubations while remaining unstained with alkaline preincubation. Conversely, type IIA fibers remain unstained with acid and intermediate preincubations, while staining dark with alkaline preincubation. Given that IC and IIC fibers are hybrids made up of MHC I and IIA, they have intermediate staining properties. IC fibers are relatively stable under acid conditions, but stain at intermediate intensity under alkaline conditions. IIC fibers are somewhat stable throughout the entire pH range. IID fibers are labile with acid preincubation, but stain moderately dark at intermediate pH and dark with alkaline preincubation. IIAD fibers stain less intensely at an intermediate pH than IID fibers, but are darker than IIA fibers. IIB fibers are labile with acid preincubation, but retain a small amount of mATPase activity at intermediate and alkaline pH. Thus IIB fibers stain lightly with intermediate and alkaline preincubations. Delineation of IIDB fibers requires close inspection of fibers as they stain slightly darker than IIB but slightly lighter than IID at intermediate and alkaline pH values. For a summary of the staining intensities of the continuum of fiber types at different preincubations and examples of stained muscle sections see Figure 13. Given the difficulty in delineating all the fast fiber subtypes and hybrids contained in small mammals, combining immunohistochemical analysis with mATPase histochemistry allowed a more precise fiber type delineation.
Figure 13. mATPase histochemistry. A. mATPase analysis of the deep red gastrocnemius muscle. B. Schematic representation of the mATPase histochemical fiber typing.
MHC Immunohistochemistry

Myosin immunohistochemical assays were utilized in conjunction with mATPase histochemistry to verify and elucidate the range of fiber types including I, IC, IIC, IIA, IIAD, IID, IIDB, and IIB. This procedure was done using M.O.M peroxidase kits (PK-2200)(Vector Laboratories, Burlingame, CA, USA) and antibodies A4.951 (anti-MHC I), N2.261 (anti-MHC I & IIa), A4.74 (anti-MHC IIa & IId), and F30 (anti-MHC IIb) from the Developmental Studies Hybridoma Bank at the University of Iowa (see Appendix B for specific steps and reagents). Using the specificity of the different antibodies, different fiber types were stained based on the antibody used.

For detailed laboratory procedures, see Appendix B. All steps were performed at room temperature. Briefly, coverslips were first re-hydrated in PBS. Samples were then treated with a blocking solution (100:1, methanol:H2O2) for 10 minutes followed by another PBS wash. Most subsequent steps in the process were separated by PBS washes as well. Next the slides were preincubated in MOM Ig block reagent for 60 minutes followed by incubation in the primary myosin antibody for 30 minutes. After another PBS wash, slides were treated with a secondary antibody solution, a PBS wash, and an ABC peroxidase reagent. After this the final staining was done with diaminobenzidine tetrahydrochloride (DAB). The procedure was completed with ethanol dehydration, a clearing reagent, and finally mounted on glass slides. The staining of muscle fibers treated with different primary antibodies was compared to discern fiber type profile using microscopic views. The antibody-treated muscle sections were also compared to serial sections of the same muscle previously stained using mATPase histochemistry to make
the final fiber type determinations (see Figure 14 for an example of immunohistochemical fiber typing).

Figure 14. Myosin immunohistochemistry from the deep red gastrocnemius muscle and schematic representation of immunohistochemical fiber typing. A = anti-MHC I (A4.951); B = anti-MHC I & IIa (N2.261); C = anti-MHC IIa & IId (A4.74); D = anti-MHC IIb (F30).
Determination of Muscle Fiber Type Composition

Triceps surae cross-sections that had been stained utilizing the methods described previously were examined microscopically to determine the location of the soleus, plantaris, DRG, and SWG muscles. The soleus muscle was identified by its central location, large proportion of type I fibers, and a clear border between it and the plantaris and gastrocnemius muscles. The plantaris muscle was identified adjacent to the soleus muscle, but clearly separated from the gastrocnemius. Subsequently, the gastrocnemius muscle was split into the DRG and SWG portions. The DRG was identified as the deepest portion of the gastrocnemius muscle and was typically the only portion containing the more oxidative fibers (type I or IIA). The SWG was identified as the most superficial area of the gastrocnemius containing predominantly type IIB fibers. The location of each muscle was also verified using an interactive anatomy atlas (National Institute of Medical Research; http://www.nimr.mrc.ac.uk/3dlimb/) of the mouse lower limb with a cross-sectional view.

Fiber type composition was determined utilizing a combination of mATPase histochemistry and MHC immunohistochemistry. Serial sections of each muscle were compared utilizing assays for mATPase activity following preincubations of pH 4.1, 4.4, and 10.0 and specific MHC antibodies A4.951, N2.261, A4.74, and F30. Digital pictures of a representative area of each muscle assayed at pre-incubation pH 4.4 were taken and then compared to the other staining assays. All fibers that were clearly visualized in each picture field were counted yielding a mean of 364 fibers per muscle. These fiber counts were used to calculate the percentage of each fiber type in each muscle.
Muscle Fiber CSA Measurement

Muscle fiber CSA of at least 50 fibers of each major fiber type per muscle was measured on the sections from mATPase histochemical staining using the NIH Image software program and a video-capture microscope. Pictures of each muscle section using a 6.3x magnification were used with a picture calibration slide of a known distance at the same magnification used to set the measurement scale. Individual muscle fibers were traced with digitizing software and CSA calculated for each of the major muscle fiber types. In muscle samples containing less than ~50 fibers of a particular fiber type, that fiber type was not included in statistical analysis. Any fibers that appeared to be sectioned at an oblique angle were not measured for muscle fiber CSA. Utilizing the aforementioned techniques allowed calculation of the percentage of each muscle fiber type for each muscle and CSA of each major muscle fiber type.

Other Measures

Other data were collected as part of the research conducted by Dr. Ed List’s group (see Figure 12). Prior to starting the respective diets, body weight and body composition were recorded for all groups. After the initial 16-week diet phase, prior to starting any hormone or placebo treatments, all groups were tested for body weight, body composition, blood glucose levels, blood insulin levels, and received an intraperitoneal glucose tolerance test (GTT). During the treatment phase, there were weekly measurements including body weight, body composition via a Bruker Minispec (The Woodlands, TX, USA), blood glucose, and plasma collection. There was also another GTT performed 3 weeks after treatment (see Figure 12). Upon sacrifice, other tissues
including fat pads, liver, additional muscle, kidney, heart, and spleen were collected by Dr. Ed List and associates for future analyses.

Statistics

Analysis of variance (ANOVA) was used to compare the effects of diet and hormone administration across groups. Multi-variate ANOVA was used to test for main effects with fiber type composition, percent fiber type area, and muscle wet weight. Univariate ANOVA was used to test for main effects with muscle fiber CSA. When significant main effects were detected, Tukey’s HSD was used for post-hoc analysis making pairwise comparisons between groups. Significance level for all tests was set at p = 0.05.
CHAPTER 3: RESULTS

Animal Characteristics

Because the triceps surae group represents only a portion of the total muscle mass in the mouse, the overall muscle growth parameters of the animal must be considered in the interpretation of muscle fiber type composition and muscle fiber CSA. Thus, the body composition data, as well as the triceps surae muscle group wet weight, provided a context for the interpretation of the other results. As part of his research, Dr. Ed List utilized a total of 49 C57BL/6J mice split into five groups as described previously. All body composition data including total body mass, LBM, fat mass, and fluid mass were provided by Dr. Ed List.

The most notable finding regarding body composition was the marked decrease in fat mass and increase in LBM in the GH and Combo groups compared to the DM, while the IGF-1 group had only a modest increase in LBM and no decrease in fat mass (Figure 15). The DM, GH, IGF-1, and Combo groups all had a significantly higher body mass and fat mass compared to the Control group. The IGF-1 group also had a significantly greater total body mass (p<0.05) than the GH group due to the IGF-1 group having a significantly greater fat mass (p<0.01). While all the other groups had a greater fat mass than the Control group (10% bodyfat), the GH group (18% bodyfat) and Combo group (19% bodyfat) had significantly less fat mass than the DM (37% bodyfat) and IGF-1 (35% bodyfat) groups. The hormone treated groups (GH, IGF-1, and Combo) all had significantly greater lean body mass LBM than the Control and DM groups. The IGF-1 group also had significantly less LBM (p<0.01) than the GH group, while the Combo
group had significantly more LBM than the GH (p<0.05) and IGF-1 (p<0.01) groups. The increase in LBM in the IGF-1 groups versus the DM group was only half that of the GH group versus the DM group (13% and 26% respectively). See Appendix C for the body composition raw data.

Figure 15. Mouse body composition performed via nuclear magnetic resonance. Data provided by Dr. Ed List. a= significantly different than Control group; b=significantly different than DM group; c=significantly different than GH group; d= significantly different than IGF-1 group

Muscle Wet Weight

The wet weight of the triceps surae was similar to LBM in that the treatment groups containing GH had the greatest hypertrophic response (Figure 16). The mean wet weight of the triceps surae muscle group was significantly greater in the GH (p<0.01) and Combo (p<0.01) groups compared to the Control group. The GH and Combo groups also...
had significantly greater triceps surae muscle wet weight than the DM and IGF-1 groups. Even with the increase in total body mass, the wet weight of the triceps surae in the DM group was not significantly different than the Control group. Even though not statistically significant, it should be noted that the magnitude of increase in the DM group versus the Control group (13%) was similar to that of the GH group versus the DM group (14%). While the hormone conditions containing GH (GH and Combo groups) had significantly greater triceps surae wet weight than the DM group, the IGF-1 group did not differ from the DM group. The Combo group also had a significantly greater triceps surae wet weight than the DM (p<0.05) and IGF-1 (p<0.01) groups. For a summary of the triceps surae muscle weight data see Figure 16.
Figure 16. Wet weight (g) of the right triceps surae muscle group (mean+SD). a = significantly different than Control group; b = significantly different than DM group; c = significantly different than GH group; d = significantly different than IGF-1 group.

Triceps surae wet weight was also calculated as a ratio of LBM to measure whether changes in triceps surae mass were proportional to changes in total LBM. The triceps surae wet weight to LBM ratio did not differ significantly between the Control, DM, and GH groups (Figure 17). The IGF-1 group had a significantly lower wet weight/LBM ratio than the DM group (p<0.05) and the Combo group was significantly lower than both the Control and DM groups (p<0.05). Based on these results, the increases in LBM in the IGF-1 and Combo groups were of a greater magnitude than the increase in wet weight of the triceps surae. See Appendix D for raw data of triceps surae wet weight and wet weight/LBM ratio.
Figure 17. Triceps surae muscle group wet weight to lean body mass ratio (mean±SD). LBM – lean body mass; a = significantly different than Control group; b = significantly different than DM group. LBM data provided by Dr. Ed List.

Because the triceps surae is a weight-bearing muscle group and there was a significant increase in total body mass with the high-fat diet, triceps surae wet weight was also calculated as a ratio of total body mass (see Figure 18). All of the groups receiving the high-fat diet (DM, GH, IGF-1, Combo) had a significantly lower triceps surae wet weight/body weight ratio than the Control group. This is likely due to a very large increase in total body mass due to increases in fat mass with a relatively smaller change in triceps surae weight (see Figures 15 and 16). Amongst the hormone-treated groups, the GH group had significantly greater wet weight/body weight ratio than the IGF-1 and DM groups. The Combo group ratio was significantly greater than the IGF-1 group, but not different than the GH and DM groups. See Appendix D for the raw data for the triceps surae wet weight/body weight ratio.
**Percent Muscle Fiber Type**

The percent fiber type data provided answers to the specific aims of the study regarding the effects of type 2 diabetes, GH, and IGF-1 on muscle fiber type composition. Overall, the DM group did not differ in muscle fiber type composition from the Control group (Figures 19, 20, 21, 22). GH treatment resulted in a fiber type shift toward type IIA fibers in the soleus muscle with a decrease in the proportion of type I and IIAD fibers (Figure 19). Overall, there was very little change in fiber type with GH treatment in the plantaris, DRG, and SWG muscles (Figures 20, 21, 22). IGF-1 treatment resulted in a fiber type shift toward type IIA fibers in the soleus muscle with a decrease in the proportion of type IIAD fibers (Figure 19). There was little change in muscle fiber

*Figure 18.* Triceps surae muscle group wet weight to body weight ratio (mean+SD). a = significantly different than Control group; b = significantly different than DM group; c = significantly different than GH group; d = significantly different than IGF-1 group. Body weight data provided by Dr. Ed List.
type composition in the plantaris, DRG, and SWG muscles with IGF-1 treatment (Figures 20, 21, 22). Combination treatment resulted in a fiber type shift toward IIA fibers in the soleus muscle with a decrease in the proportion of type I and IIAD fibers (Figure 19) and very little change in fiber type profile in the plantaris, DRG, or SWG muscles.

There were no significant differences in fiber type profile in the soleus muscle for the DM versus the Control groups (Figure 19). The GH group demonstrated a significant reduction in the percentage of type I fibers compared to the Control group (p<0.01). The Combo group also had a significantly lower percentage of type I fibers than the Control (p<0.01), DM (p<0.01), and IGF-1 (p<0.05) groups. IGF-1 administration did not yield any significant difference in the percentage of type I fibers compared to the Control and DM groups. Both GH and Combo groups also had a lower proportion of IC fibers than the Control group. Administration of GH, IGF-1, or both resulted in a significant increase in the percentage of type IIA fibers in the soleus compared to both the DM and Control groups (p<0.01). All three hormone conditions (GH, IGF-1, and Combo) also resulted in a significant decrease in the percentage of type IIAD fibers compared to the DM group.
Figure 19. Percent fiber type in the soleus muscle (mean±SD). a = significantly different than Control group within fiber type; b = significantly different than DM group within fiber type; c = significantly different than GH group within fiber type; d = significantly different than IGF-1 group within fiber type.

In the plantaris muscle there was no significant difference in the percentage of type I, IIA, IIA, and IIB fibers between any of the conditions (Figure 20). The Combo group had a significantly lower percentage of type IID fibers than the Control group (p<0.01). For the type IIDB fibers, the GH (p<0.01) and Combo (p<0.05) groups had a significantly lower percentage of fibers than the DM group. Amongst the IIDB fibers, the IGF-1 group trended lower than the DM group but did not reach significance (p=0.74).
Figure 20. Percent fiber type in the plantaris muscle (mean+SD). a = significantly different than Control group within fiber type; b = significantly different than DM group within fiber type.

In the DRG there were no significant differences in fiber type percentage among the type IIA, IIAD, IIDB, and IIB fiber populations (Figure 21). The DM and GH groups had a significantly lower percentage of type I fibers than the Control group. There was a lower proportion of IID fibers (p<0.05) in the IGF-1 group than the Control group. There was also a lower percentage of IID fibers in the Combo group than the Control (p<0.01) and GH (p<0.05) groups.
Figure 21. Percent fiber type in the deep, red gastrocnemius muscle (DRG) (mean±SD). a = significantly different than Control group within fiber type; c = significantly different than GH group within fiber type.

In the SWG there was little fiber type variation between any of the groups (Figure 22). The percentage of IID and IIDB fibers were not different between any of the groups. There was a significantly greater percentage of type IIB fibers in the SWG in the Combo group (94%) compared to the Control group (97%) (p<0.05). The raw data for all the fiber type percentages are contained in Appendix E.
Figure 22. Percent fiber type in the superficial, white gastrocnemius muscle (SWG) (mean±SD). a = significantly different than Control within fiber type.

Muscle Fiber Cross-Sectional Area

The muscle fiber CSA data also provided answers for other parts of the specific aims of the study. The DM group had a greater muscle fiber CSA than the Control group across fiber types in the soleus, plantaris, DRG, and SWG muscles (Figures 23, 24, 25, 26). GH treatment appeared to cause preferential hypertrophy of fast fiber types the soleus, plantaris, DRG, and SWG muscles, with no significant effect on type I muscle fiber CSA in the soleus (Figures 23, 24, 25, 26). Conversely, IGF-1 treatment resulted in greater muscle fiber CSA in the slower fiber types within each muscle compared to the DM group (Figures 23, 24, 25, 26). Combination treatment resulted in a greater muscle fiber CSA in some fast fiber types in the soleus, plantaris, DRG, and SWG muscles, but not as consistently as GH treatment (Figures 23, 24, 25, 26).
In the soleus muscle, the DM group averaged a 25% greater muscle fiber CSA than the Control group. This increase was statistically significant for all fiber types (Figure 23). The GH, IGF-1, and Combo groups all had a significantly greater mean type I fiber CSA than the Control group. Only the IGF-1 group had a significantly greater type I fiber CSA than the DM group (p<0.001). The IGF-1 group type I CSA was also significantly greater than the GH group (p<0.01) and the Combo group (p<0.001). Amongst the type IIA fibers all three hormone treated groups (GH, IGF-1, and Combo) had a greater type IIA fiber CSA than the DM group. Overall, the GH group had a 14% greater muscle fiber CSA than the DM group in type IIA and IIAD fibers. There were no significant differences between the hormone groups in type IIA fiber CSA. The CSA of type IIAD fibers was greater in the GH and Combo groups than the DM group and Control groups. IGF-1 administration alone did not yield significantly greater muscle fiber CSA in type IIAD fibers than the DM group but was greater than the Control group.
Figure 23. Muscle fiber cross-sectional area in the soleus muscle (mean μm²±SD). a = significantly different than Control group within fiber type; b = significantly different than DM group within fiber type; c = significantly different than GH group within fiber type; d = significantly different than IGF-1 group within fiber type.

Like the soleus muscle, the mean muscle fiber CSA in the plantaris muscle was greater in the DM than the Control group across all major fiber types with an average increase of 21% compared to the Control group. The three hormone conditions (GH, IGF-1, and Combo) all yielded a significantly greater type IIA fiber CSA than the DM group (Figure 24). Although significantly greater than the DM group, the type IIA fiber CSA was significantly less in the Combo group than the GH and IGF-1 groups. In the IID fiber population, the three hormone treatments (GH, IGF-1, and Combo) resulted in a significantly greater type IID CSA than the DM group with no significant differences between the three hormone treatment groups. With the IIDB fibers, the GH group was the only group with a greater fiber CSA than the DM group (p<0.001). Both the IGF-1
and Combo group had a significantly smaller IIDB fiber CSA than the GH group. The GH and Combo groups, but not the IGF-1 group, had a greater type IIB fiber CSA than the DM group. Overall, the GH treatment resulted in an average 12% increase in muscle fiber CSA in the plantaris.

![Plantaris](image)

**Figure 24.** Muscle fiber cross-sectional area in the plantaris muscle (mean µm²±SD). a = significantly different than Control group within fiber type; b = significantly different than DM group within fiber type; c = significantly different than GH group within fiber type; d = significantly different than IGF-1 group within fiber type.

In the DRG, the DM group again had a greater muscle fiber CSA (average 18%) than the Control group in the major fiber types examined (IIA, IID, IIB) (Figure 25). In type IIA fibers, all three hormone treatments (GH, IGF-1, and Combo) resulted in a
significantly greater fiber CSA than the DM group. Amongst the hormone treated
groups, the GH and Combo groups had significantly larger type IIA fibers than the IGF-1
group. In the type IID fibers, the GH and Combo groups had a significantly larger mean
CSA than the DM and IGF-1 groups (p<0.001). The type IIB fibers were similar with the
GH and Combo groups having a greater CSA than the DM and IGF-1 groups. The GH
group also had a significantly greater type IIB fiber CSA than the Combo group (p<0.05).
Again, GH treatment had the greatest overall effect with an average 20% increase in
muscle fiber CSA across fast fiber types in the DRG.

Figure 25. Muscle fiber cross-sectional area in the deep red gastrocnemius muscle (mean
µm²+SD). a = significantly different than Control group within fiber type; b =
significantly different than DM group within fiber type; c = significantly different than
GH group within fiber type; d = significantly different than IGF-1 group within fiber
type.
In the SWG, the DM group had a 34% greater muscle fiber CSA in type IID, IIDB, and IIB fibers compared to the Control group. For type IID fibers, all three hormone conditions had a greater fiber CSA than the Control group, but only the GH group had a significantly greater fiber CSA than the DM group (p<0.05). The GH group did have a significantly greater type IID fiber CSA than the IGF-1 group (p<0.01). Similar to the IID fibers, in the type IIDB fiber population only the GH group had a greater CSA than the DM group (p<0.001), while the IGF-1 and Combo groups did not differ from the DM group. While both the IGF-1 and Combo groups had a lower IIDB fiber CSA than the GH group, the Combo group fiber CSA was significantly greater than the IGF-1 group (p<0.05). For the type IIB fibers, the GH group was again the only group that had a greater fiber CSA than the DM group (p<0.001). The IGF-1 group IIB fiber CSA was actually lower than the DM group (p<0.05), while the Combo group was greater than the IGF-1 group (p<0.001) and less than the GH group (p<0.001) (see Figure 26 for an overview of the SWG fiber CSA data). Like the DRG, GH induced the greatest increase in muscle fiber CSA in the SWG with an average 24% increase. The raw data for muscle fiber CSA in all muscle groups are contained in Appendix F.
Figure 26. Muscle fiber cross-sectional area in the superficial white gastrocnemius muscle (mean $\mu$m$^2$+SD). $a$ = significantly different than Control group within fiber type; $b$ = significantly different than DM group within fiber type; $c$ = significantly different than GH group within fiber type; $d$ = significantly different than IGF-1 group within fiber type.

Other Measurements

The percentage of total muscle fiber area was also calculated for each of the soleus, plantaris, DRG, and SWG muscles. These results mirrored the percent fiber type data for each muscle and thus, did not provide any additional insight into fiber type transformations (see Appendix G for percent muscle fiber area data). In addition to the data detailed previously, other pertinent data were provided by Dr. Ed List. A summary of plasma concentrations of mouse-specific IGF-1 provided by Dr. List is contained in Appendix H. Plasma insulin and glucose concentration data provided by Dr. List are summarized in Appendix I.
CHAPTER 4: DISCUSSION

Key Findings

There were several notable findings in the current investigation that provided important insight into the effects of type 2 diabetes, GH, and IGF-1 on skeletal muscle in mice. The primary findings were as follows: 1) Muscle fiber type composition shifts did not appear to play a prominent role in the development of type 2 diabetes; 2) GH treatment in diabetic mice produced a robust hypertrophic response; 3) IGF-1 treatment in diabetic mice produced a less robust hypertrophic response than GH treatment; 4) GH preferentially increased muscle fiber CSA in type II fibers; 5) IGF-1 preferentially increased muscle fiber CSA in the slower fiber types within each muscle; 6) Both GH and IGF-1 caused a shift toward type IIA fibers in the soleus. These results are important because they enhance our understanding of the changes in skeletal muscle associated with type 2 diabetes, GH, and IGF-1. This insight could prove valuable in exploring future treatments for type 2 diabetes including GH and IGF-1.

Muscle Hypertrophy

The LBM, triceps surae wet weight, and muscle fiber CSA data can be taken together to provide a picture of overall muscle hypertrophy in each of the groups. Using both LBM and wet weight to measure muscle hypertrophy can be limited since other components besides skeletal muscle mass may be included in these measures. Examining muscle fiber CSA in conjunction with these measures can provide a more complete picture of muscle hypertrophy because the CSA of individual muscle fibers provides a more specific measure of muscle hypertrophy. Overall, these data suggest that the DM group
did experience some muscle hypertrophy based on muscle fiber CSA increases ranging from 18-34% compared to the Control group, even though the increases in LBM and triceps surae wet weight were non-significant. The hypertrophic response in the DM group compared to the Control group appears to be somewhat preferential to the triceps surae. The wet weight of the triceps surae was 13% greater in the DM group with a substantial increase (18-34%) in CSA across all fiber types and muscles, while overall LBM was only 9% greater in the DM group. It seems likely this triceps surae-specific response is due to increased loading from substantial increases in overall body mass (Figure 15). GH treatment in the GH group promoted a robust hypertrophic response with a 26% increase in LBM, 14% increase in triceps surae wet weight, and a 12-24% increase in muscle fiber CSA compared to the DM group. Surprisingly, IGF-1 administration yielded a smaller overall hypertrophic response with a 13% increase in LBM, a 3% decrease in triceps surae wet weight, and variable effects on muscle fiber CSA. Despite variable effects on muscle fiber CSA, the Combo demonstrated a 37% increase in LBM and 15% increase in triceps surae wet weight compared to the DM group. These data suggest that GH does indeed induce significant muscle hypertrophy. It also suggests that the hypertrophic response from GH may not be solely due IGF-1 given that IGF-1 administration yielded a less robust hypertrophic response.

*Animal Body Composition and Triceps Surae Wet Weight*

The DM group had a significantly greater total body mass compared to the Control group primarily due to increases in fat mass from the high-fat diet. GH administration increased LBM, which was similar to previous research on GH in the
same diabetic mouse strain (List et al., 2009). IGF-1 administration also resulted in significantly greater LBM compared to Control mice, but not to the same extent as GH (Figure 15). Both groups that received GH (GH and Combo) had the greatest LBM compared to the other groups. Overall, these results are consistent with previous research that has demonstrated a significant increase in LBM with administration of GH, IGF-1, or both to mice, rats, or humans (Aroniadou-Anderjaska, Lemon, & Gilloteaux, 1996; Daugaard et al., 1998; Daugaard et al., 1999; Kim et al., 2005; Lewis et al., 1997a; Lewis et al., 1998; Libera et al., 2004; List et al., 2009; Liu et al., 1993; Musaro et al., 2001; Schuenke et al., 2008). Again, these data confirm that GH administration does induce muscle hypertrophy.

The wet weight of the triceps surae muscle group was not significantly different in the Control and DM groups (Figure 16). It should be noted that the triceps surae wet weight in the DM group was 13% greater than the Control group, although this difference was not statistically significant. Given that the triceps surae are a weight-bearing muscle group, some hypertrophy might be expected due to the increased loading due to a greater total body mass. Despite the non-significant increase in triceps surae wet weight, the muscle fiber CSA data suggest there was some muscle hypertrophy in the DM group compared to the Control group. GH administration resulted in a 14% increase in triceps surae wet weight compared to the DM group, which was statistically significant. The IGF-1 group did not differ significantly from the Control or DM groups while the Combo group had the greatest mean triceps surae wet weight suggesting GH has a greater anabolic effect than IGF-1. GH exposure has previously been shown to result in
hypertrophy of the soleus, gastrocnemius, and triceps surae muscles (Aroniadou et al., 1996; Kim et al., 2005; McCall et al., 1998; Roy et al., 1996; Schuenke et al., 2008). It is also possible that GH therapy resulted in increased fluid retention in the muscle as GH has been noted to increase total body water and ECW (Carrol et al., 1998; Ehrnbor et al., 2005; Llu et al., 2008; Meinhardt et al., 2010; Moller, 2003). Increases in intracellular fluid volume have been noted with GH replacement in GH deficiency, but not necessarily with GH administration to healthy subjects (Moller). Because fluid content of the triceps surae muscle group was not specifically measured or calculated, it is impossible to rule out fluid retention as a contributor to the increased wet weight with GH therapy. Given that the total fluid content in the mice was not different between the DM group and GH and Combo groups, it seems unlikely that increases in triceps surae muscle group wet weight were solely due to fluid retention. Muscle fiber CSA was also greater in all fiber types except type I fibers with GH treatment, which also suggests that increases in LBM and triceps surae wet weight were not solely due to fluid shifts. Although IGF-1 did increase total LBM compared to Control, there was no significant increase in triceps surae muscle mass. One possible explanation is that IGF-1 administration increased muscle mass in muscles other than the triceps surae muscle group. Since the wet weight/LBM ratio was significantly less in the IGF-1 and Combo groups it seems IGF-1 administration may disproportionately increase overall LBM, which includes a variety of tissue as well as skeletal muscle mass, more than triceps surae mass (Figure 17). A more detailed analysis of all aspects of LBM would be necessary to determine if IGF-1 administration preferentially targets lean tissue besides skeletal muscle.
Although most previous research has indicated increases in muscle weight or CSA with IGF-1 exposure, Roy et al. (1996) found IGF-1 administration to hypophysectomized rats increased mass in the vastus intermedius muscle, but not the soleus muscle. In contrast, IGF-1 administration to laminin deficient mice resulted in an increase in soleus muscle mass (Lynch et al., 2001). Direct infusion of IGF-1 into the tibialis anterior muscle of rats increases muscle mass as well (Adams & McCue, 1998). The differential response to IGF-1 may be related to the difference in animal models and different IGF-1 administration methods utilized in the current investigation compared to previous investigations. It is plausible that a direct infusion into to a muscle would deliver a greater dose of IGF-1 to the muscle tissue than a subcutaneous injection that enters systemic circulation. Although optimal dosing of exogenous GH and IGF-1 may differ, the current study utilized the maximum tolerable dose of IGF-1 based on pilot work. This suggests that the lack of response in triceps surae muscle mass and LBM with IGF-1 was not due to insufficient dosing.

An unknown factor is the localized exposure of skeletal muscle to IGF-1 with the different hormone treatments. GH is known to induce IGF-1 expression in a paracrine or autocrine fashion in skeletal muscle (Davey et al., 2001; Dominici et al., 2005; Woelfle et al., 2003). It is unclear if systemic IGF-1 delivered via subcutaneous injection (as in the current study) results in the same local concentrations of IGF-1 in skeletal muscle as the autocrine/paracrine response to GH. It is also possible that the biologically active portion of IGF-1 is altered with systemic IGF-1 administration. There appears to be some
compensation of IGF-1 binding proteins to maintain unbound IGF-1 concentrations when liver IGF-1 expression is suppressed (LeRoith, Scavo, & Butler 2001).

There are six known IGF binding proteins (IGFBP) that have been characterized (Firth & Baxter, 2002; Hwa, Youngman, & Rosenfeld, 1999). They are composed of amino acid chains ranging from 216 to 289 amino acids with core molecular mass ranging from 22.8 to 31.4 kDa (Firth & Baxter, Hwa et al.; Rajaram, Baylink, & Mohan, 1997) (see Table 7). In general, IGFBPs are thought to stabilize circulating IGF-1 by preventing degradation or sequester a reserve “supply” of IGF-1 (Ezzat, Duncan, Wheatcroft, & Kearney, 2007; Firth & Baxter; Hwa et al.; Rajaram et al.). IGF-1 has a greater affinity for IGFBPs than the IGF-1 receptor and thus can modulate IGF-1 action (Ezzat et al.). IGFBPs can potentiate or inhibit the effects of IGF-1 depending on the IGFBP and/or the phosphorylation status, proteolysis, and cell surface association of the IGFBP (Rajarma et al.). The majority of circulating IGF-1 is bound to IGFBP-3 along with the acid labile subunit (ALS) (Ezzat et al., Firth & Baxter; Mauras & Haymond, 2005; Rajaram et al.). In circulation, unbound IGF-1 makes up approximately 1% of the total circulating IGF-1 (Ezzat et al., Firth & Baxter; Rajaram et al.).
Table 6.

**Characteristics of IGF Binding Proteins**

<table>
<thead>
<tr>
<th>No. of AA</th>
<th>Molecular mass (kDa)</th>
<th>IGF affinity</th>
<th>Modulation of IGF action</th>
<th>Biological source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>234</td>
<td>25.3</td>
<td>I = II</td>
<td>Inhibition and/or potentiation</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>289</td>
<td>31.4</td>
<td>I &lt; II</td>
<td>Inhibition</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>264</td>
<td>28.7</td>
<td>I = II</td>
<td>Inhibition and/or potentiation</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>237</td>
<td>25.9</td>
<td>I = II</td>
<td>Inhibition</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>252</td>
<td>28.5</td>
<td>I &lt; II</td>
<td>Potentiation</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>216</td>
<td>22.8</td>
<td>I &lt; II</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

Adapted from Rajaram, Baylink, & Mohan (1997).

Therefore, IGF-1 administration may not effectively increase muscle IGF-1 exposure if it is quickly degraded or if the IGFBP kinetics inhibit IGF-1 action.

Interestingly, IGF-1 seems to be eliminated more quickly in normal human subjects compared to those who are GH deficient (Mauras, Quamby, & Bloedow, 1999).

Alterations in IGFBP-3 and ALS expression have been found to affect IGF-1 stimulated bone growth and therefore it seems plausible that alterations may also affect skeletal muscle (Yakar et al., 2009). It is also noteworthy that delivery of IGF-1 combined with IGFBP-3 has shown promise in mimicking a physiologic profile if IGF-1 kinetics (Mauras & Haymond, 2005; Savage, Camacho-Hubner, & Dunger, 2004). There are also some questions regarding the laboratory methods for testing IGF-1 blood concentrations.
and whether it is more physiologically relevant to test total IGF-1 or free IGF-1 (Frystk, 2010). One limitation of the current study is that muscle IGF-1 content was not tested, and thus it is unclear what amount of IGF-1 effectively reached the triceps surae muscle group in each of the treatment conditions.

**Muscle Fiber Cross-Sectional Area**

Muscle fiber CSA appears to be significantly affected by both diabetes and the administration of GH and IGF-1. The DM group had significantly greater muscle fiber CSA across the major fiber types in the soleus, plantaris, DRG, and SWG muscles compared to the Control group (Figures 23, 24, 25 and 26). It is interesting to note that while muscle fiber CSA was consistently greater in the DM group, the muscle wet weight of the entire triceps surae muscle group did not differ significantly from the Control group (Figure 16). Initially, these results seem incongruent as one would expect consistent increases in muscle fiber CSA in all muscles to yield a significantly larger overall muscle mass. However the magnitude of increase in triceps surae wet weight with the DM group versus the Control group was nearly the same as the GH group versus the DM group (13% vs. 14% respectively). Perhaps with a larger sample size the difference in triceps wet weight between the DM group and Control group would be significant. One explanation is that muscle fiber CSA examines hypertrophy on a much smaller scale than gross wet weight of the three different muscles together. Because wet weight is a less precise way to measure hypertrophy there may be more room for measurement error. There are also other components such as connective tissue, blood vessels, and fat that contribute to the total wet weight that are not accounted for in muscle
fiber CSA measurements. However, increased fluid retention within the muscle cannot be completely ruled out. Given this confounding variable, muscle fiber CSA may be a more reliable indicator of the degree of muscle hypertrophy than triceps surae wet weight in the current investigation.

There is also the possibility that more intramuscular lipid is stored in the muscle of diabetic mice that is not readily visible with the fiber type staining procedures utilized here. Skeletal muscle in type 2 diabetes has been characterized by overall increases in intramuscular lipid storage (He et al., 2001; Lee et al., 2002; Levin et al., 2007; Malenfant, et al., 2001; Timmers et al., 2008). Although not directly measured, it is plausible that the DM group had an increase in muscle lipid storage since a high-fat diet has previously been shown to increase muscle lipid storage in rats (Lee et al.). Given that lipid is less dense than the proteins that make up the muscle structure, it could disproportionately increase fiber CSA more than muscle wet weight. However, substantial pockets of adipose tissue in the muscle fiber cross-sections were not seen.

One potential reason for the observed increase in muscle fiber CSA is simply because of the overload of the entire triceps surae muscle group due to the increased overall bodyweight in the DM group versus the Control group (Figure 15). It is well known that overloading a muscle or group of muscles induces hypertrophy with corresponding increases in muscle fiber CSA (Campos et al., 2002; Gollnick, Timson, Moore, & Riedy, 1991; Hikida et al., 2000; Hostler et al., 2001; McCall et al., 1998; Mikesky et al., 1991; Staron et al., 1991; Timson 1990). An overload stimulus sufficient to induce hypertrophy is often sufficient to induce muscle fiber type shifts as well,
particularly shifts in the fast fiber subtypes (Campos et al.; Hikida et al., 2000; Hostler et al.; Pette 2001; Pette & Staron 2001; Staron et al., 1991). Because there were no significant fiber type shifts noted with the DM group, it is possible that the observed muscle fiber CSA increase was not due to overload as fiber type shifts may accompany hypertrophy. Another possibility is that the overload was promoting a fast-to-slow fiber type shift and the underlying diabetes a slow-to-fast fiber type shift. These two opposing stimuli could have been offsetting each other, resulting in no net fiber type shift while still yielding an increase in muscle fiber CSA.

Another potential reason for the observed increase in muscle fiber CSA in the DM group is an increase in IGF-1 concentrations due to overfeeding. Caloric excess, higher fat intake, and obesity have been associated with increased blood IGF-1 concentrations (Crowe et al., 2011; Holmes, Pollack, Willett, & Hankinson, 2002; Kaklamani et al., 1999). Increases in circulating IGF-1 concentrations could conceivably lead to increases in muscle fiber CSA as increased IGF-1 exposure has often been shown to induce muscle hypertrophy (Barton-Davis et al., 1999; Chakravarthy et al., 2000; Chakravarthy, Booth, & Spangenburg, 2001; Lewis et al., 1997a; Lewis et al., 1997b; Lewis et al., 1998; Lynch et al., 2001; McCall et al., 1998; Semarian et al., 1999; Song et al., 2005). However, this appears unlikely because plasma IGF-1 concentrations were not substantially higher in the DM group compared to the Control group (see Appendix H for data provided by Dr. Ed List).

Administration of GH to diabetic mice in the GH and Combo groups consistently resulted in a significant increase in muscle fiber CSA of all fast twitch fiber subtypes
(IIA, IIAD, IID, IIDB, IIB) across all three muscle groups (Figures 23, 24, 25, and 26). GH did not significantly increase muscle fiber CSA of type I fibers in the soleus muscle (Figure 23). The soleus muscle was the only muscle group that had a sufficient number of type I fibers for statistical analysis of muscle fiber CSA. It appears that GH may even inhibit the effects of IGF-1 on type I fibers because the Combo group had a smaller type I fiber CSA in the soleus muscle than the IGF-1 or GH group.

Interestingly, IGF-1 administration consistently resulted in increases in muscle fiber CSA in type I and IIA fibers. Only in the IID fiber population of the plantaris muscle did IGF-1 administration induce significant increases in muscle fiber CSA in a fiber type faster than IIA fibers (Figure 24). IGF-1 did not increase muscle fiber CSA in type IIDB or IIB in any muscle group examined. It is also noteworthy that there is a less robust effect on fast fiber CSA in the Combo group than the GH group (Figures 23, 24, and 26). These data suggest that IGF-1 administration may offset some of the growth promoting effects of GH on these fast fiber types. It seems that GH may have a preferential effect on fast fiber subtypes when it comes to increases in muscle fiber CSA in diabetic mice. It may also inhibit any growth effects of IGF-1 on type I fibers. These data also suggest that IGF-1 may preferentially increase fiber CSA in the slower fiber types (e.g. I, IIA) within any particular muscle, while not influencing the faster fiber types (e.g. IID, IIB). More research is needed to determine if these effects are the result of GH and/or IGF-1 administration alone or administration in conjunction with diabetes. Without lean control mice that also received GH and/or IGF-1, it is impossible to tell if
the differential effects of GH and IGF-1 are related to intrinsic properties of the hormones or an interaction with diabetes.

As with the fiber type transformation, opposite trends in muscle fiber CSA have been noted with increased GH exposure (Schuenke et al., 2008). GH consistently increased muscle fiber CSA in all fiber types except IIB and in some cases IIDB (Schuenke et al.). There was a significant increase in type I muscle fiber CSA in the soleus and DRG muscles with bGH overexpression, but no significant effect on IIB fiber CSA in the plantaris, DRG, and SWG muscles (Schuenke et al.). As discussed previously, these varied results are likely due to the different GH exposure model being used and/or the underlying type 2 diabetes in the GH-treated animals in the current investigation. Interestingly, one other investigation found a significant increase in muscle fiber CSA in type II fibers but not type I fibers in the soleus muscle of rats given GH injections (Aroniadou et al., 1996). Given these studies and the current investigation it seems that the method of GH exposure (injection vs. overexpression) may influence the observed effects on muscle fiber characteristics.

Although IGF-1 is generally thought to promote muscle hypertrophy and increases in muscle fiber CSA, there is limited information regarding IGF-1 administration and muscle fiber CSA broken down by specific muscle fiber types. IGF-1 increased the muscle fiber CSA of type IIA and IID fibers while GH only increased fiber CSA of type IIA fibers in the diaphragm muscle of rats (Lewis et al., 1997b). When the same group examined the diaphragm muscle of nutrient-deprived rats, they found that both GH and IGF-1 administration for 3-7 days increased the muscle fiber CSA of type I,
IIA, and IID fibers (Lewis et al., 1997a; Lewis et al., 1998). These data suggest that nutritional status may influence the effects of IGF-1 and GH on muscle fiber CSA. However this is not a direct parallel to the current investigation given the difference in animal model, absence of diabetes, different muscle group being examined, and duration of hormone administration.

The overall picture of increases in LBM and muscle fiber CSA are novel in that GH and IGF-1 have differential effects on diabetic mice. These differences are difficult to explain given that many attribute the muscle hypertrophy effects of GH to IGF-1 (Butler & LeRoith, 2001; Davey et al., 2004; Dos Santos et al., 2010; Hameed et al., 2004). Because GH administration in the current investigation induced significant increases in circulation IGF-1 concentrations (Appendix H), it is difficult to completely isolate the effects of GH and IGF-1. Even if there are some GH-independent effects on skeletal muscle, previous research suggests IGF-1 plays an important role in muscle development and hypertrophy. In fact, absence of the IGF-1 gene in mice results in severely underweight mice with substantially less muscle mass that rarely survive more than few days after birth (Liu et al., 1993; Liu et al., 1998; Powell-Braxton et al., 1993). Those that do survive have severely stunted growth (Liu et al., 1993; Liu et al., 1998; Powell-Braxton et al.). GH binding and signaling leads to increased expression of IGF-1 in the liver and in skeletal muscle (Davey et al., 2004; Dominici et al., 2005; Woelfle, Chia, & Rotwein, 2003). IGF-1 binding to its membrane bound receptor in skeletal muscle activates a signaling pathway that includes PI3K, Akt, mTOR, and p70S6K leading to increased translation and ultimately, increased protein synthesis and
hypertrophy (see Figures 8 and 9 for an overview) (Bodine et al., 2001; Chakravarthy et al., 2000; Dardevet et al., 1996; Latres et al., 2005). Blocking the Akt/mTOR signaling pathway has been shown to prevent muscle hypertrophy (Bodine et al., 2001). IGF-1 also appears to play a role in activation and proliferation of satellite cells via the PI3K/Akt pathway (Chakravarthy et al., 2000; Chakravarthy, Booth, & Spangenburg, 2001; Jacquemin et al., 2004). Proliferation of satellite cells is thought to be one of the primary factors in modulating muscle hypertrophy (Barton-Davis et al., 1999; Jacquemin et al., 2004; Semsarian et al., 1999). Using cluster analysis, Petrella, Kim, Mayhew, Cross, & Bamman (2008) found that greater satellite cell proliferation was associated with a greater hypertrophy response from resistance training presumably due to the ability to add a greater number of myonuclei. It should be noted that not all of the hypertrophy inducing effects of IGF-1 can be attributed to satellite cells as hypertrophy is attenuated, but not completely blocked, with inactivation of satellite cells (Barton-Davis et al., 1999; Jacquemin et al., 2004). Based on this, it would be expected that IGF-1 administration would promote muscle hypertrophy much like GH. As mentioned earlier, the local concentrations of IGF-1 in the various conditions were unknown. Thus it cannot be ruled out that IGF-1 administration did not provide as much skeletal muscle local exposure to IGF-1 as GH administration.

Another intermediate in the hypertrophy process that may be affected with GH administration is myostatin. Myostatin is a known inhibitor of muscle hypertrophy. GH treatment in GH deficient humans has been shown to decrease myostatin mRNA with an accompanying increase in LBM (Liu et al., 2003). Myostatin deficient mice have greater
than normal LBM and are very lean. They also have lower blood glucose and better insulin sensitivity than normal control mice (Guo et al., 2009). The improved glucose metabolism with decreased myostatin expression appears likely to be related to favorable body composition although other mechanisms cannot be definitively ruled out. Therefore if GH decreased myostatin expression it could lead to muscle hypertrophy and fat loss. It is not clear whether there is any preferential effect on myostatin with GH versus IGF-1 administration.

There is also noteworthy evidence that local production of IGF-1 may be more important for muscle growth than systemic IGF-1 levels. Mice that have the IGF-1 gene knocked out specifically in the liver, which reduces systemic IGF-1 concentrations by ~75%, do not have any significant impairment in muscle growth parameters (LeRoith, Scavo, & Butler, 2001; Sjogren et al., 1999; Yakar et al., 1999). It should also be noted that in these IGF-1 liver gene knockout mice IGF-1 binding proteins are also affected. The changes were profound enough that despite a 75% reduction in total IGF-1 concentrations, the “free IGF-1” concentrations (unbound to IGF binding proteins) were not significantly different than wild-type mice (LeRoith et al., 2001). There are two potential explanations for these results. One explanation is that paracrine IGF-1 production stimulated in skeletal muscle by systemic GH is sufficient for muscle growth. The other explanation is that free IGF-1 concentrations are more important for skeletal muscle growth than total IGF-1 levels. The current investigation also suggests that the endocrine function of IGF-1 is not as important as the paracrine function because GH
stimulated more muscle hypertrophy than systemic doses of IGF-1, although systemic alterations in IGF-1 binding proteins cannot be ruled out.

It was previously thought that IGF-1 could promote hypertrophy via a calcium dependent pathway that involves calcineurin (Semsarian 1999). More recent evidence has shown that calcineurin is not required for hypertrophy (Bodine et al., 2001; Naya et al., 2000; Parsons et al., 2004; Serrano et al., 2001). Again, activation of this pathway has been implicated in increasing MHC Iβ expression (Chin et al., 1998; Delling et al., 2000; Koulmann et al., 2008; Meiβner et al., 2001; Naya et al., 2000; Torgan et al., 2001; Vescovo et al., 2005). This may explain why IGF-1 administration led to type I fiber hypertrophy in the soleus muscle as increased MHC Iβ expression could lead to hypertrophy of existing type I fibers. It does not explain why GH did not promote type I fiber hypertrophy as it would presumably stimulate IGF-1 expression and thus, activate the calcineurin pathway as well.

Neuronal activity also cannot be ruled out regarding the seemingly preferential effects of IGF-1 on slower fiber types and CSA. Again, both GH and IGF-1 have been implicated in influencing neuron size and growth. GH may increase motor neuron size (Chen et al., 1997; Parsons et al., 2002) and IGF-1 appears to stimulate nerve sprouting (Messi & Delbono, 2003). If GH or IGF-1 had a differential effect on the motor neurons supplying the different muscles of the triceps surae group, it could conceivably influence which fiber types were most affected.

The primary IGF-1 isoform may also differ in the IGF-1 group compared to the GH group. GH in the GH and Combo groups stimulated large increases in circulating
concentrations of mouse-specific IGF-1 (see Appendix H, data provided by Dr. Ed List). The IGF-1 group had much lower levels of mouse-specific IGF-1 presumably due to the human IGF-1 suppressing GH release and thus, mouse-specific IGF-1 production. It is possible that human IGF-1 preferentially acts on slower muscle fiber types, while mouse-specific IGF-1 acts on the faster fiber types. It should also be noted that in general humans have a significantly slower fiber type profile in a given muscle compared to small rodents (Staron et al., 2000; Klover et al., 2009).

There is emerging evidence that specific isoforms of IGF-1 can differ in their overall biological effects or degree of action. Due to alternate splicing at both the 5’ and 3’ ends of the IGF-1 gene there are multiple forms of IGF-1 (Barton, 2006). Different isoforms can result in different E-peptide extension on the IGF-1 propeptide (Barton, 2006). Different isoforms of murine IGF-1 have been found to have different effects depending the age of the mice examined (Barton, 2006). To further support this notion, IGF-1 isoforms with different carboxy-terminal E-peptide extensions have been found to cause differential hypertrophy responses (Barton, DeMeo, & Lei, 2010). Some E-peptides may be necessary for a local hypertrophic response (Barton et al., 2010). IGF-1 isoforms activated some common pathways, but also some unique pathways (Barton et al., 2010). It is also unclear if IGF-1 isoforms from a different species will react with or influence IGF-1 binding proteins differently and thus, alter the amount of biologically active IGF-1 available. Given that different IGF-1 isoforms appear to have different effects within the same species, it certainly seems plausible that an IGF-1 isoform from a different species could have different effects compared to a native IGF-1 isoform.
Another factor that could influence the differential effects of GH and IGF-1 is GH receptor content and/or density. The lowest GH receptor mRNA expression has been noted in the pig longissimus dorsi muscle, which had the fastest fiber type profile studied (Katsumata, Catteneo, White, Burton, & Dauncey, 2000). The greatest GH receptor mRNA content was in the pig soleus muscle, which was the slowest muscle examined. Interestingly they did not find any significant correlation between type I fiber content and GH receptor mRNA, but did find a significant relationship between oxidative fiber content and GH receptor mRNA (Katsumata et al.). Conversely, in rats there does not appear to be a difference in GH receptor density in muscles with different fiber type profiles. The EDL had greater overall GH receptor mRNA content compared to the soleus muscle, but there were no differences in GH receptor mRNA content when adjusted for differences in CSA (Casse et al., 2003). Given the limited evidence available and conflicting results, it is difficult to draw any conclusions regarding GH receptor density in the different muscle fiber types.

Muscle Fiber Type Changes

Fiber type composition in the soleus, plantaris, DRG, and SWG muscles was not significantly different between the DM and Control groups with the exception of a lower proportion of type I fibers in the DRG of the DM group (Figures 19, 20, 21, and 22). Overall, this suggests that diet-induced type 2 diabetes does not significantly alter fiber type in the triceps surae muscles of C57BL/6J mice. These data also suggest that alterations in fiber type profile are not necessarily responsible for insulin resistance or diabetes in the context of obesity and a caloric surplus. Based on these results, the
physiological changes associated with insulin resistance do not appear to be directly related to MHC based fiber type as the DM group had greater blood glucose and insulin concentrations despite no significant difference in fiber type (see Appendix I for summary of insulin and glucose data provided by Dr. Ed List). However, we cannot rule out fiber type profile changes in other muscles.

These results are consistent with previous evidence that diabetes or blood glucose concentrations are not associated with alterations in fiber type (Cederholm et al., 2000; He et al., 2001; Moller et al., 1996). Disruption in insulin receptor function does not alter muscle fiber type despite decreased insulin signaling and increased blood glucose concentrations in transgenic mice (Moller et al., 1996). Some previous research suggesting a difference in fiber type with diabetes did not utilize the MHC-based fiber typing methods used in the current research. Gaster et al. (2001) used only immunohistochemistry to classify slow and fast twitch fibers, while Oberbach et al. (2006) used metabolic enzyme classification for fiber typing. Thus, it is difficult to draw a direct parallel to the current investigation.

Conversely, others have found a lower type IIA percentage in obese versus lean subjects (Malenfant et al., 2001), or an increased IID fiber type percentage in subjects with type 2 diabetes (Mogensen et al., 2007). These differences could be due to differences in chronic activity or exercise patterns and not necessarily directly related to obesity or diabetes. In addition, the specific muscle group investigated may also influence the results. Alterations in fiber type with a high-fat diet in mice appear to depend on the muscle being investigated (Shortreed et al., 2009). Increases in percentage
of IIA fiber and decreased IIB fibers in the plantaris and gastrocnemius muscles and increases in soleus muscle type I and IIA percent fiber area have also been noted (Shortreed et al.). The same investigation also found no change in the fiber type of the tibialis anterior muscle (Shortreed et al.). An increase in the percentage of type IIA fibers and corresponding decrease in IIB fibers in the EDL of diabetic mice compared to their non-diabetic littermates have also been reported (Klueber, Feczko, Schmidt, & Watkins, 1989). Similar findings have been noted in mice with streptozotocin-induced diabetes (Klueber & Feczko, 1994). They again found a greater percentage of IIA fibers with fewer IIB fibers in the EDL of the diabetic mice compared to non-diabetic littermates (Klueber & Feczko, 1994). Given the variable results of the available research, no clear pattern of fiber type alterations emerges with diabetes and/or obesity.

There is also some evidence that the metabolic activity and glucose uptake can vary significantly within the same fiber type. Succinate dehydrogenase and oxidative enzyme activity differed in all fiber types in diabetic and obese subjects compared to lean subjects despite no significant differences in mATPase fiber type profile in one investigation (He et al., 2001). Succinate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activity have been shown to follow a fiber type continuum, but the activity of these enzymes overlapped between fiber types in rats (Rivero, Talmadge, & Edgerton, 1999). These data suggest metabolic enzyme activity is not necessarily reflective of MHC-based muscle fiber type.

There also appear to be some general trends in GLUT4 content of the different muscle fiber types, however the current study demonstrates that development of insulin
resistance does not require or result in fiber type shifts. The available evidence regarding GLUT4 content generally suggests that red muscle or muscle with a slower fiber type profile has greater GLUT4 content (Daugaard & Richter 2001; Gaster et al., 2000; Gaster, Vach, Beck-Nielsen, & Schröder 2002; Goodyear et al., 1991; Hayaski et al., 2001; Martte et al., 1992; Roy, Johannsson, Bonen, & Marette, 1997; Stuart et al., 2010; Stuart et al., 2006). Only Marette et al. (1992) examined fast twitch fiber subtypes, demonstrating that IIA fibers have greater GLUT4 content than IIX fibers in rats. Although this evidence suggests a correlation between muscle fiber type and glucose transport, the glucose disposal properties can vary substantially within the same fiber type. The glucose uptake of IIA fibers in the gastrocnemius muscle of mice can differ based on the specific region within the muscle (Hayaski et al., 2001). There is also evidence that demonstrates GLUT4 content can increase without any corresponding change in muscle fiber type (Daugaard et al., 2000). Based on these results and the results of the current investigation, there appears to be a wide range in glucose disposal and metabolic properties possible without any alteration in muscle fiber type profile. Furthermore, total GLUT4 content is not necessarily altered with obesity and type 2 diabetes (Friedman et al., 1992; Handberg, Vaag, Damsbo, Beck-Nielsen, & Vinten 1990; Pedersen, Bak, & Andersen, 1990; Zorzano et al., 2005). Therefore, changes in GLUT4 content associated with fiber type changes may not necessarily be related to glucose disposal and any link between fiber type, GLUT4 content, and glucose disposal is tenuous at best.
The primary effects of GH and/or IGF-1 on fiber type were seen in the soleus muscle (Figure 18). In general, GH administration in the GH and Combo groups caused a decrease in type I fibers with a concomitant increase in type IIA fibers in the soleus muscle. On the other hand, IGF-1 treated mice demonstrated an increased percentage of type IIA fibers without an alteration in type I fiber percentage. Although this suggests a shift to a faster fiber type profile, it should be noted that all hormone treatments in this study resulted in a shift in the opposite direction from IIAD hybrid fibers to IIA fibers. Overall in the soleus muscle, there was a shift toward type IIA fiber type expression with GH and/or IGF-1. With GH the shift appears to be both slow to fast and fast to slow given that both type I and type IIAD fiber proportions decreased in the soleus muscle. However, the fiber type transitions with IGF-1 appear to be faster to slower (IIAD to type IIA). Lynch et al. (2001) found an increase in the percentage of type IIA fibers, but also a decrease in type I fibers when laminin deficient mice were given IGF-1.

In general, a fast to slow fiber type transformation would appear to be clinically beneficial in type 2 diabetes. In the current investigation, GH induced a shift toward IIA fibers in the soleus while also lowering blood glucose concentrations. Therefore, it appears this fiber type composition shift was not detrimental to glucose metabolism. While it appears that type II fibers have lower GLUT4 content and less glucose transport capability than type I fibers, most investigations did not examine specific fast fiber subtypes (Bonen et al., 1981; Daugaard & Richter, 2001; Gaster et al., 2000; Gaster et al., 2001; Goodyear et al., 1991; Stuart et al., 2001). There is evidence that GLUT4 content in rat soleus muscle is similar in type I and IIA fibers (Marette et al., 1992). This
suggests that a shift toward IIA fibers in the soleus is not necessarily detrimental to glucose metabolism. It should also be noted that in rats and mice it is very difficult to induce fiber type shifts to type I fibers (Pette & Staron, 2000; Pette & Staron, 2001; Pette & Vrbova, 1999). Even with long-term chronic low frequency stimulation (CLFS) that mimics the neural firing pattern of slow motor neurons, fiber type transitions to type I fibers are very difficult to achieve in mice and rats (Pette & Staron, 2000; Pette & Staron, 2001; Pette & Vrbova, 1999). Other mammals such as rabbit demonstrate an essentially complete fiber type transformation to type I fibers with CLFS (Pette & Staron, 2000; Pette & Staron, 2001; Pette & Vrbova, 1999). This suggests that transitions to type IIA fibers in mice may be an advantageous in terms of oxidative capacity. Interestingly, fiber type transitions to type I fibers in the soleus of mice have been noted with bGH overexpression (Schuenke et al., 2008). The fiber type transition to toward IIA fibers in the soleus seems unlikely to be a substantial contributor in improved glucose metabolism since IGF-1 increase IIA fiber expression without decreasing blood glucose concentrations (see Figure 19 and Appendix H).

These results differ from those of Schuenke et al. (2008) who utilized bGH overexpression mice. They found that bGH overexpression shifted the fiber type profile from fast to slow in the soleus, plantaris, and gastrocnemius muscles. There are some notable differences between the bGH transgenic mice utilized and the current investigation including mode of hormone exposure, presence or absence of type 2 diabetes, and gender of the animals. Schuenke et al. utilized transgenic mice that were giant due to overexpression bGH or dwarf due to disruption of the GH receptor. This is
different than the current investigation comparing GH and IGF-1 administration in mice with pre-existing obesity and type 2 diabetes. Also, two large daily injections of GH for three weeks may result in different hormone kinetics or IGF-1 concentrations than chronic overexpression found in bGH mice. The pattern of GH secretion from the pituitary gland is pulsatile and thus, the pattern of GH exposure may influence the effects of the hormone. There is evidence that smaller, more frequent doses of GH are more effective at increasing blood IGF-1 concentrations than larger less frequent doses in humans (Jaffe, Turgeon, Lown, Demott-Friberg, & Watkins, 2002). A similar trend has been demonstrated in rats where pulsatile GH infusions were more effective at increasing skeletal muscle IGF-1 mRNA content than continuous GH infusion (Isgaard, Carlsson, Isaksson, & Jansson, 1988). It is also possible that a longer period of exposure to elevated GH concentrations would change the results, although it seems unlikely that a reversal of the fiber type changes noted in the current investigation would occur.

Schuenke et al. (2008) also used female mice while the current investigation used male mice, although it is uncertain if this would significantly alter muscle fiber type profile. However, gender does not appear to significantly affect fiber type profile (Staron et al., 2000). One notable gender difference is a difference in circulating testosterone concentrations with males having significantly higher levels than females (Pang & Tang, 1984). Testosterone, acting through the androgen receptor, may promote MHC Iβ expression in the quadriceps of mice (Altuwaijri et al., 2004). Alternately, when testosterone was administered to female rats it resulted in a significant decrease in the proportion of type I fibers in the soleus muscle while showing a trend toward an increase
in type 2 fibers in the red portion of the gastrocnemius muscle (Holmang, Svedburg, Jennische, & Björntorp, 1990). Testosterone administration has not been shown to affect fiber type in the vastus lateralis of older men (Sinha-Hakim et al., 2002).

GH may also have a different effect on IGF-1 concentrations and body composition in males versus females. There is very compelling evidence that GH replacement for GH deficient humans increases serum IGF-1 concentrations more in men than women (Barbosa et al., 2010; Bengtsson et al., 1999; Hayes, Fiad, & McKenna, 1999; Soares et al., 2004; Span, Pieters, Sweep, Hermus, & Smals, 2001). This results in men responding more favorably in terms of increases in LBM and decreases in adipose tissue compared to women (Franco et al., 2009; Hayes et al.; Span et al.). Even with what is known about GH and testosterone regarding muscle fiber type composition, it is difficult to know if the gender of the mice directly influenced specific fiber type characteristics. With the available evidence, GH exposure, duration, dose, and timing appear to be most likely responsible for the differences in fiber type expression.

Some literature suggests GH maintains a normal fiber type profile in both rats and humans. As such, GH deficiency often results in a faster fiber type or MHC profile. Daugaard et al. (1998) noted an increase in the type I fiber percentage and a decrease type IIA in the soleus muscle when GH deficient rats were given GH, whereas there were no changes detected in the extensor digitorum longus (EDL). Similarly, humans with GH deficiency were also shown to have less overall MHC Iβ and greater MHC IId in the vastus lateralis muscle than normal controls (Daugaard et al., 1999). In rats with congestive heart failure, which is associated with a shift in fast fiber subtypes $\text{IIA} \rightarrow \text{IIB}$,
GH administration shifted fiber type profile back toward a normal IIA fiber percentage (Libera et al., 2004). GH has also been shown to promote a fast to slow shift back toward normal when given to hypophysectomized rats (Ayling et al., 1989).

Other investigations have reported different results. Roy et al. (1996) found no change in fiber type profile with GH administration to hypophysectomized rats. Likewise, supraphysiological exposure to GH or IGF-1 in non-hormone deficit animals did not appear to result in any significant fiber type shift (Aroniadou et al., 1996; Florini & Ewton, 1989; Hikida et al., 1995; Lewis et al., 1997a; Lewis et al., 1997b; Lewis et al., 1998). These data suggest that GH or IGF-1 may help maintain “normal” fiber type profiles, while giving supraphysiological doses of GH or IGF-1 to normal animals may not promote further fiber type profile changes.

The results of the current investigation are different than those of most of the current literature with the exception of Lynch et al. (2001) who found an increase in IIA fiber type percentage in the soleus muscle following IGF-1 administration. These data are supported by others who have reported an association between mechano-growth factor (MGF), an IGF-1 isoform, and increases in MHC Ila mRNA expression and decreases in MHC Iβ mRNA expression (Dai, Wu, Yeung, & Li, 2010; Maricic et al., 2008). The difficulty in reconciling the existing literature with the current investigation is that there are no known previous studies investigating the effects of GH or IGF-1 on muscle fiber type composition in diabetic mice. As previously outlined, potential confounding factors include species, strain, muscle being examined, hormone delivery method, hormone dose, fiber typing method, and presence or non-presence of diabetes.
The previous work of List et al. (2009) provided a basis for the current investigation, but they did not examine muscle fiber characteristics. They also used GH but not IGF-1 in that investigation (List et al.). Others have utilized the same strain of mice in their control group, but utilized a transgenic bGH overexpression model in non-diabetic mice (Schuenke et al., 2008). While type 2 diabetes alone did not have an effect on fiber type, it is unknown if there was an interaction between GH or IGF-1 and diabetes that affected fiber type transformations.

The mechanisms behind these fiber type changes were not directly examined in the current investigation, but there are some possible intermediates at work. Both calcineurin, a phosphatase stimulated by calcium, and PGC-1α, a nuclear transcription factor, are associated with increases in MHC Iβ expression (Delling et al., 2000; Handschin et al., 2007; Koullmann et al., 2008; Kramer et al., 2006; Naya et al., 2000; Russell, 2005; Torgan et al., 2001; Vescovo et al., 2005). Calcineurin is also likely an intermediate involved in calcium-dependent signaling that is associated with activity-induced muscle fiber type switching (Serrano et al., 2001). PGC-1α has been implicated in promoting MHC IIa expression, but does not appear absolutely necessary for a shift toward MHC IIa expression (Geng et al., 2010; Handschin et al.). Expression of both calcineurin and PGC-1α may be induced by GH or IGF-1 (Musaro et al., 1999; Semsarian et al., 1999; Vescovo et al.). Diabetes, on the other hand, is associated with decreased expression or action of PGC-1α (Barres et al., 2009; Bonen et al., 2009; Olesen et al., 2010; Roberts-Wilson et al., 2010; Russell). Thus in the GH, IGF-1, and Combo groups the presence of diabetes could be working to decrease calcineurin and PGC-1α.
expression, while the GH and/or IGF-1 could be working to enhance calcineurin and PGC-1α expression.

If indeed calcineurin and/or PGC-1α are responsible for maintaining MHC Iβ expression and type I fiber type expression, type 2 diabetes may not decrease the expression of calcineurin or PGC-1α enough to alter MHC I expression based on the results of the current investigation. If calcineurin and PGC-1α expression were increased with GH or IGF-1 administration one would expect an increase in the percentage of type I fibers or preferential hypertrophy of type I fibers given the evidence that both promote MHC Iβ expression (Delling et al., 2000; Handschin et al., 2007; Koulmann et al., 2008; Kramer et al., 2006; Naya et al., 2000; Russell, 2005; Torgan et al., 2001; Vescovo et al., 2005). In the current investigation, the results are not that clear cut. One possible explanation is that PGC-1α expression was increased in the hormone conditions, but calcineurin was not. Because PGC-1α has been shown to increase MHC Iβ and IIa expression, perhaps the MHC IIa expression was dominant.

While calcineurin has generally been shown to increase MHC Iβ expression, the effects of calcineurin on fast MHC isoforms (IIa, IId, IIb) appear to be variable. Blocking calcineurin does not appear to have any effect on MHC IIa or IId mRNA expression (Meißner et al., 2001). Conversely, Koulmann et al. (2008) blocked calcineurin activity in rats resulting in a fiber type shift with fewer type I fibers and more type IIA fibers in the soleus. Calcineurin Aα has been identified as the dominant form of calcineurin in skeletal muscle (Parsons et al., 2004). In calcineurin Aα knockout mice, no significant fiber type shift in the soleus muscle has been noted (Parsons et al., 2004).
Interestingly the same investigation found significant shifts in fiber type with fewer type I fibers and more type II fibers in other muscles with a faster overall fiber type profile (Parsons et al., 2004). Another confounding piece of evidence reported similar calcineurin mRNA levels in the vastus lateralis of humans with significantly different percentages of type I fibers (Kramer et al., 2006). Even with what is known about calcineurin and PGC-1α regarding muscle fiber type, clearly the entire scope of the interaction between these factors and fiber type composition have not been fully elucidated.

While calcineurin and PGC-1α have been associated with fiber type expression, they may not be responsible for fiber type shifts in all situations. There are also other potential pathways and intermediates at work. Using PGC-1α knockout mice Geng et al. (2010) found that PGC-1α is not necessary for IIB to IIA fiber type shifts with exercise. Another potential mediator of fast MHC expression is mitogen-activated protein kinase 6 (MKK6). MKK6 is part of the MAPK family of signaling molecules that can promote cell differentiation. MKK6 is associated with promoting fast MHC expression in C2C12 cell cultures (Delling et al., 2000). These results suggest that not all fiber type modulation is associated with PGC-1α or calcineurin.

Another factor that greatly influences muscle fiber type is nervous system activity and stimulation (Grossman, Roy, Talmadge, Zhong, & Edgerton, 1998; Gundersen, Leberer, Lomo, Pette, & Staron, 1988; Hamalainen & Pette, 1996; Huey, Haddad, Qin, & Baldwin, 2003). Motor neurons provide almost constant stimulation of postural muscles (Chin et al., 1998). Calcineurin is also likely involved in this process via activation due
to an increase in intracellular calcium concentrations (McCullagh et al., 2004; Serrano et al., 2001). GH has been shown to promote proliferation of VSC4.1 neuronal hybrid cells (Lyuh et al., 2007). It has also been implicated in promoting growth of adult neurospheres from GH deficient mice (McLenachan, Lum, Waters, & Turnley, 2009). Motor neurons have also been shown to increase in size with exposure to GH (Chen, Lung, Burgess, Rudisch, & Mellwain, 1997; Parsons et al., 2003) and IGF-1 has been shown to promote nerve sprouting (Messi & Delbono, 2003). Therefore, it is possible that motor neuron size or activity affects the action of GH or IGF-1 on particular fiber types. Based on the available evidence, it seems likely that any preferential action would influence type I fibers because most postural muscles are rich in type I fibers. The shift toward type IIA fibers in the soleus muscle with GH or IGF-1 administration is the opposite of what might be expected. Ultimately, the shift in fiber type toward IIA fibers in the soleus muscle with GH and IGF-1 treatment cannot be fully explained using known pathways and intermediates, suggesting a complex interplay involving a variety of physiological factors.

In the plantaris muscle the only significant difference in fiber type composition between the groups was a lower percentage of type IID fibers in the Combo group compared to the Control group and fewer IIDB fibers in the GH and Combo groups compared to the DM group (Figure 19). Because there were no other significant fiber type differences, it is difficult to draw any conclusions about the direction of fiber type shifts in the plantaris muscle with GH or Combo administration. The overall fiber type
profile of the plantaris muscle was not substantially altered with diabetes or administration of GH or IGF-1.

In the DRG, the DM group had a significantly lower percentage of type I fibers than the control group (Figure 21). Treatment with GH did not reverse this, but the treatments containing IGF-1 (IGF-1 and Combo groups) appeared to restore the proportion of type I fibers to that of the Control group. Treatment with IGF-1 also resulted in fewer type IID fibers in the IGF-1 and Combo groups. Overall these data suggest IGF-1 may promote a fast to slow fiber type shift with increased type I fiber expression and decreased type IID expression in the DRG of diabetic mice. It should be noted that in the process of sectioning and staining it was observed that the type I fiber content of the DRG varied substantially if the muscle sections were near the ends of the muscle group compared to sections in the mid-muscle belly. Analysis was done on sections taken from the mid-muscle belly, but it is impossible to guarantee that all sections were precisely in the same location. Serial sections from the same mid-muscle belly area did have fibers that could be tracked throughout a series of serial sections, thus the muscle sections analyzed did appear to represent comparable areas of the DRG across mice.

The fiber type profile of the SWG was nearly identical across groups (Figure 22). The only exception was a significantly higher percentage of IIB fibers in the Combo group compared to the Control. Although statistically significant, this difference does not appear to represent a physiologically relevant difference as the percentage of type IIB fibers was 94% in the Control group vs. 97% in the Combo group. Even with this
difference, type IIB fibers were almost the exclusive fiber type represented in the SWG in all groups.

In summary, obesity and type 2 diabetes do not appear to substantially alter fiber type expression in the triceps surae muscle group of C57BL/6J mice. With the administration of GH and/or IGF-1 in diabetic mice, the most striking fiber type shift was in the soleus muscle resulting in an increase in the proportion of type IIA fibers and a concomitant decrease in IIAD fibers. GH also appears to decrease the proportion of type I fibers in the soleus muscle. There were very few fiber type differences in the plantaris muscle or gastrocnemius muscle with these hormone treatments. Because the major fiber type shifts were in the soleus muscle, which has the slowest fiber type profile, the effects of GH and IGF-1 in fiber type transformations in diabetic mice appear to be somewhat muscle dependent. Pathways implicated in fiber type expression and transformation include the intermediates calcineurin and PGC-1α, but the known effects of diabetes, GH, and IGF-1 on these intermediates and pathways do not fully account for the observed fiber type changes.
Conclusions

In conclusion, several important findings can be drawn from the data in this investigation. These data provide insight into muscle characteristics in the development of type 2 diabetes, the muscle hypertrophic response to GH and IGF-1 treatment in diabetes, and muscle fiber type transformations with GH and IGF-1 treatment of type 2 diabetes. The most profound muscle fiber type transformations occurred in the soleus muscle (see Figure 27 for an overview). Despite minimal fiber type shifting in the plantaris and gastrocnemius muscles, muscle fiber CSA did respond to hormone treatment in these muscles (see Figure 28 for an overview).
Figure 27. Summary of muscle fiber type composition and muscle fiber CSA in the soleus muscle of C57BL/6J mice. GH and combination treatment shifted toward a type II A fiber type with decreases in type I and type IIAD fibers. IGF-1 shifted toward a type II A fiber type with a decrease in type IIAD fibers. Type IC and IIC fibers were not included because they comprised less than five percent of the total fibers present. There were an insufficient number of type IID fibers to do statistical analysis for muscle fiber CSA. CSA = cross-sectional area; % = percent muscle fiber type.
Figure 28. Overview of muscle fiber CSA changes in the plantaris and gastrocnemius muscles in C57BL/6J mice. Diabetic mice consistently had a greater muscle fiber CSA across muscles and fiber types compared to lean control mice. GH preferentially induced hypertrophy in all fast fiber types. IGF-1 preferentially induced hypertrophy in the slower fiber types of each particular muscle. There were an insufficient number of IIAD fibers in any of the muscles to do statistical analysis on muscle fiber CSA.

Type 2 diabetes does not appear to alter fiber type composition of the soleus, plantaris, or gastrocnemius muscles in C57BL/6J mice. This suggests that fiber type composition is not a critical factor in the development of type 2 diabetes. It also indicates that insulin resistance can develop independently of muscle fiber type profile. Given these results, muscle fiber type composition does not appear to be a valuable predictor of insulin resistance. Even if muscle fiber type composition does not contribute to the
development of type 2 diabetes, promoting a slower muscle fiber type profile could still prove valuable in the treatment of the disease.

The current investigation confirms that GH does induce muscle hypertrophy. This hypertrophy does not appear to be the result of fluid retention given that muscle fiber CSA increased significantly with GH administration in most fiber types examined. The hypertrophic response to IGF-1 treatment was smaller than with GH. The increase in LBM with IGF-1 was approximately half of what it was with GH (13% vs. 26%). The overall response of muscle fiber CSA to IGF-1 was also not as profound as with GH. This suggests that GH may have independent actions or that IGF-1 kinetics are different with GH administration compared to IGF-1 administration.

Both GH and IGF-1 appeared to preferentially induce hypertrophy in some fiber types but not others. GH appears to mediate preferential hypertrophy of fast fiber types while IGF-1 mediates preferential hypertrophy of the slower muscle fiber types of a given muscle (see Figure 29). GH did not affect type I CSA in the soleus muscle. GH did increase muscle fiber CSA in all the fast fiber subtypes (IIA, IID, IIDB, IIB) in all three muscle groups compared to the DM group. This was a novel finding and does not necessarily fit into any known mechanism regarding muscle hypertrophy with GH or IGF-1 administration.
Growth Hormone

↔ I ↑↑↑IIA ↑↑↑IIAD ↑↑↑IID ↑↑↑IIDB ↑↑↑IIB

IGF-1

↑↑↑I ↑↑↑IIA ↔ IIAD ↔ IID ↓IIDB ↓IIB

Figure 29. Summary of the effects of growth hormone and IGF-1 on muscle fiber CSA in the triceps surae muscle group of diabetic C57BL/6J mice. Overall, growth hormone induced hypertrophy of all fast fiber types while IGF-1 induced hypertrophy of the slower fibers types within each muscle. In some cases IGF-1 induced a small increase in type IIAD and IID fiber CSA, but overall IGF-1 induced little change when the results of all muscle groups were summarized. ↑ = increase in muscle fiber CSA. ↓ = decrease in muscle fiber CSA. ↔ = no overall change in muscle fiber CSA.

Treatment with GH in diabetic mice resulted in a significant fiber type shift in the soleus muscle toward type IIA fibers with fewer IIAD fibers and a trend toward decreased type I fibers (see Figure 30). IGF-1 treatment in diabetic mice also resulted in a significant fiber type shift in the soleus muscle away from type IIAD fibers and toward type IIA fibers although the proportion of type I fibers was maintained (Figure 30). Combination treatment with GH and IGF-1 resulted in a muscle fiber type profile in the soleus similar to that of GH alone. It is difficult to gauge whether these fiber type shifts had a significant impact on glucose metabolism. If a shift toward IIA fibers in the soleus were favorable for glucose metabolism, it might be expected that blood glucose concentrations would be very similar in the GH and IGF-1 groups because they had a similar proportion of IIA fibers. Based on data from Dr. List, blood glucose concentrations were lower with GH than IGF-1 (Appendix 1). This suggests any fiber
type transformations with GH are not necessarily related to reductions in blood glucose. This refutes our original hypothesis that muscle fiber type transformations were a key factor in the improvements in glucose metabolism observed with GH treatment of diabetic mice. There were very few alterations in muscle fiber type profile in the plantaris or gastrocnemius muscles with GH, IGF-1, or Combination treatment. Overall the current investigation suggests that muscle fiber type composition and muscle fiber CSA are not directly involved in the development or treatment of type 2 diabetes. Based on these results, we rejected the null hypotheses 1-9 since diabetes and/or hormone treatments yielded significant differences between groups in fiber type profile or muscle fiber CSA.

**Figure 30.** Muscle fiber type composition changes in the soleus of diabetic C57BL/6J mice with GH or IGF-1 treatment. GH induced a shift from both type I and type IIAD fibers toward increased expression of type IIA fibers. IGF-1 induced a fiber type shift from type IIAD fibers to IIA fibers. $\leftrightarrow$ = fiber type transition in the direction of the arrow. $\leftrightarrow$ = no net change in muscle fiber type profile.

When taken together, the results of the current investigation also suggest that GH and IGF-1 administration have different effects on overall muscle hypertrophy, muscle fiber CSA, and muscle fiber type composition (see Figures 29 and 30 for an overview of
the differences between GH and IGF-1). This makes it questionable whether the effects of GH on skeletal muscle are completely mediated by IGF-1. The difficulty in the interpretation of these results is that the independent effects of GH and IGF-1 cannot be fully elucidated based on the current investigation. GH administration induced large increases in circulating IGF-1, thus the results in the GH group cannot be completely attributed to GH. Nonetheless, the current investigation suggests that GH may have effects on skeletal muscle that IGF-1 alone does not.

Future Directions

While the current investigation provided some novel insight into the effects of GH and IGF-1 on muscle fiber phenotype in mice with diet-induced diabetes, it also provided the genesis for other questions to be investigated in the future. Given the results of the current investigation and recent work by List et al. (2009), further study of GH as a treatment of type 2 diabetes is warranted. IGF-1 should also continue to be examined at least until the mechanisms behind its lack of effectiveness are better understood. A better understanding of the unique effects of different IGF-1 isoforms could also be valuable and still holds promise in the development of an IGF-1-based diabetes treatment. It cannot be ruled out that a different IGF-1 isoform would yield different results.

Regarding muscle fiber phenotype and GH or IGF-1, a remaining question is whether the effects of GH and IGF-1 noted here are due to the hormones alone or if they have an interaction with diabetes. Ideally a similar study could be performed with administration of GH and IGF-1 to lean control mice and diabetic mice. This would allow for comparisons between lean and diabetic mice of the same genotype given GH
or IGF-1. It would also prove valuable to elucidate if the effects noted in the current investigation are gender specific by doing similar research in female mice.

Given the relative ineffectiveness of IGF-1, further research is warranted to determine if this is true for IGF-1 in general or only for specific isoforms. Because different IGF-1 isoforms can have unique effects depending on the age of the animal, it seems reasonable that the unique physiological environment of diabetes may yield differential effects depending on the isoform (Barton, 2006; Barton et al., 2010). It may also be worth investigating whether administration of species-specific IGF-1 isoforms would yield a more robust response.

To further elucidate the role of IGF-1 in diabetes treatment, it would be useful to know what level of IGF-1 exposure is actually achieved in skeletal muscle with systemic delivery of IGF-1. Because it is not clear whether the lack of muscle response to IGF-1 was due to lack of exposure or lack of response at the muscle level, testing muscle levels of IGF-1 for multiple isoforms would be useful. This would also provide more insight into whether the autocrine/paracrine IGF-1 response to GH yields greater muscle exposure to IGF-1 than subcutaneous injections of IGF-1. Based on the current investigation it appears GH has more promise as a treatment agent for type 2 diabetes compared to IGF-1 but some of these questions need to be answered before ruling out IGF-1 as a viable treatment option.

Given that GH has been proven useful in improving glucose metabolism in type 2 diabetes, further study of the signaling events associated with GH in the context of type 2 diabetes could prove useful. Because increases in the p85α subunit of PI3K have been
implicated in contributing to insulin resistance with GH, studying this intermediate with GH in diabetes may provide novel insight into the signaling events under these circumstances (Dominici et al., 2005). Although in the present study the GH group likely had insulin resistance given little change in plasma insulin concentrations with GH administration (Appendix H), it would also be worthwhile to examine insulin signaling with GH administration in diabetic mice. Given the proposed role of PGC-1α in insulin resistance and diabetes, it would also be interesting to explore whether GH stimulates PGC-1α expression in the context of type 2 diabetes. Because PGC-1α has been implicated in other metabolic aspects related to diabetes, it holds some promise as a mediator or predictor of the glucose lowering effects of GH in diabetes. The other signaling pathway worthy of further study in this context is the MAPK pathway that promotes cell proliferation. Especially in the case of IGF-1, this pathway has been thought by many to play a role in the development of multiple types of cancers with IGF-1 receptor inhibitors being studied for potential cancer treatment (Grimberg & Cohen, 2000; Gualberto & Pollack, 2009; LeRoith & Roberts, 2003).

Because of the potential link between muscle lipid accumulation and insulin resistance and type 2 diabetes, it seems reasonable to examine the lipid storage in muscle with GH administration for type 2 diabetes. Ideally, the total lipid content, lipid storage pattern and location, and lipid type should all be examined. If indeed lipid toxicity is related to or responsible for insulin resistance, then perhaps the lipolytic effects of GH could be beneficial in insulin signaling by decreasing or altering lipid storage in skeletal muscle. Enhancing our understanding of skeletal muscle lipid storage patterns with type
2 diabetes and GH treatment could provide key information regarding the effects of GH on skeletal muscle and ultimately treatment of type 2 diabetes.

Further human trials examining the effects of GH or IGF-1 in type 2 diabetes are also warranted given the promise of GH for diabetes treatment and the limited published research regarding human studies of GH in type 2 diabetes. Future trials should be of sufficient length to ensure that the full effects of GH and/or IGF-1 on body composition have an opportunity to manifest themselves. It may also be beneficial to examine muscle characteristics and GH, IGF-1, and insulin signaling as part of these human trials to better understand the etiology of the interaction between GH and IGF-1 with diabetes. Further research using both animal and human models will be important to realize the full potential for GH or IGF-1 as possible treatments for type 2 diabetes.

Given the anabolic effects of GH on muscle mass noted here and in previous investigations, GH also has promise for use in situations beyond type 2 diabetes. GH could prove beneficial in situations associated with muscle atrophy including aging, spaceflight, muscle-wasting diseases, immobility, nerve and spinal cord injury, and musculo-skeletal rehabilitation. One of the most often cited problems with the use of GH as a therapeutic agent is its negative effects on insulin resistance. A more thorough understanding of this effect may provide insight regarding ways to offset or minimize the negative side effects of GH therapy.

Many of the effects of GH and IGF-1 found here are not readily explained by known mechanisms and appear inconsistent with much of the available literature. The biggest confounding factor in the current investigation is the presence of type 2 diabetes.
To the author’s knowledge there are no previous investigations that have examined muscle fiber phenotype with GH or IGF-1 in diabetic mice. Given the great promise shown by GH as a therapeutic agent in type 2 diabetes future research should continue to focus on elucidating the effects of GH on body composition, muscle characteristics, and signaling events at work. Even though muscle characteristics do not appear to be directly related to the favorable effects of GH on type 2 diabetes, further research is warranted to confirm these findings and better understand the physiological events at work.
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APPENDIX A: MYOSIN ATPASE HISTOCHEMISTRY

<table>
<thead>
<tr>
<th>Alkaline Preincubation</th>
<th>Time</th>
<th>Acid Preincubation</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10% formalin fixation (4°C)</td>
<td>5 min</td>
<td>1. No Step</td>
<td></td>
</tr>
<tr>
<td>2. Solution 2 rinse</td>
<td>1 min</td>
<td>2. Preincubation</td>
<td>7 min</td>
</tr>
<tr>
<td>3. Preincubation</td>
<td>20 min</td>
<td>3. Solution 2 rinse</td>
<td>1 min</td>
</tr>
</tbody>
</table>

(remaining steps are identical for all preincubation pH values)

4. Incubation solution | 45 min |
5. 1% CaCl₂ rinse | 3 x 3 min |
6. 2% CoCl₂ incubation | 2 x 1.5 min |
7. 0.01M $C_8H_{11}N_2O_3Na$ rinse | 6 x 30 sec |
8. $dH_2O$ rinse | 30 sec |
9. 2% $(NH_4)_2S$ incubation | 45 sec |
10. Tap water rinse | 5 min |
11. Dehydration
   - 70% ethanol | 2 min |
   - 90% ethanol | 2 min |
   - 95% ethanol | 2 min |
   - 100% ethanol | 2 min |
12. Clear with CitriSolve | 2 x 2 min |
13. Mount with Canadian Balsam

Acid preincubation (pH 4.1, 4.4)
Solution A
- Na Acetate | 1.94 g |
- $C_8H_{11}N_2O_3Na$ | 2.94 g |
- Add $dH_2O$ to yield 1L

Alkaline Preincubation (pH to 10.0)
Ammonium Sulfide
- $(NH_4)_2S$ | 2 ml |
- $dH_2O$ | 100 ml |

Solution 2 (pH to 7.8)
Tris | 12.1 g |
100 ml 0.18M CaCl₂
Add $dH_2O$ to yield 1L

Incubation solution (pH 9.4)
Sigma 221 | 4.3 ml |
0.18M CaCl₂ | 6 ml |
$dH_2O$ | 45 ml |
ATP | 0.15 g |
APPENDIX B: MYOSIN IMMUNOHISTOCHEMISTRY

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1 min</td>
</tr>
<tr>
<td>Blocking Solution</td>
<td>10 min</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>MOM Ig blocking reagent</td>
<td>60 min</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>MOM diluent</td>
<td>5 min</td>
</tr>
<tr>
<td>Myosin Ab (diluted in MOM diluent)</td>
<td>30 min</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>MOM Biotinylated IgG reagent</td>
<td>10 min</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>ABC reagent</td>
<td>5 min</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>DAB</td>
<td>15 min</td>
</tr>
<tr>
<td>PBS</td>
<td>10 min</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>CitriSolve</td>
<td>2 x 2 min</td>
</tr>
<tr>
<td>Mount</td>
<td></td>
</tr>
</tbody>
</table>

**Blocking Solution**
- Methanol 40 ml
- H2O2 0.4 ml

**MOM Mouse Ig Blocking Reagent**
- Stock solution 2 drops
- PBS 2.5 ml

**MOM diluent**
- Protein concentrate 600 µl
- PBS 7.5 ml

**MOM Biotinylated Anti-Mouse IgG**
- Stock solution 10 µl
- MOM diluent 2.5 ml

**ABC reagent**
- Reagent A 2 drops
- PBS 2.5 ml (mix)
- Reagent B 2 drops (mix)

Allow to stand for 30 min
APPENDIX C: BODY COMPOSITION DATA

_Animal Body Composition. Values are Mean±SD._

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Mass (g)</th>
<th>Lean Body Mass (g)</th>
<th>Fat Mass (g)</th>
<th>Fluid Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.4±2.2</td>
<td>20.6±1.7</td>
<td>2.6±0.7</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>DM</td>
<td>42.2±5.9(^a)</td>
<td>22.6±1.6</td>
<td>15.9±4.1(^a)</td>
<td>2.2±0.3(^a)</td>
</tr>
<tr>
<td>GH</td>
<td>39.6±3.9(^a)</td>
<td>28.3±1.6(^a,b)</td>
<td>7.1±2.1(^a,b)</td>
<td>2.3±0.4(^a)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>46.5±6.5(^a,c)</td>
<td>25.4±2.0(^a,b,c)</td>
<td>16.4±4.7(^a,c)</td>
<td>2.6±0.5(^a)</td>
</tr>
<tr>
<td>Combo</td>
<td>45.2±3.2(^a)</td>
<td>30.9±1.2(^a,b,c,d)</td>
<td>8.5±1.7(^a,b,d)</td>
<td>2.8±0.4(^a)</td>
</tr>
</tbody>
</table>

\(^a\) = significantly different than Control group  
\(^b\) = significantly different than DM group  
\(^c\) = significantly different than GH group  
\(^d\) = significantly different than IGF-1 group

Data provided by Dr. Ed List.
APPENDIX D: TRICEPS SURAE WET WEIGHT RATIOS

Triceps surae wet weight, lean body mass (LBM), wet weight/LBM ratio, total body weight, and wet weight/body weight ratio. Values are (mean±SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet Weight (g)</th>
<th>Lean Body Mass (g)</th>
<th>Wet Wt/LBM ratio</th>
<th>Body Mass (g)</th>
<th>Wet Wt/Body Wt ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14±0.02</td>
<td>20.6±1.7</td>
<td>0.0068±0.0011</td>
<td>26.4±2.2</td>
<td>0.0053±0.0009</td>
</tr>
<tr>
<td>DM</td>
<td>0.16±0.02</td>
<td>22.6±1.6</td>
<td>0.0070±0.0006</td>
<td>42.2±5.9</td>
<td>0.0038±0.0004</td>
</tr>
<tr>
<td>GH</td>
<td>0.18±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.3±1.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.0064±0.0004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.6±3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0046±0.0004&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.15±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.4±2.0&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.0060±0.0006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.5±6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0033±0.0004&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Combo</td>
<td>0.18±0.01&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>30.9±1.2&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td>0.0059±0.0002&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>45.2±3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0040±0.0002&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = significantly different than Control group within respective variable  
<sup>b</sup> = significantly different than DM group within respective variable  
<sup>c</sup> = significantly different than GH group within respective variable  
<sup>d</sup> = significantly different than IGF-1 group within respective variable  

Lean body mass data provided by Dr. Ed List.
APPENDIX E: MUSCLE FIBER TYPE PERCENTAGES

Muscle fiber type percentages. Values are means ± SD.

<table>
<thead>
<tr>
<th></th>
<th>% I</th>
<th>% IC</th>
<th>% IIC</th>
<th>% IIA</th>
<th>% IIAD</th>
<th>% IID</th>
<th>% IIDB</th>
<th>% IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.64±4.2</td>
<td>1.72±0.9</td>
<td>1.62±0.8</td>
<td>43.06±5.4</td>
<td>8.79±4.0</td>
<td>2.93±1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>38.32±4.8</td>
<td>1.43±1.6</td>
<td>1.30±1.0</td>
<td>41.21±8.6</td>
<td>13.48±6.3</td>
<td>2.91±3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>34.36±3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93±0.8</td>
<td>54.95±5.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>6.47±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.14±1.7</td>
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</tr>
<tr>
<td>IGF-1</td>
<td>37.85±4.1</td>
<td>0.68±0.5</td>
<td>0.85±0.8</td>
<td>53.49±6.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>4.69±2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.25±1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combo</td>
<td>31.65±1.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.36±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72±0.4</td>
<td>58.07±3.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.61±2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.89±1.3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>% I</th>
<th>% IC</th>
<th>% IIC</th>
<th>% IIA</th>
<th>% IIAD</th>
<th>% IID</th>
<th>% IIDB</th>
<th>% IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plantaris</strong></td>
<td></td>
<td></td>
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<sup>a</sup> = significantly different than Control group; <sup>b</sup> = significantly different than DM group; <sup>c</sup> = significantly different than GH group; <sup>d</sup> = significantly different than IGF-1 group
APPENDIX F: MUSCLE FIBER CROSS-SECTIONAL AREA

Muscle fiber cross-sectional area (µm²). Values are mean±SD.

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a = significantly different than Control group; b = significantly different than DM group; c = significantly different than GH group; d = significantly different than IGF-1 group
APPENDIX G: PERCENT FIBER AREA

Percent fiber area. Values are mean±SD.

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a = significantly different than Control group; b = significantly different than DM group; c = significantly different than GH group; d = significantly different than IGF-1 group
Mouse-specific IGF-1 concentrations (mean±SE). This is only a measure of mouse specific IGF-1 and does not include human IGF-1. a = significantly different than Control group; b = significantly different than DM group; c = significantly different than GH group.

Data provided by Dr. Ed List
APPENDIX I: SERUM INSULIN AND GLUCOSE

Serum Insulin

Fasting serum insulin concentrations after three weeks of placebo or hormone treatment (mean±SE).

Serum Glucose

Fasting serum glucose concentrations after three weeks of treatment (mean±SE). Insulin and glucose data provided by Dr. Ed List.
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