ALTERING MUSCLE HYPERTROPHY AND FAT ACCUMULATION IN MYOSTATIN NULL MICE

BY

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DISSERTATION

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ABSTRACT

Myostatin is a negative regulator of muscle growth. Loss of myostatin function results in a dramatic phenotype in several species including cattle, mice, dogs and humans. Myostatin mutants exhibit wide-spread hypermuscularity and decreased fat accumulation. Though the phenotype of myostatin mutant animals is very striking, the question remains whether the loss of myostatin function fundamentally changes growth potential, metabolism, or inherent regulation in tissues, or if it is simply an effect independent of other factors. To that end, we sought to increase muscle growth even further in myostatin null mice through the use of the β-adrenergic agonist clenbuterol. The effects of clenbuterol treatment and the loss of myostatin function are quite similar and, in this study, were completely additive. Clenbuterol treatment increased muscle weights and protein accretion and reduced fat accumulation in both wild type and myostatin null mice. There was a significant interaction of genotype and clenbuterol treatment for gastrocnemius muscle weight, but the magnitude of the differences in weight gain between myostatin null and wild type was very small. We also aimed to alter fat accumulation and metabolism in myostatin null mice by feeding diets high in fat. Other research indicated that myostatin null mice were resistant to fat accumulation induced by high-fat feeding. In our study, fat accumulation was increased in both wild type and myostatin null mice fed high-fat diets, but the magnitude of the increase in myostatin null mice was less than that of wild type mice. This suggests that myostatin null mice are partially resistant to high-fat diet-induced obesity. Markers of metabolic syndrome often induced by high-fat feeding in mice were also improved in myostatin null compared with wild type mice. Liver fat content, serum triglycerides and serum leptin

levels were lower in high-fat fed myostatin null mice than similarly fed wild type mice. Glucose tolerance, however, was altered in both genotypes by high-fat feeding and did not show an improvement in myostatin null mice. Given the partial resistance to high-fat induced fat accumulation, we investigated expression of adaptive thermogenic genes and found that some genes were upregulated specifically in high-fat fed myostatin null mice. These changes, however, likely do no fully account for the resistance to fat gain in myostatin null mice. Overall, we conclude from these two studies that, while the loss of myostatin function does have a dramatic effect in increasing muscle growth and reducing fat deposition, it does not represent maximal muscle or minimal fat accumulation. Muscle and fat tissues do respond to both beta-adrenergic agonists and to increased dietary fat and calories.

This dissertation is dedicated to my husband, Ryan, and my family who inspire me to start earlier, work harder and keep better records.

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CHAPTER 1

REVIEW OF LITERATURE

Introduction

In the approximately 10 years since the identification of myostatin, a growth factor involved in inhibiting muscle growth, much has been made about the impressive increase in muscle mass resulting from mutations or inhibition of this protein. Several people have speculated whether myostatin inhibition may provide possible treatments for muscular dystrophy, sarcopenia or even lead to enhanced athletic performance. Similarly, the apparent leanness of these animals has also led to speculation regarding the use of myostatin inhibitors for the treatment of obesity and metabolic syndrome. The goal of experiments comprising this thesis was to determine if muscle growth due to deletion of the myostatin gene would render muscle and fat tissue unresponsive to further alterations. Clenbuterol treatment was used as an additional supplement to increase muscle growth, and high-fat diet-induced obesity was employed as a means of increasing fat accumulation.

Myostatin

Discovery and Characteristics of Myostatin-mutant Animals

Myostatin, originally termed growth and differentiation factor (GDF)-8, was identified in 1997 by McPherron, Lawler and Lee (McPherron *et al.*, 1997) due to its homology with other members of the transforming growth factor (TGF)-β superfamily. To determine its' biological function, this group disrupted GDF-8 in embryonic stem cells of mice, preventing the protein from being cleaved for activation. The resulting

mice were hypermuscular with individual muscles weighing double that of wild type mice. For several years prior to this discovery, double-muscled cattle had been noted for their hypermuscularity and the gene involved in this phenomenon had been mapped to chromosome 2 and termed the *mh* locus for its' involvement in muscle hypertrophy (Charlier *et al.*, 1995; Dunner *et al.*, 1997). Soon after the discovery of myostatin, it was determined that the *mh* locus was indeed the myostatin gene (Grobet *et al.*, 1997; Grobet *et al.*, 1998; Kambadur *et al.*, 1997; McPherron and Lee, 1997). The unifying features of myostatin mutants, which have now been identified or created in several species, include pronounced increases in muscle mass due to both hyperplasia and hypertrophy accompanied by decreased fat accumulation.

Several different mouse models involving myostatin have been identified or generated including the original myostatin null mouse (McPherron *et al.*, 1997). When compared to wild type littermates, myostatin null mice are 30% heavier. This additional body weight is the result of widespread increases in muscle mass. Individual muscles of myostatin null mice weigh two to three times that of wild type mice. The increase in body weight observed at 3 months of age is attributed primarily to an increase in muscle weight because the myostatin null, as a proportion of body weight, has less adipose tissue than the wild type. At its widest point, the tibalis anterior (TA) muscle of the myostatin null contains 86% more muscle fibers than wild type controls. Per gram of tissue, the TA muscle contains 50% more DNA. Additionally, the mean cross sectional area of individual fibers is increased 7% and 22% in the TA and gastrocnemius muscles, respectively. Furthermore, the protein:DNA ratio is increased in the myostatin null

animal when compared to the wild type. These changes are observed in both male and female myostatin null mice (McPherron *et al.*, 1997).

However, other mouse models involving myostatin do exist. A hypermuscular line of mice developed from long-term selection for growth possess a mutation in the propeptide region of myostatin which may impact dimerization of the protein (Szabo *et al.*, 1998). Transgenic expression of myostatin inhibitors (Lee and McPherron, 1999) or overexpression of the propeptide of myostatin (Yang *et al.*, 2001) result in hypermuscularity, though increased muscle mass in the propeptide overexpressing mice results from increased hypertrophy and not altered hyperplasia. Despite the presence of these other models, the original myostatin null mouse continues to be the most-commonly used model for research into myostatin function.

First described in 1807 (Culley, 1807), double-muscling has been reported in several breeds of cattle including Belgian Blue, Blonde d'Aquitaine, Charolais, Gasconne, Limousin, Maine-Anjou, Parthenaise, Asturiana, Rubia Gallega, and Piedmontese. The double-muscling phenomenon in all these breeds except Limousin and Blonde d'Aquitaine results from loss-of-function mutations in myostatin (Grobet *et al.*, 1998). Similar to other myostatin mutants, double-muscled cattle have increased muscle mass and reduced fat and bone mass, thought to reflect an alteration in nutrient partitioning (Shahin and Berg, 1985). Double-muscled cattle have leaner carcasses than normal cattle and are more efficient at converting food to body tissue (Arthur, 1995). These animals have an increased amount of saleable meat product and therefore, have the potential to increase profits. However, the use of double-muscled cattle is somewhat difficult in a production system due to reduced fertility and the need for calving

assistance due to increased dystocia. Meat from double-muscled cattle, reflective of leaness throughout the body, is often lacking in intramuscular fat, or marbling. However, it has been reported that double-muscled cattle have less connective tissue within the muscle and therefore, are more tender (Hanset, 1991).

The bully whippet is another example of a naturally occurring myostatin mutation. Whippets normally resemble greyhounds, but the bully whippet, possessing a two-base-pair deletion in the third exon of myostatin, exhibits a wide-spread increase in muscling. Like greyhounds, whippets are often used in racing; dogs heterozygous, but not homozygous, for the mutation show increased race performance being significantly faster than normal whippets (Mosher *et al.*, 2007). In contrast to other myostatin mutants, Texel sheep do not have a mutation in the coding region of myostatin. Texel sheep, renowned for their meatiness, posses a mutation in the 3' untranslated region of the myostatin gene which affects the binding of two microRNAs which are strongly expressed in muscle. This results in downregulation of myostatin translation and reduced circulating myostatin by two-thirds contributing to their increased muscle hypertrophy (Clop *et al.*, 2006). Finally, there is one human with a confirmed mutation of myostatin; this German boy exhibited increased muscle mass and strength from birth (Schuelke *et al.*, 2004).

The myostatin gene has been mapped to chromosome 2 in humans and in other species including cattle, mice and sheep (Charlier *et al.*, 1995; Clop *et al.*, 2006; Szabo *et al.*, 1998). The human gene contains 3 exons which encode for 125, 124 and 126 amino acids each (Gonzalez-Cadavid *et al.*, 1998). The promoter region of the myostatin gene has multiple response elements for glucocorticoids, androgens, monocyte enhancing

factor, peroxisome proliferator-activated receptor gamma, and nuclear factor κ-β (Ma *et al.*, 2001).

Myostatin mRNA is primarily expressed in muscle and is detected as early as 9.5d post-coitus in mice (McPherron et al., 1997) and 21d post-conception in swine (Ji *et al.*, 1998). Myostatin, however, has also been documented in the heart (Sharma *et al.*, 1999), the lactating mammary gland of swine (Ji *et al.*, 1998), brain and ovarian tissue in fish (Roberts and Goetz, 2001; Rodgers *et al.*, 2001), and at low levels in adipose tissue in mice (Allen *et al.*, 2008; McPherron *et al.*, 1997). Expression is influenced by several factors including strength training (Roth *et al.*, 2003), disuse (Reardon *et al.*, 2001) glucocorticoid treatment (Ma *et al.*, 2003), obesity (Allen *et al.*, 2008), and the administration of other myogenic factors such as MyoD (Spiller et al., 2002).

Myostatin is a 376 amino acid peptide that is proteolytically processed into a 15 kDa active protein. It contains all the hallmarks of growth factors belonging to the TGF-β superfamily and includes a proteolytic processing site, a signal sequence for secretion, and an active carboxy-terminal site with a highly conserved series of cysteine knots (McPherron et al., 1997). The carboxy-terminal end possesses 100% homology across mouse, rat, human, swine, chicken and turkey forms of the growth factor, suggesting that the function of myostatin is well-conserved (McPherron and Lee, 1997). Post-translational cleavage frees the active protein from the propeptide, though the two parts remain associated and the propeptide acts as an endogenous inhibitor of myostatin activity (Hill *et al.*, 2002). Transgenic overexpression of the myostatin propeptide in mice leads to a phenotype similar to that of myostatin null mice (Jinzeng Yang, 2006).

The majority of circulating myostatin is associated with its propeptide to form a latent complex unable to bind to receptors (Hill et al., 2003). Myostatin is also inhibited by follistatin, follistatin related gene proteins (FRG-P) and GDF-associated serum protein (GASP-1) (Hill *et al.*, 2002; Hill *et al.*, 2003; Lee and McPherron, 2001). The mechanism of myostatin activation *in vivo* is unknown. Once free, myostatin binds to activin type II receptors (ActIIB) leading to the activation of type I receptors and the Smad proteins (Lee and McPherron, 2001).

Impact on Muscle Development, Growth and Atrophy

In C_2C_{12} cells, a well-known mouse myoblast cell line (Del Aguila *et al.*, 1999; Milasincic *et al.*, 1996; Semsarian *et al.*, 1999), myostatin treatment decreased proliferation as well as DNA and protein synthesis (Rios *et al.*, 2002; Taylor *et al.*, 2001; Thomas *et al.*, 2000). Furthermore, myostatin treatment increased the number of myoblasts observed in the G_1 and G_2 phase of the cell cycle and decreased the number of myoblasts in the S phase (Joulia et al., 2003). These data indicate that myostatin may modulate muscle development by targeting the cell cycle.

Cell cycle progression from the G_1 to the S phase requires phosphorylation of the retinoblastoma susceptibility gene product (Rb) by cyclin-cyclin dependent kinase (Cdk) complexes. Hypophosphorylated Rb binds to transcription factors important in S phase gene activation such as E2F-DPI and prevents progression into the S phase. Phosphorylation of Rb by G_1 Cdks releases these factors allowing passage through the G_1/S checkpoint (La Thangue, 1996).

In response to myostatin signaling, there is an increase in the expression of the cyclin kinase inhibitor p21 and a decrease in both the level of Cdk 2 and its activity

resulting in accumulation of hypophosphorylated retinoblastoma. This leads to an arrest of myoblasts in the G_1 phase of the cell cycle (Thomas *et al.*, 2000). In the absence of myostatin, high levels of p21 are not present and Cdk2 remains active. Thus, the Rb protein becomes hyperphosphorylated and the cells enter the S phase of the cell cycle. In the case of animals with loss-of-function mutations, the absence of myostatin signaling ultimately leads to increased proliferation of myoblasts during development and an increased number of mature muscle fibers. Normal myostatin signaling results in cell cycle arrest of myoblasts thereby negatively influencing muscle development.

Myostatin can also inhibit myoblast differentiation. Myostatin treatment of C_2C_{12} cells decreased MyoD and myogenin mRNA and protein levels and downregulated the activity of their downstream target, creatine kinase (Joulia et al., 2003; Rios et al., 2002). Langley and co-workers (2002) observed increased Smad3 expression and association with MyoD in response to myostatin treatment. This resulted in a decrease in myoblast differentiation that could not be rescued with MyoD treatment, suggesting that the effect of myostatin may be mediated through modulation of muscle regulatory factor activity. This theory is supported by the finding that double-muscled bovine fetuses have higher levels of MyoD than wild type fetuses (Oldham et al., 2001). However, Spiller and others (2002) showed that MyoD induced myostatin expression suggesting myostatin and MyoD may reciprocally regulate the others' expression. Furthermore, though both myostatin and MyoD expression peak during the G_1 phase of the cell cycle, myostatin was expressed at high levels during the G_1/S-phase and G_0 phases. This suggests that myostatin expression is controlled independently of MyoD in those phases.

In addition to prenatally regulating myoblast proliferation and differentiation, myostatin may also regulate the postnatal growth of muscle. Specific postnatal inhibition of myostatin leads to muscle hypertrophy (Grobet et al., 2003; Whittemore et al., 2003). In contrast, systemic or muscle-derived overexpression of myostatin leads to a decrease in muscle mass (Reisz-Porszasz *et al.*, 2003; Zimmers *et al.*, 2002). Postnatally, muscle growth is the result of satellite cell proliferation, differentiation and fusion with existing muscle fibers to maintain a relatively constant DNA:protein ratio (Snow, 1990). Myostatin expression has been detected in proliferating satellite cells in chicken and in culture (Kocamis *et al.*, 2001). Additionally, the number of satellite cells per unit length and the number of active satellite cells was higher in myostatin null mice when compared to wild type. Myostatin increases p21 levels in satellite cells similar to proliferating myoblasts and, therefore, may hold satellite cells in a quiescent state. Thus, when myostatin is not present, increased satellite cell activation leads to hypertrophy (McCroskery *et al.*, 2003).

Increased myostatin expression has been observed in several models of muscle atrophy (Dasarathy *et al.*, 2004; Gonzalez-Cadavid *et al.*, 1998; Ma *et al.*, 2003; Reardon *et al.*, 2001; Yarasheski *et al.*, 2002). Furthermore, muscle atrophy caused by glucocorticoids was prevented by when the myostatin gene was deleted (Gilson *et al.*, 2007). Some have suggested that myostatin upregulation reduced satellite cell activity (Dasarathy *et al.*, 2004; McCroskery *et al.*, 2003). However, overexpression of myostatin reduced muscle mass (Reisz-Porszasz *et al.*, 2003) and led to down-regulation of Akt/mTOR signaling (Amirouche *et al.*, 2009). This suggests that the role of myostatin in

muscle atrophy may be due to alterations in protein synthesis in addition to muscle regeneration through satellite cell activity.

Involvement in Adipose Tissue Regulation

In addition to regulating muscle development and growth, it has been suggested that myostatin, or the lack thereof, alters adipose development or fat accumulation. As previously discussed, one unifying feature of myostatin mutant animals is reduced body fat accumulation. Myostatin null mice, unlike wild type mice, do not continue to gain weight after 6 months of age. Adipose tissue pad weight, adipocyte number and size are all reduced in myostatin null mice compared with age-matched wild type mice (McPherron and Lee, 2002). Serum leptin and triglyceride levels are also reduced in myostatin null mice (Guo *et al.*, 2009). Decreased fat accumulation in myostatin null mice does not correspond with reduced feed intake, differences in body temperature regulation, or uncoupling protein (UCP) expression (McPherron and Lee, 2002). However PPARγ and C/EBPα are reduced in myostatin null animals at 12 weeks of age suggesting not only decreased lipid filling of adipocytes, but also decreased adipogenesis (Lin *et al.*, 2002).

Myostatin mutant animals are also resistant to fat gain under conditions which normally produce obesity. When the myostatin null mutation was interseminated with the agouti obese (A^y/a) and leptin obese (ob/ob), mutations, researchers observed reduced obesity and improved glucose metabolism (McPherron and Lee, 2002). Furthermore, it has been suggested that myostatin null and mice overexpressing the myostatin propeptide are resistant to high-fat diet induced obesity (Hamrick *et al.*, 2006; Jinzeng Yang, 2006;

Zhao *et al.*, 2005). This resistance to fat accumulation, however, is not always observed. Incorporating myostatin null alleles into mice deficient for the leptin receptor (db/db) did not reduce fat accumulation in 9 week old mice (Dilger, 2002). Furthermore, when fed a high-energy diet for several months, a double-muscled steer accumulated fat similar to a normal steer (Novakofski and Kauffman, 1981).

In vitro, myostatin promotes (Artaza *et al.*, 2005; Feldman *et al.*, 2006) or inhibits (Kim *et al.*, 2001; Zimmers *et al.*, 2002) adipogenesis depending on the cell type used, suggesting myostatin may function differently during determination and differentiation. Direct injection of myostatin has produced conflicting results (Stolz *et al.*, 2008; Zimmers *et al.*, 2002) with some researches noting whole body cachexia including both muscle atrophy and fat depletion, while others note no change in body composition. These conflicting results may be dose-related as cachexia was induced by a super-physiological dose.

Though predominantly expressed in muscle tissue, myostatin is detectable in fat tissue as well, and is thought to be produced by adipocytes themselves and not simply by stromal cells associated with fat (Allen *et al.*, 2008; McPherron *et al.*, 1997). Myostatin is also upregulated in adipocytes in response to obesity while it is unchanged or slightly downregulated in muscle cells under the same conditions (Allen *et al.*, 2008). Mice overexpressing myostatin specifically in adipocytes have normal body fat mass despite having smaller adipocytes, but have increased energy expenditure and are resistant to diet-induced obesity (Feldman *et al.*, 2006). Mice with adipose-specific knock-out of myostatin did not have altered body composition or fat cell size, but were also not resistant to diet-induced obesity (Guo *et al.*, 2009). These findings suggest that myostatin

production by adipocytes may be one mechanism by which fat attempts to limit its' own growth in obesigenic conditions.

If myostatin does indeed limit adipocyte cell growth, then it is contradictory that myostatin mutant animals, with little or no functional myostatin, accumulate less fat. Thus, the mechanism of resistance to fat accumulation in myostatin mutant animals may be an indirect effect of myostatin on whole-body metabolism as opposed to a direct effect of myostatin on adipocytes. This paradigm would be similar to other mutations which increased muscle hypertrophy, and therefore, resulted in less fat mass and resistance to diet-induced obesity (Izumiya *et al.*, 2008; Lai *et al.*, 2004; Musarò *et al.*, 2001; Sutrave *et al.*, 1990;). While the direct role of myostatin in altering body metabolism cannot be excluded, it seems plausible that changes in fat accumulation in myostatin mutant animals is secondary to altered muscle mass.

Clenbuterol

Generalized β-adrenergic agonist activity

Synthetic β-adrenergic receptor agonists (βAA), such as clenbuterol, bind to β-adrenergic receptors (β-AR) and elicit responses similar to endogenous βAA, epinephrine and norepinephrine. Upon binding, the agonist-receptor complex activates G_s proteins which activate adenylate cyclase producing cyclic adenosine monophosphate (cAMP). Levels of cAMP orchestrate many intracellular events. For example, cAMP binds the regulatory subunit of protein kinase A, allowing the catalytic subunit to phosphorylate other proteins. Some of these proteins are transcription factors, such as cAMP response element (CREB) which binds to and increases the transcription of several genes. Other

proteins phosphorylated by protein kinase A are enzymes; some enzymes, like hormone-sensitive lipase, are activated by phosphorylation while others like acetyl co-A carboxylase are inhibited by phosphorylation (Mersmann, 1998). Thus, binding of βAA to its' receptor has both enzymatic effects and influences gene transcription.

There are three subtypes of β-AR labeled β_1-AR, β_2-AR, and β_3-AR with most tissues containing multiple β-AR subtypes (Minneman *et al.*, 1979). Subtype distribution differs both between and within tissues, and also between species. In humans, heart is the prototypic tissue that responds to β_1-AR agonists, lung for β_2-AR and fat for β_3-AR. However, bovine adipocytes are thought to have more β_2-AR (Houseknecht *et al.*, 1995; Sillence and Matthews, 1994) while swine adipocytes have β_1-AR as their primary transcript (McNeel and Mersmann, 1999). These subtype expression differences in adipocytes may also relate to animal age as β_3-AR expression is enriched in brown adipose tissue of which adult swine and cattle have little (Strosberg, 1997).

Several agonists have been used in human medicine and in livestock production to activate β-AR signaling. While it is convenient to imply that one compound is more selective for one type of β-AR than another, the literature does not always support such sweeping conclusions. Moreover, findings are often confounded by the use racemic mixtures of βAA compounds and differences in receptor distribution in tested tissues. There is some evidence to suggest, however, that clenbuterol is more selective for β_2-AR though it is an agonist for the other subtypes as well (Sillence *et al.*, 1991).

Clenbuterol Alters Body Composition

Oral administration of clenbuterol increased protein:fat ratio in carcasses of several species including cattle, sheep and chickens (Baker *et al.*, 1984; Rehfeldt *et al.*,

1997; Ricks *et al.*, 1984). Additionally, clenbuterol attenuated muscle atrophy caused by hind-limb unloading (Yimlamai *et al.*, 2005), denervation (Zeman *et al.*, 1987), and glucocorticoid treatment (Pellegrino *et al.*, 2004). Skeletal muscle protein accretion is increased by clenbuterol treatment by two actions: first, a transient increase in muscle protein synthesis (Maltin *et al.*, 1989) and second, a more prolonged decrease in muscle protein degradation (Bohorov *et al.*, 1987; Reeds *et al.*, 1986). Clenbuterol treatment causes slow to fast transition of fiber types (Oishi *et al.*, 2004).

In mice, clenbuterol increases muscle hypertrophy mediated by β_2-AR (Hinkle *et al.*, 2002) leading to downstream activation of protein kinase A as described above. However, β_2-AR binding by clenbuterol also activates the $G_i\alpha$-$G\beta\gamma$-phosphatidylinositol 3-kinase (PI3K) /Akt signaling pathway resulting in transiently increased phosphorylation of eukaryotic initiation factor (eIF)4E binding protein-1 and $p70^{S6k}$ (Sneddon *et al.*, 2001). Treatment with rapamycin, the specific inhibitor of m-TOR, reduced clenbuterol-induced muscle hypertrophy and blocked clenbuterol-induced muscle sparing during hindlimb unloading (Kline *et al.*, 2007). Regarding the more long-term reduction in protein degradation, clenbuterol treatment reduced activation of ubiquitin-proteosome (Yimlamai *et al.*, 2005).

In addition to increasing protein accretion, clenbuterol administration reduces fat accumulation. As previously detailed, β-AR binding leads to a signaling cascade resulting in protein kinase A activation which in turn phosphorylates hormone-sensitive lipase, responsible for lipolysis in adipocytes. Protein kinase A also phosphorylates acetyl co-A carboxylase, thereby inhibiting the first step of fatty acid synthesis. As a consequence, clenbuterol reduces adipocyte number and size in cattle (Miller *et al.*, 1988) and overall

fat pad weight in mice (Choo *et al.*, 1992; Page *et al.*, 2004). Others have suggested that clenbuterol induces adipocyte apoptosis as measured by DNA fragmentation, but further study is warranted (Page *et al.*, 2004).

High-fat Diet-induced Obesity

Human obesity, defined as a body mass index in excess of 30 kg/m^2, is increasing in developed nations, and in the United States, over 30% of adults are considered obese (Ogden *et al.*, 2006). Thus, models to study obesity are important and ways to reduce fat accumulation and offset negative health consequences associated with excess weight and body fat are sought. Associated with obesity, though sometimes occurring independently, is metabolic syndrome, a cluster of risk factors for cardiovascular disease of which insulin resistance is thought to be an underlying cause (Kahn *et al.*, 2005). Skeletal muscle is the largest tissue responsible for insulin-stimulated glucose uptake (DeFronzo *et al.*, 1985), and thus, is also at the root of insulin resistance. In skeletal muscle, insulin signaling leads to translocation of GLUT-4 receptors to the cell surface where they transport glucose into cells. Insulin resistance, or the failure of insulin to stimulate glucose uptake, is related to obesity.

The overall purpose of adipose tissue is to store fuel in times of food excess to increase survivability during times of food shortage. Diet-induced obesity disrupts lipid storage by increasing lipids in tissues other than adipose. If a normal individual consumes too many calories, their adipocytes expand and produce more leptin. This leptin encourages fatty acid oxidation, decreased storage of lipids in tissues, and helps to maintain a normal level of lipids in lean tissue. However, chronic overconsumption leads

to a build-up of lipid in lean tissues including muscle partly because efficiency of fatty acid uptake is increased in skeletal muscle of animals fed diets high in fat (Hegarty *et al.*, 2002).

Insulin resistance may be related to the increased lipid content of muscle cells in high-fat diet-induced obesity. In muscle tissue, palmitoyl coA, a fatty acid, combines with serine to produce ceramide. Increased ceramide decreases phosphorylation of insulin receptor substrate 1 (IRS-1) thereby reducing its' activity and decreasing insulin signaling (Kanety *et al.*, 1996). Independent of ceramide, fatty acyl coA and diacylglycerols lead to inhibitory phosphorylation of IRS-1 (Yu *et al.*, 2002), which also reduces insulin signaling. The formation of ceramide is also toxic to muscle cells as evidenced by increased apoptosis (Turpin *et al.*, 2006). This lipotoxicity is not limited to muscle cells; ceramide also induces apoptotic factors in pancreatic β-cells responsible for insulin production. This loss of insulin producing capability further decreases insulin signaling in obesity.

Increased circulating levels of triglycerides, insulin and glucose, and increased liver fat content are also all correlated with metabolic syndrome (Marchesini *et al.*, 2003; Taskinen, 2003). Leptin levels are reflective of lipid content in mice, and increased leptin is associated with obesity (Frederich *et al.*, 1995). Though the action of leptin is to increase metabolic rate and decrease food intake, increased leptin levels in obesity do not result in these actions indicating leptin resistance (Lin *et al.*, 2000). Conversely, adiponectin sensitizes cells to insulin and is decreased in obese or insulin resistant individuals (Yamauchi *et al.*, 2001). Thus, aberrations in leptin, insulin and adiponectin all contribute to the obesigenic state and related insulin resistance.

In rodents, high concentrations of dietary fat are associated with increased weight gain and body fat content (West et al., 1992) similar to human obesity. Increased weight gain is due in part to hyperphagia, or an overconsumption of calories. However, in studies where intake was controlled, the weight and body fat of animals are still increased (West and York, 1998). Rats fed isocalorically with an increasing amount of calories from fat showed increased body fat in a dose-dependent manner (Boozer *et al.*, 1995) suggesting that fat in the diet, in particular, contributes to obesity.

Not all rodents, however, are equally susceptible to diet-induced obesity. Mice of the C57BL/6J inbred strain are particularly sensitive to high-fat diet-induced obesity (Surwit *et al.*, 1995). When diets with high sucrose and high fat were fed for 4 months, mice exhibited increased weight gain, fat pad weight and adipocyte hyperplasia, most evident in mesenteric fat. However, increases were more pronounced in mice on combined high fat/high sucrose diets than those on low fat/ high sucrose diets despite the diets being isocaloric. Furthermore, mice of the same strain fed isocalorically with animals on a lower fat diet also had increased body fat and impaired glucose metabolism (Petro *et al.*, 2004). These findings illustrate that obesity-induction by high fat diets results from both increased caloric consumption and increased fat consumption Furthermore, the use myostatin null mice of a C57BL/6J inbred background seem the most appropriate model for exploring the susceptibility of myostatin null mice to high-fat diet induced obesity.

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CHAPTER 2

THE MYOSTATIN NULL MUTATION AND CLENBUTEROL ADMINISTRATION ELICIT ADDITIVE EFFECTS IN MICE

Abstract

In mice, the myostatin null mutation and treatment with clenbuterol both increase muscle growth and decrease fat mass. Our objective was to determine if mechanistic overlap exists by administering clenbuterol to myostatin null mice. Male myostatin null and wild type mice of similar genetic backgrounds received either 0 (control) or 20 ppm clenbuterol in tap water free choice for 14 days. Several traits were measured to estimate muscle and fat growth. The myostatin null mutation resulted in increased body and empty carcass weight, increased muscle weights, and decreased fat pad weights. Fat content was reduced and protein content increased in the empty carcasses of myostatin null mice. Similarly, treatment with clenbuterol resulted in increased body and empty carcass weight, increased muscle weights and reduced fat pad weights. Fat content of empty carcasses and viscera was reduced and protein content of empty carcasses was increased with clenbuterol treatment. A significant interaction of genotype and clenbuterol treatment would indicate an altered responsiveness of myostatin null mice to clenbuterol. However, only the weight of gastrocnemius muscles exhibited a significant ($P = 0.01$) interaction of genotype and clenbuterol treatment, indicating myostatin null mice were less responsive to clenbuterol compared with wild type mice. Thus, for all other traits, the impact of myostatin null mutation and clenbuterol treatment were completely additive.

These data suggest that disruption of myostatin function does not alter the response of mice to β-adrenergic agonists.

Introduction

Several strategies are available to alter muscle and fat growth in animals with the ultimate goal of increasing efficiency of livestock production. The use of β-adrenergic agonists (βAA) to increase muscle mass and decrease fat mass in several species is well-established (Beermann, 2002; Mersmann, 1998; Ricks *et al.*, 1984). Clenbuterol, though not approved for commercial use in livestock, increases muscle growth in mice and other species (Yang and McElligott, 1989). Another strategy to alter animal growth is to introduce mutations into the myostatin (Mstn) gene as occurs naturally in double-muscled cattle. These cattle exhibit striking increases in muscle growth and reductions in fat mass (Grobet *et al.*, 1997; McPherron and Lee, 1997; Kambadur *et al.*, 1997; Grobet *et al.*, 1998). Transgenic Mstn null mice can be used as a model for this effect as this species also exhibits increased muscularity and decreased fat deposition (McPherron *et al.*, 1997; McPherron and Lee, 2002). Furthermore, disruption of Mstn function is thought to confer resistance to several types of obesity development (McPherron and Lee, 2002; Zhao *et al.*, 2005).

Though the results of Mstn disruption are similar to those of treatment with βAA, namely increased muscle deposition and decreased fat mass, the proposed mechanisms of each are quite different. Clenbuterol is a selective β-2 agonist (Hinkle et al., 2002) and results in alterations in protein deposition possibly through changes in protein synthesis or degradation rates, but a specific mechanism is, as of yet, unclear. Clenbuterol has also

been shown to reduce adipose tissue mass through apoptosis of adipocytes (Page et al., 2004). In contrast to βAA, Mstn functions in cell cycle regulation with the loss of Mstn function resulting in increased proliferation of embryonic and fetal myoblasts causing hyperplasia (Thomas et al., 2000) and greater activity in satellite cells contributing to increased hypertrophy (McCroskery et al., 2003). Mechanisms behind the reduced adiposity of Mstn null mice and their resistance to obesity are less clear. It may simply be a result of increased energy needs of muscle tissue, thus reducing the energy available for fat deposition, or may result from altered adipocyte function due to the loss of Mstn activity.

Due to the disparate mechanisms of increased muscle and reduced fat growth from alteration of Mstn or the use of βAA, we hypothesized that the Mstn null mutation would not alter responsiveness to βAA treatment, though such an interaction has not previously been tested. It is possible, however, that increased muscle growth due to loss of Mstn function is so extensive that further increases are not possible, and in this scenario, one would expect that Mstn null mice would be unresponsive to clenbuterol treatment. Simply put, muscles of Mstn null animals may have reached their maximal size and cannot grow any larger. To test this hypothesis, clenbuterol was administered to Mstn null and wild type mice. Several measures of muscle mass and adiposity were used to determine the impact of clenbuterol treatment on muscle and fat growth in these mice.

Materials and Methods

Animals, Observations and Materials

Five-week-old male wild type (n=32) and Mstn null (n=24) mice from an internal C57BL6/J colony were housed singly in solid-bottom cages and given ad libitum access to pelleted rodent chow (Teklad F6 rodent diet, Harlan, Indianapolis, IN) and water. Ambient room temperature was maintained with a 12 hour light/dark cycle. Mice received free access to either tap water (control) or tap water with 20 ppm clenbuterol (Sigma-Aldrich, St. Louis, MO, item # C5423) for two weeks. Body, food and water weights were all measured on day 1 (trial initiation), day 8, and day 15 (trial conclusion). Efficiency was calculated by dividing weight gain by food intake. Animals were sacrificed on day 15 by carbon dioxide asphyxiation and cervical dislocation. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Dissection and Proximate Composition

A subset of mice (12 wild type and 10 Mstn null) were dissected to estimate muscle and fat deposition. Inguinal, retroperitoneal, and epididymal fat pads were harvested and weighed. Skin, feet, head, tail and viscera were removed and the remaining carcass (i.e., empty carcass) was weighed. Tibialis anterior, triceps brachii, biceps femoris and gastrocnemius muscles were removed from the right side and weighed.

Proximate composition of empty carcass and viscera were determined on a subset of mice (20 wild type and 14 Mstn null) to estimate muscle and fat deposition. Empty

carcasses and viscera were weighed, dried in a 60ºC oven for at least 48 hours, re-weighed and ground with a commercial coffee grinder (SmartGrind, Black and Decker, Towson, MD). Ground samples were then dried to a constant weight in a 110ºC oven. Lipid content was calculated by weight difference after extraction with 4:1 chloroform:methanol solution (Novakofski et al., 1989). Crude protein content (nitrogen x 6.25) was determined by the combustion method (AOAC, 2000; method 990.03) with a Leco model FP2000 (Leco Corp., St. Joseph, MI), using EDTA as an internal standard.

Statistical Analysis

Statistical analyses were performed using SAS version 9.1 (SAS Institute Inc, Cary, NC). Analysis of variance was used to determine the significance of genotype, clenbuterol treatment and their interaction employing the Mixed procedure of SAS. For body weight, analysis included repeated measures with an autogressive variance/covariance structure. Data are presented as lsmeans ± S.E.M with P-values for the main effects of genotype, clenbuterol treatment and their interaction. Of particular interest is the interaction term, as lack of significant interaction indicates that main effects are completely additive, and response to clenbuterol treatment is similar between the two genotypes.

Results

Body Weight, Food and Water Intake, Efficiency and Dosage

There were no differences in body weight between the genotypes on day 1. After 7 days on trial, however, Mstn null mice were 1.8 g heavier ($P < 0.01$) than wild type

mice and mice treated with clenbuterol were 1.3 g heavier ($P = 0.01$) than control (Figure 1). These effects continued until the end of the trial (d 15) when Mstn null mice were 3.5 g heavier ($P < 0.01$) than wild type mice and clenbuterol treatment increased ($P = 0.02$) body weight by 1.3 g. There was no interaction ($P > 0.64$) between genotype and clenbuterol treatment for body weight at any time point. Similarly, gain (mg/d) was higher ($P < 0.01$) in Mstn null mice compared with wild type and was increased ($P < 0.01$) by clenbuterol treatment (Table 2.1). Food intake (g/d) was also higher ($P = 0.01$) in Mstn null compared with wild type but was not impacted by clenbuterol treatment ($P = 0.11$). Efficiency (mg gain: g food), however, was higher ($P < 0.01$) in Mstn null mice compared with wild type and was also increased ($P = 0.02$) by clenbuterol treatment. There was no interaction of genotype and clenbuterol treatment for gain, food intake or efficiency ($P > 0.21$). Water intake was increased ($P < 0.01$) in clenbuterol-treated mice compared with control but was not impacted by genotype. Given the water intake of these mice, clenbuterol intake was estimated to be 0.148 mg/d for wild type and 0.135 mg/d for Mstn null mice.

Proximate Composition of Carcass and Viscera

To establish the composition of increased body weight in Mstn null mice and in mice treated with clenbuterol, proximate analyses of the empty carcass and the viscera were performed (Table 2.2). Empty carcass weight was approximately 4 g higher ($P < 0.01$) in Mstn null mice compared with wild type and was increased ($P < 0.01$) approximately 1 g by clenbuterol treatment. There was no interaction of genotype and clenbuterol treatment for empty carcass weight ($P = 0.99$). Furthermore, indicative of

increased muscle growth, protein content of empty carcasses was 0.5 percentage units higher ($P = 0.04$) in Mstn null mice compared with wild type, and was increased 0.7 percentage units ($P < 0.01$) by clenbuterol treatment. Conversely, fat content of the empty carcass was 2 percentage units lower ($P < 0.01$) in Mstn null mice compared with wild type, and was decreased 1.3 percentage units ($P < 0.01$) by clenbuterol treatment, indicative of lower fat deposition. Weight of viscera was not different between the treatment groups, but fat content of the viscera was decreased 2 percentage units ($P < 0.01$) by clenbuterol treatment. For all proximate composition traits, there was no interaction of genotype and clenbuterol treatment ($P > 0.24$).

Muscle and Fat Pad Weights

To further establish the concept of increased muscle and decreased fat deposition in Mstn null mice and mice treated with clenbuterol, several muscles and fat pads were dissected and weighed (Table 2.3); these data were normalized to day 15 body weights (Figure 2.1). Muscle weights of Mstn null mice were 1.5 – 2 times heavier ($P < 0.01$) than those of wild type mice. Clenbuterol treatment also increased the weight of tibialis anterior, triceps brachii, and gastrocnemius muscles ($P < 0.05$) but did not alter the weight of the bicep femoris. For the gastrocnemius, however, there was an interaction ($P = 0.01$) of genotype and clenbuterol treatment indicating a decreased response to clenbuterol treatment in Mstn null mice. In wild type mice, gastrocnemius weight was increased 0.12 percentage units (4.5 mg) while in Mstn null mice it was increased only 0.07 percentage units (2.8 mg).

Inguinal fat pad weight was not altered by genotype or clenbuterol treatment. Retroperitoneal and epididymal fat pad weights were lowered ($P < 0.01$) 0.08 and 0.47 percentage units, respectively, in Mstn null mice compared with wild type and were also reduced ($P < 0.05$) by clenbuterol treatment. The interaction of genotype and clenbuterol treatment was tending towards significance ($P = 0.12$) for the epididymal fat pad. Similar to the gastrocnemius muscle, fat pads from Mstn null mice were not as responsive to clenbuterol as wild type mice. Epididymal fat pad weight was decreased 0.48 percentage units in wild type mice while it was only reduced 0.14 percentage units in Mstn null mice.

Discussion

Both the Mstn null mutation and clenbuterol administration result in increased muscle mass and decreased fat mass in animals, including mice. Though phenotypic outcomes are similar, the mechanisms by which each impacts these tissues are quite different. Therefore, we hypothesized that myostatin null mice would respond to clenbuterol treatment similarly to wild type mice. This interaction of βAA treatment and the loss of myostatin function has not previously been reported. Although the extreme muscling phenotype observed in Mstn null animals may represent near maximal muscle mass, it is imperative to establish whether the Mstn null mutation and clenbuterol treatment elicit mutually exclusive effects.

Disruption of Mstn functionality results in increased muscle mass, decreased adiposity and improved efficiency (McPherron and Lee, 1997; McPherron and Lee, 2002; Mitchell and Wall, 2007; Szabo *et al.*, 1998; Yang *et al.*, 2001). Mstn null mice often

exhibit muscle weights 1.5- to 2-times greater than their wild type counterparts (McPherron et al., 1997) while weights of individual fat pads are reduced (McPherron and Lee, 2002). In our experiment, the Mstn null mutation resulted in the expected phenotype. Body weight and empty carcass weight were both increased, 3.5 and 4 g, respectively, in Mstn null mice compared with wild type. The majority of weight gain was either in muscle or bone tissue as indicated by the large increase in empty carcass weight. While viscera weight was not reduced in Mstn null mice compared with wild type, it did constitute a smaller proportion of total body mass. This finding is similar to reports of smaller organ sizes in double-muscled cattle (Arthur, 1995), the mechanism of which is unclear.

Food intake was also increased in Mstn null mice compared with wild type mice contrary to other reports (Lin *et al.*, 2002; McPherron and Lee, 2002). Compared with previous research, mice in our study consumed less food overall. This may reflect slight site-to-site and strain-to-strain differences in Mstn null mice. However, efficiency (gain:food) was increased in Mstn null mice compared to wild type indicating that, though they consumed more food than their wild type counterparts, Mstn null mice were still more efficient at converting nutrients into body tissue. As others have shown increased weight gain in Mstn null mice despite a lack of change in food intake, increased efficiency was expected.

Muscle mass was increased in Mstn null mice compared with wild type. Weights of individual muscles were 1.5- to 2-times higher in Mstn null mice compared with wild type similar to previous reports (McPherron et al., 1997). Further evidence of increased

muscularity in Mstn null mice included the 0.5 percentage unit increase in empty carcass protein compared with wild type. Conversely, fat deposition was reduced by the Mstn null mutation. We observed a 2 percentage unit decrease in empty carcass fat, and a reduction in fat pad weight of 33-38% in Mstn null compared with wild type mice, similar to the findings of McPherron and Lee (2002). There was no observed decrease in visceral fat content in Mstn null mice. From these data, we conclude that the loss of Mstn function increased muscularity and decreased adiposity as expected.

Clenbuterol treatment, similar to several other βAA, improves feed efficiency, increases protein accretion and therefore muscle mass, and decreases fat mass (Beermann, 2002; Mersmann, 1998; Ricks *et al.*, 1984; Yang and McElligott, 1989). As a selective β-2 agonist (Hinkle et al., 2002), clenbuterol likely alters protein degradation (Reeds *et al.*, 1986; Yimlamai *et al.*, 2005) to increase protein accretion but may also alter protein synthetic rates. Alternatively, reductions in fat mass may result from energy repartitioning or direct effects on adipocyte apoptosis (Page et al., 2004). In our experiment, clenbuterol treatment increased muscle deposition and reduced fat mass as expected. Body and empty carcass weight were increased 1.3 g and 1 g, respectively, in clenbuterol treated mice compared with control. The large proportion of body weight gain retained in the empty carcass was indicative of increases in muscle and bone mass as opposed to increased visceral or fat mass. Previous reports have indicated that heart muscle weight is increased with clenbuterol treatment (Cubria *et al.*, 1998; Petrou *et al.*, 1995). Thus, we would have expected increased viscera weight, but in our study, viscera weight was unchanged by clenbuterol treatment. It is possible that cardiac hypertrophy occurred in our study but was unnoticeable as the entire visceral mass, and not the heart

alone, was weighed. Increased muscularity of clenbuterol-treated mice, as suggested by increased empty carcass weight, was also observed. Muscle weights of clenbuterol treated mice were 1.2-times heavier than those of control mice. Clenbuterol treatment also resulted in a 0.7 percentage unit increase in empty carcass protein content. Furthermore, clenbuterol treatment reduced fat mass as expected. Fat pad weight was decreased 23-25% and empty carcass fat content was reduced by 1.3 percentage units. In contrast to the lack of effect due to the Mstn null mutation, visceral fat content was also lowered by clenbuterol treatment. Therefore, we conclude from these data that clenbuterol treatment increased muscle growth and decreased fat mass similar to previous reports.

Given that results of both clenbuterol treatment and Mstn null mutation were as expected, we were able to determine the validity of our hypothesis that Mstn null mice respond similarly to wild type mice when administered clenbuterol. A lack of significant interaction between genotype and clenbuterol treatment would, therefore, validate our hypothesis. The only trait with a significant interaction was gastrocnemius weight. The increase in gastrocnemius muscle weight from clenbuterol treatment was less in Mstn null mice than wild type. There was also a tendency for the decrease in epididymal fat with clenbuterol treatment to be less in Mstn null mice than in wild type. Thus, in these instances, it is possible that the phenotypic effects of the Mstn null mutation were so extreme (i.e., greatly increased weight of the gastrocnemius and reduced weight of the epididymal fat pad), that additional changes from clenbuterol treatment were minimal. However, both of these tissues did experience numerical changes in weight in Mstn null mice treated with clenbuterol, indicating the capacity to respond was intact but somewhat

blunted. For all other traits measured, there was no significant interaction, indicating the effect of Mstn null mutation and clenbuterol treatment were completely additive. Thus, Mstn null mice experienced increased muscle deposition and reduced fat mass due to clenbuterol treatment to the same extent as wild type mice.

Given their diverse mechanisms of action, it is logical to conclude that βAA treatment and Mstn disruption would not interact. However, with identification of Mstn (McPherron et al., 1997) and its association to the well-characterized double-muscling phenotype in cattle (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron and Lee, 1997), the idea of a muscle chalone—an inhibitory molecule secreted by the muscle which ultimately governed its size (Bullough, 1962; Bullough, 1965)—was revived (Lee and McPherron, 1999). If Mstn was this muscle chalone, the loss of its function to regulate the size of muscle independent of other factors would allow muscle to grow to its maximal possible size. The "doubling" observed in mice and cattle lacking Mstn activity certainly seems to represent a type of maximal size which muscle could attain and unresponsiveness to other means of increasing muscle growth would be expected. The working hypothesis that Mstn was the elusive muscle chalone, however, has been disproven as supported by evidence from our study and others. Whereas the increase in muscle mass of Mstn null mice is large, there is now sufficient evidence to suggest that maximal muscle growth has not yet been attained. Mice with postnatal transgenic overexpression of Mstn propeptide fed high-fat diets exhibit increased muscle growth compared to similar transgenic mice fed lower fat diets (Yang and Zhao, 2006). Furthermore, follistatin overexpression in Mstn null mice also results in increased muscle growth compared with normal Mstn null mice—nearly a quadrupling of muscle mass

compared with wild type mice (Lee, 2007). Our study, in which Mstn null mice exhibited increased muscle growth following clenbuterol treatment, provides additional evidence contradicting the hypothesis that Mstn may serve as a muscle chalone and indicates that Mstn null mice respond to treatment with βAA similar to wild type mice. Given the additive effects of Mstn and clenbuterol, it may be possible to exploit these apparently distinct modes of action to control muscle mass in animals.

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Tables and Figures

Figure 2.1. Body weight during clenbuterol administration (dashed lines) to wild type (■) and myostatin null (▲) mice with standard errors displayed.

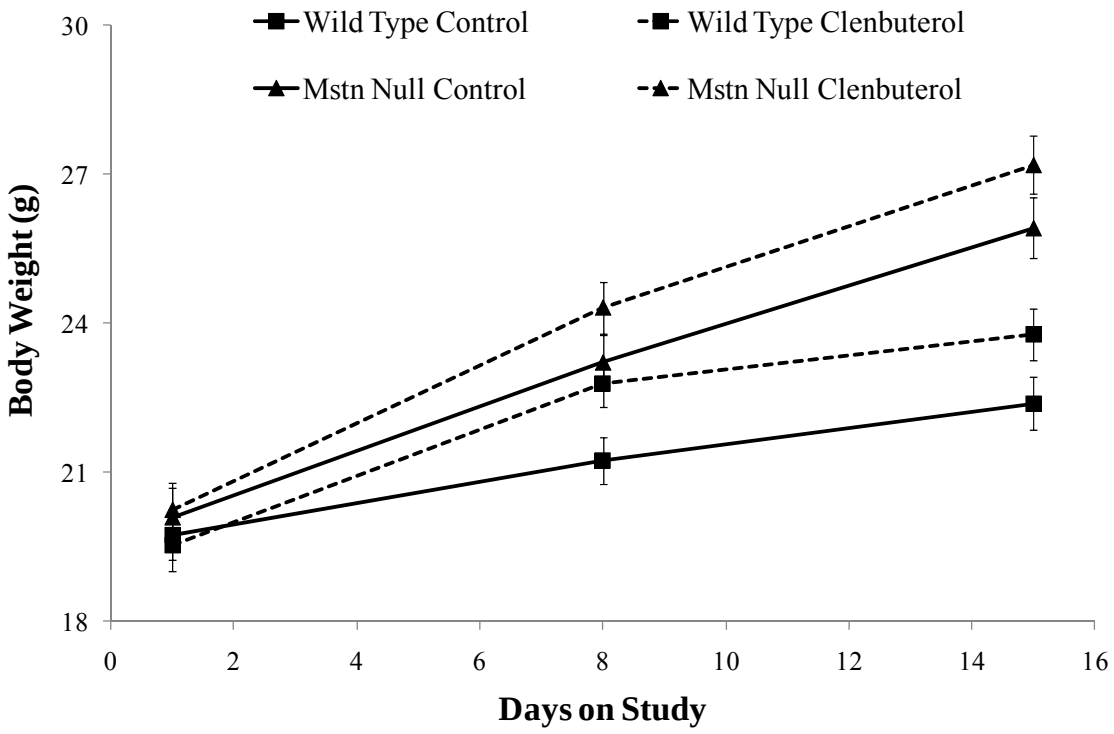

Table 2.1. Effects of genotype (G) and clenbuterol treatment (T) and their interaction (GxT) on daily gain, food intake, water consumption and clenbuterol dose of wild type and myostatin null mice[1]

	Wild Type		Myostatin Null		P-value		
	Control	Clenbuterol	Control	Clenbuterol	G	T	GxT
Number of mice	16	16	11	13			
Gain (mg/d)	189 ± 27.2	304 ± 27.2	419 ± 32.8	497 ± 30.2	<0.01	<0.01	0.53
Food intake (g/d)	3.5 ± 0.16	3.6 ± 0.16	3.8 ± 0.20	4.2 ± 0.18	0.01	0.11	0.33
Efficiency (mg/g)[2]	562 ± 10.1	958 ± 10.1	111 ± 12.2	123 ± 11.2	<0.01	0.02	0.21
Water intake (ml/d)	6.1 ± 0.27	7.4 ± 0.27	5.9 ± 0.32	6.7 ± 0.30	0.16	<0.01	0.42
Clenbuterol intake (mg/d)[3]	.	0.148 ± 0.005	.	0.135 ± 0.006	.	.	.

[1] Mice were given ad libitum access to tap water with 20 ppm clenbuterol (Clenbuterol) or without (Control). Values are lsmeans ± S.E.M.

[2]Efficiency was calculated by dividing weight gain by food intake.

[3]P-values are not provided for clenbuterol intake as it was a numerical calculation based on water intake and a dose of 20 parts per million clenbuterol in the water.

Table 2.2. Effects of genotype (G) and clenbuterol treatment (T) and their interaction (GxT) on empty carcass weight and composition and viscera weight and composition of wild type and myostatin null mice[1]

	Wild Type		Myostatin Null		P-value		
	Control	Clenbuterol	Control	Clenbuterol	G	T	GxT
Number of mice	15	15	11	12			
Empty carcass weight (g)[2]	8.8 ± 0.29	10 ± 0.29	12.9 ± 0.34	14.1 ± 0.32	<0.01	<0.01	0.99
Number of mice	10	10	6	8			
Empty carcass fat (%)	7.1 ± 0.30	5.4 ± 0.30	4.7 ± 0.38	3.8 ± 0.33	<0.01	<0.01	0.24
Empty carcass protein (%)	20.9 ± 0.22	21.7 ± 0.22	21.5 ± 0.28	22.1 ± 0.24	0.04	0.01	0.69
Viscera weight (g)	6.0 ± 0.19	6.0 ± 0.19	6.1 ± 0.24	5.6 ± 0.21	0.52	0.17	0.27
Viscera fat (%)	9.8 ± 0.47	8.3 ± 0.47	10.7 ± 0.6	8.2 ± 0.52	0.49	<0.01	0.40

[1] Mice were given ad libitum access to tap water with 20 ppm clenbuterol (Clenbuterol) or without (Control). Values are lsmeans ± S.E.M.

[2] Empty carcass is carcass remaining after skin, head, tail, feet and viscera were removed.

Table 2.3. Effects of genotype (G) and clenbuterol treatment (T) and their interaction (GxT) on muscle and fat pad weights (% of body weight) of wild type and myostatin null mice[1]

	Wild Type		Myostatin Null		P-value		
	Control	Clenbuterol	Control	Clenbuterol	G	T	GxT
Number of mice	6	6	5	5			
Muscles							
Tibalis Anterior	0.20 ± 0.015	0.23 ± 0.015	0.37± 0.016	0.40 ± 0.016	< 0.01	0.03	0.94
Triceps Brachii	0.38 ± 0.020	0.43 ± 0.020	0.57 ± 0.022	0.67 ± 0.022	< 0.01	< 0.01	0.26
Biceps Femoris	0.51 ± 0.044	0.58 ± 0.044	0.72 ± 0.048	0.71 ± 0.048	< 0.01	0.58	0.42
Gastrocnemius	0.51 ± 0.010	0.63 ± 0.010	0.80 ± 0.011	0.87 ± 0.011	< 0.01	< 0.01	0.01
Fat Pads							
Inguinal	0.89 ± 0.071	0.72 ± 0.071	0.78 ± 0.078	0.71 ± 0.078	0.45	0.13	0.55
Retroperitoneal	0.23 ± 0.025	0.16 ± 0.025	0.13 ± 0.027	0.09 ± 0.027	< 0.01	0.05	0.53
Epididymal	1.40 ± 0.101	0.92 ± 0.101	0.76 ± 0.111	0.62 ± 0.111	< 0.01	< 0.01	0.12

[1] Mice were given ad libitum access to tap water with 20 ppm clenbuterol (Clenbuterol) or without (Control). Values are lsmeans ± S.E.M.

CHAPTER 3

HIGH-FAT DIET-INDUCED OBESITY IN MYOSTATIN NULL MICE

Abstract

High-fat diet-induced obesity is one model to examine obesity in humans, and several mutations have been identified which confer some degree of resistance to obesity development. The objective of this experiment was to examine the resistance to diet-induced obesity of myostatin (Mstn) null mice and determine if genes involved in thermogenesis were altered in these mice. Four-week-old male Mstn null and wild type mice were given ad libitum access to diets with 10 or 60% kcal from fat for 12 weeks. Glucose tolerance was determined after 0, 4, 8 and 12 weeks on diet. Body composition, muscle and fat pad weights, as well as other markers of metabolic syndrome were recorded and mRNA expression of several genes was examined in muscle, fat and liver. High-fat fed mice consumed more calories and deposited weight and calories more efficiently than control mice. After 12 weeks of high-fat feeding, body weight, body fat, fat pad weight, fasting blood glucose, serum insulin and leptin expression in adipose tissue were increased in both wild type and Mstn null mice. However, for all these parameters, the increase in Mstn null mice was less pronounced than in wild type mice indicating partial resistance to fat gain with excessive caloric consumption. While some genes involved in thermogenesis were increased in Mstn null mice, it does not appear that

increased thermogenesis accounts for differences in body fat content between Mstn null and wild type mice.

Introduction

Myostatin (Mstn), a member of the TGF-β superfamily, has been well-characterized as a negative regulator of muscle growth. Mutations that inactivate or inhibit Mstn result in hypermuscularity in several species including: mice (Lee and McPherron, 1999; Marcq, 1998; McPherron *et al.*, 1997; Yang and Zhao, 2006), cattle (Grobet *et al.*, 1997; Grobet *et al.*, 1998; Kambadur *et al.*, 1997; McPherron and Lee, 1997), dogs (Mosher *et al.*, 2007) and humans (Schuelke *et al.*, 2004). For example, Mstn mutations are responsible for the "double-muscled" phenotype long observed in Belgian Blue and Piedmontese cattle (Kambadur *et al.*, 1997). Increased muscle mass results from both hyperplasia and hypertrophy, and muscles from affected individuals can have nearly twice as many muscle fibers as normal individuals.

In addition to its role in muscle, a biological function of Mstn has been suggested in adipose tissue. Body fat accumulation is decreased in Mstn null mice (McPherron and Lee, 2002) when compared with wild type mice of the same chronological age. This reduction in body fat is a manifestation of reductions in both adipocyte number and size in Mstn null mice (McPherron and Lee, 2002) and is accompanied by an increased respiratory exchange rate and enhanced utilization of carbohydrates (Guo *et al.*, 2009). Furthermore, this reduced adiposity is coupled with a reduction of adipogenic proteins in fat cells including PPARγ and C/EBPα, markers of terminal differentiation in adipocytes (Lin *et al.*, 2002a).

In several rodent models of obesity, Mstn null mice have proven to be less sensitive to obesity induction. Diets high in fat cause overeating and induce obesity in mice (West and York, 1998; Woods *et al.*, 2003), especially in the obesity-prone C57BL6/J inbred strain (Surwit *et al.*, 1995). However, several authors have demonstrated a reduced propensity for fat gain of Mstn null mice fed high-fat diets (Guo *et al.*, 2009; Hamrick *et al.*, 2006). Similarly, mice with a transgenic overexpression of the Mstn propeptide fed similar diets were also resistant to high-fat diet-induced obesity (Yang and Zhao, 2006; Zhao *et al.*, 2005). When incorporated into mice with genetic forms of obesity (agouti yellow, *ob/ob*), Mstn null alleles provided protection against the development of obesity and insulin resistance (McPherron and Lee, 2002). Taken together, these data suggest that inactivation or inhibition of Mstn results in resistance to fat accretion.

The direct impact of Mstn on adipocytes has recently been explored. Myostatin, the Mstn receptor (activin type 2B receptor) and one Mstn inhibitor were expressed in adipose tissue though at levels much lower when compared with skeletal muscle (Allen *et al.*, 2008). Myostatin expression increased in adipose tissue with high-fat feeding and in *ob/ob* mice. In other work, mice with transgenic overexpression of Mstn in adipocytes were resistant to developing insulin resistance and obesity induced by high-fat feeding (Feldman *et al.*, 2006). These results indicate that Mstn may be important in adaptation of adipose tissue to limit its own growth and resist the development of obesity. However, if Mstn plays a role in resisting obesity development, it is plausible that Mstn null mice would be more, and not less, sensitive to obesity induction. Thus, the role Mstn plays in obesity development is unclear. Therefore, the objective of this study was to determine

the response of Mstn null mice to consumption of a very high-fat diet and elucidate the mechanism of obesity resistance in these animals.

Materials and Methods

Animals, Observations and Materials

All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Replicate 1: Four-week-old male Mstn null (n=21) and wild type mice (n=22) from an internal C57BL6/J colony were genotyped by PCR-based genotyping described by McPherron et al. (1997). Mice were housed singly in solid-bottom cages and given ad libitum access to pelleted rodent chow and water. Ambient room temperature was maintained with a 12 hour light/dark cycle. Mice were assigned to either a normal or high-fat diet obtained from Research Diets, Inc. (New Brunswick, NJ). The high fat diet (Product number: D12492) contained 5.24 kilocalories per gram of food while the normal low fat diet (Product number: D12450B) contained 3.85 kilocalories per gram of food. Lard was the fat source for both diets. Body, food and water weights were all measured at the initiation of the trial and then weekly for 12 weeks.

Replicate 2: An additional set of four-week-old male Mstn null mice from the internal colony (n=10) and wild type mice (n=10) obtained from Jackson Labs (Bar Harbor, ME) were assigned to normal and high fat diets. These mice were housed in groups of 5 animals per cage with environmental conditions identical to those described above and

given ad libitum access to food and water. Body weight was measured weekly on individual mice for 12 weeks.

Animals from both replicates were sacrificed after 12 weeks of dietary treatment (16 weeks of age) by carbon dioxide asphyxiation and cervical dislocation after a 16 h fast. Blood, collected by cardiac puncture, was allowed to coagulate, and serum was obtained after centrifugation at 14,000xg for 10 minutes at 4ºC and stored at -80ºC.

Glucose Tolerance Test

In the first replicate, a single glucose tolerance test was performed at approximately 16 weeks of age (2 days prior to sacrifice). Food was removed 16 hour prior to the glucose tolerance test. Mice were weighed and a small drop of blood was obtained from the end of the tail. Blood glucose was determined using an Accu-chek Active glucose meter (Roche Diagnostics Corp., Indianapolis, IN). Mice were then administered 1 mg dextrose/g body weight by intraperitoneal injection. Blood glucose was measured at 30, 60, 90 and 120 min post-injection. In the second replicate, glucose tolerance tests were performed as described above at 8, 12 and 16 weeks of age. Area under the curve was calculated using the trapezoidal method with the baseline of each mouse subtracted.

Dissection, Sample Collection and Proximate Composition

All mice were dissected to estimate muscle and fat deposition. Inguinal, retroperitoneal, subscapular and epididymal fat pads were collected and weighed. Skin, feet, head, tail and viscera were removed and the remaining carcass (i.e., empty carcass)

was weighed. Tibialis anterior, triceps brachii, biceps femoris and gastrocnemius muscles were removed from the right side and weighed. From the first replicate, sub-samples of liver, epididymal fat pad, gastrocnemius muscle and bicep femoris muscle were flash-frozen in liquid nitrogen and stored at -80ºC.

Mice from the second replicate were used to estimate body composition. Fat and protein content were determined from the skin, carcass, viscera and liver. Samples were weighed and dried to a constant weight in a 60ºC oven, re-weighed and ground with a commercial coffee grinder (SmartGrind, Black and Decker, Towson, MD). Ground samples were then dried to a constant weight in a 110ºC oven. Lipid content was calculated by weight difference after extraction with 4:1 chloroform:methanol solution (Novakofski *et al.*, 1989). Crude protein content (nitrogen x 6.25) was determined by the combustion method (AOAC, 2000; method 990.03) with a Leco model FP2000 (Leco Corp., St. Joseph, MI), using EDTA as an internal standard. Total body fat and protein were calculated by summing the fat or protein from each sub-sample and dividing by total body weight.

Serum Analysis

A subset of animals (n=4-6 for each genotype by diet combination) were selected from the first replicate for serum analysis. Serum insulin was determined in duplicate by ELISA (Linco Research Inc, St. Charles, MO) according to manufacturer directions. Serum triglycerides were determined by the enzymatic measurement of glycerol after lipase digestion of serum triglycerides following the procedures of a Serum Triglyceride

Determination Kit (Sigma-Aldrich Company, St. Louis, MO). Free glycerol was also measured and used to correct for true triglyceride content. *Gene expression*

A subset of animals (n=4-8 from each genotype by diet combination) were selected from the first replicate for gene expression analysis of liver, biceps femoris(fast, glycolytic muscle), gastrocnemius muscle (more intermediate in fiber type) and epididymal fat.

Total RNA was prepared by disrupting tissues in TRIzol reagent (Invitrogen, Carlsbad, CA) with a TissueLyzer (Qiagen, Valencia, CA) following manufacturer's instructions. Concentration and RNA purity was determined spectrophotometrically and samples with 260:280 ratios of less than 1.8 were repurified (RNeasy Mini kit; QIAGEN, Valencia, CA). One microgram of RNA was treated with DNAase before reverse transcription into cDNA (Quantitect Reverse Transcription kit, Qiagen, Valencia, CA). Expression of specific mRNAs was quantitated by Taqman real-time reverse transcriptase-polymerase chain reaction (RT-PCR) normalized to GAPDH. The Taqman primer/probe sets for all genes examined were obtained from Applied Biosystems (Foster City, CA). Data are presented as mean fold change compared with wild type mice fed normal diets (control).

Statistical Analysis

Statistical analyses were performed using SAS version 9.1 (SAS Institute Inc, Cary, NC). Analysis of variance was used to determine the significance of genotype (Mstn null or wild type), diet (normal or high fat) and their interaction employing the

Mixed procedure of SAS. Replicate was used as a random variable in the analysis of body and tissue weights and glucose measurements from 16 weeks of age while plate was used a random variable for gene expression analysis. For body weight and food intake, analysis included repeated measures with an autogressive variance/covariance structure. Week of age was included in the model for the analysis of glucose data from replicate 2 which included glucose tolerance tests at 8, 12 and 16 weeks of age. Data are presented as lsmeans ± S.E.M with P-values for the main effects of genotype, diet and their interaction where appropriate.

Results and Discussion

Weight, Body Composition, and Efficiency

Body weight of Mstn null and wild type mice did not differ at the beginning of the trial (4 weeks of age). Body weight increased for the remainder of the trial and was impacted by genotype, diet and their interaction (Figure 3.1). Mstn null mice gained more weight than wild type mice ($P < 0.05$). Furthermore, mice fed high-fat diets were heavier than mice fed control diets. The interaction of genotype and diet was also significant from weeks 6-12 of dietary treatment. While consumption of fed high-fat diets increased body weight of Mstn null mice, this diet effect was lower than similarly fed wild type mice. After 12 weeks of feeding, high-fat fed Mstn null mice were 11% heavier than control-fed Mstn null mice while high-fat fed wild type mice were 39% heavier than control-fed wild type mice.

From the second replicate, mice were used to estimate body fat and protein content and liver fat content (Table 3.1). Prior to diet initiation, Mstn null mice had less body fat than wild type but protein was not different. After 12 weeks of dietary treatment, on diets, body protein was increased ($P < 0.05$) and body fat was decreased ($P < 0.05$) in Mstn null mice compared with wild type mice. Furthermore, high-fat fed mice had more body fat than control mice ($P < 0.05$). However, there was a significant interaction of genotype and diet for body fat ($P = 0.04$). Body fat more than doubled (109% increase) in high-fat fed wild type mice compared with control wild type mice. In Mstn null mice, however, body fat increased 44% in high-fat fed animals compared with control. The interaction of genotype and diet was also significant for body protein content ($P = 0.02$). In high-fat fed wild type mice body protein was reduced compared with control wild type mice, while in Mstn null mice, it was unchanged by high-fat feeding.

Body composition was used to estimate body energy content using the conversions of 4.27 kcal/g protein and 8.79 kcal/g fat. Body energy content was not different between the two genotypes either prior to start of the study or after 12 weeks of dietary treatment. High-fat feeding, however, did increase body energy compared with the control diet, and similar to body fat content, the increase in body energy content with high-fat feeding was less pronounced in Mstn null mice compared with wild type. Body energy increased 40% in high-fed fed Mstn null mice while it increased 130% in similarly fed wild type mice compared with respective genotype controls.

Food intake was also impacted by genotype, diet and their interaction, but did not differ with time. Food intake was increased ($P < 0.05$) in Mstn null mice fed control diets

compared with similarly fed wild type mice. However, food intake was reduced ($P < 0.05$) in mice fed high-fat diets compared with control diets (Table 3.2) of both genotypes. The reduction in food intake in high-fat fed Mstn null mice was greater than in wild type mice (28% compared with 16%). Efficiency of gain (mg gain/ g food consumed) was not impacted by genotype but was higher in high-fat fed mice than control mice. The interaction of genotype and diet was also significant as efficiency of gain doubled in high-fat fed wild type mice but was only increased 44% in Mstn null mice compared with control-fed mice of each genotype, respectively.

Though food intake was reduced in high-fat fed mice, the high-fat diet was more calorically dense than the control diet, therefore, caloric intake of high-fat fed mice was greater ($P < 0.05$) in mice receiving high-fat diet compared with control diet. Calorie consumption in wild type mice increased 17% with high-fat feeding compared with the control diet. Increased calorie consumption was not as pronounced in Mstn null mice (increased less than 10%). Caloric gain was estimated from body energy calculations (Table 3.1) and used to calculate caloric efficiency. Similar to efficiency of gain, caloric efficiency was increased in high-fat fed mice. However, unlike efficiency of gain, Mstn null mice were less efficient at depositing food calories into body energy than wild type mice. Furthermore, there was an interaction of genotype and diet as the increased caloric efficiency of high-fat fed mice was less pronounced in Mstn null compared with wild type mice.

Similar to body fat content, fat pads from Mstn null mice weighed less ($P < 0.01$) than those of wild type mice and high-fat fed mice had heavier fat pads ($P < 0.01$) than

control mice (Table 3.3). Additionally, there was a significant interaction of genotype and diet as high-fat feeding caused a greater increase in fat pad weights of wild type mice compared with Mstn null mice. However, the interaction of diet and genotype was not significant for muscle weights, and the effect of diet was only significant for the biceps femoris muscle (Table 3.3). High-fat fed mice had heavier ($P < 0.05$) gastrocnemius muscle than control mice. Overall, muscles of Mstn null mice were heavier ($P < 0.05$) than muscles of wild type mice.

These data are similar to previous studies (Guo *et al.*, 2009; Hamrick *et al.*, 2006; McPherron and Lee, 2002) though mice in our study were subjected to a higher fat diet (60% kcal from fat compared with 45%) for a longer period of time (12 weeks compared with 8-10 weeks). Furthermore, our study is the first to provide critical information on body composition analyses of Mstn null mice fed control and high-fat diets. It should be noted, however, that Mstn null mice are not completely resistant to fat accumulation under conditions of excess caloric intake. Other mice with modified Mstn function, those with transgenic overexpression of Mstn pro-peptide, exhibited increased muscularity, but after 9 weeks of feeding a diet with 45% kcal from fat, no increase in fat pad weight was observed (Yang and Zhao, 2006; Zhao *et al.*, 2005). This discrepancy may result from older mice being used in the transgenic overexpression study, 9 weeks old compared with 4 weeks old in this study. It is also possible that the pro-peptide of Mstn has a role separate from the inhibition of Mstn directly, and thus further inhibits fat accumulation in the presence of excess calories. These data confirm that the Mstn null mutation only partially blunts the development of high-fat diet induced obesity as body weight gain and body fat accumulation were less pronounced in Mstn null mice compared with similarly

fed wild type mice. Furthermore, efficiency of weight and caloric gain were increased to a lesser degree in Mstn null mice compared with high-fat fed wild type mice.

Glucose Tolerance and Markers of Metabolic Syndrome

Frequently, diet-induced obesity is accompanied by insulin resistance, and mutations or compounds which confer resistance to diet-induced obesity often also improve insulin resistance. Furthermore, reduced fasting blood glucose has been noted in Mstn null mice previously (Guo *et al.*, 2009). However, in our study, fasting blood glucose was not different between wild type and Mstn null mice at any time point of dietary treatment (Figure 3.2 A) There was also no interaction of genotype and diet for fasting glucose at any time point. However, fasting blood glucose was greater ($P < 0.05$) in high-fat fed mice than in control mice after 4 weeks on high-fat diet and this increase persisted through the end of the study though as time on high-fat diet increased, fasting blood glucose decreased. Blood glucose was higher ($P < 0.05$) in high-fat fed mice than in control mice at all time points post-glucose challenge resulting in a greater area under the curve in high-fat fed compared with control-fed mice (12 week data, Figure 3.2 B). Glucose tolerance tests at 4 and 8 weeks on diet followed a similar pattern with that of 12 weeks on diet described above (data not shown). This resulted in increased area under the curve for high-fat fed mice with no effect of genotype or interaction at any time point (Figure 3.2 C). As time on high-fat diet increased, however, area under the curve also increased indicative of progressing insulin resistance. Given the results of previous studies (Guo *et al.*, 2009; McPherron and Lee, 2002; Zhao *et al.*, 2005), the lack of

improvement in glucose metabolism in high-fat fed Mstn null mice compared with similarly fed wild-type mice was surprising.

Serum triglyceride content, serum insulin levels and liver fat content are all correlated with metabolic syndrome of which insulin resistance is a common feature (Marchesini *et al.*, 2003; Taskinen, 2003). Given the insulin resistance present in high-fat fed mice of both genotypes, increased insulin, triglycerides and liver fat were expected outcomes. However, in our study, insulin and triglycerides were increased in serum of high-fat fed wild type mice but not in high-fat fed Mstn null mice (Figure 3.3 A,B). Furthermore, liver fat content was increased with high-fat feeding in wild type mice, but decreased in high-fat fed Mstn null mice (Table 3.1). Finally, several adipokines are related to obesity and insulin resistance. Leptin levels are reflective of lipid content in mice, and increased leptin is associated with obesity (Frederich *et al.*, 1995). Leptin expression in adipose tissue was increased with high-fat feeding in this study, but was unaffected by genotype (Figure 3.4). Conversely, adiponectin is decreased in obese or insulin resistant individuals (Yamauchi *et al.*, 2001). In this study, adiponectin expression in epididymal fat of high-fat fed wild type mice is unaltered, but it is increased in Mstn null mice fed high-fat diets (Figure 3.4). This would suggest improve insulin sensitivity which is not reflected in the glucose data.

Mechanism of Obesity Resistance

There are several possible mechanisms for the resistance to fat accumulation observed in Mstn null mice. Increased lipid mobilization and thermogenesis was the focus of the gene expression analysis in our study. By uncoupling oxidative

phosphorylation from ATP production, uncoupling proteins (UCP) allow energy to be dissipated as heat (Ricquier and Bouillaud, 2000). Increased UCP expression in adipose (Kopecky *et al.*, 1995) or muscle (Li *et al.*, 2000) tissue has been shown to counteract diet-induced obesity and therefore, it is tempting to speculate that the reduced fat gain in Mstn null mice may result from increased UCP expression. Previous reports have noted no changes in UCP expression in fat or muscle tissue of Mstn null mice compared with wild type mice (McPherron and Lee, 2002). Similarly, in this study, UCP 3 expression was not different between Mstn null and wild type mice in muscle tissue (Table 3.4). Recently, it was suggested that UCP 1 expression in intermuscular brown adipocytes explained differences in obesity-susceptibly between two strains of mice (Almind *et al.*, 2007). In this study, the muscle form of UCP, UCP 3, was quantified, thus it is unknown if ectopic expression of UCP 1 in Mstn null mice may explain their resistance to fat accumulation.

Two other candidate genes to explain the reduced fat accumulation in Mstn null mice are PGC 1α and PGC 1β. These co-activators are involved in regulating mitochondrial oxidative metabolism. Expressed in several tissues with a high oxidative capacity, the specific roles of PGC 1β are less well-understood than PGC 1α, though their functions overlap in many tissues. In other obesity-resistant mouse models, PGC 1 expression is increased (Lau *et al.*, 2008; Toh *et al.*, 2008) corresponding to increased mitochondrial activity in adipose tissue. Increased expression of PGC 1β results in obesity resistance (Kamei *et al.*, 2003) while expression of PGC 1α is depressed in morbidly obese subjects. Furthermore, subjecting chicks to cold conditions reduced Mstn expression while increasing PGC 1α expression in sartorius muscle (Ijiri *et al.*, 2009).

Therefore, an increased expression of PGC 1 was expected in Mstn null mice. Conversely, reduced expression of PGC 1β in biceps femoris and gastrocnemius muscles and reduced expression of PGC 1α in gastrocnemius muscles of Mstn null mice was observed (Table 3.4). This phenomenon, however, may be more related to muscle fiber type in Mstn null mice than to proposed alterations in oxidative metabolism. Though not directly observed in our study, Mstn null mice are thought to possess more fast-twitch muscle fibers than wild type mice (Girgenrath *et al.*, 2005). This can be inferred in our data due to the decrease in MHC I expression in both biceps femoris and gastrocnemius muscles (Table 3.4). On the other hand, PGC 1α expression drives the formation of slow twitch fibers as evidenced by mice with transgenic PGC 1α expression (Lin *et al.*, 2002b). Similar transgenic expression of PGC 1β drives the formation of oxidative fibers expressing MHC IIx (Arany *et al.*, 2007). Thus, the reduction in MHC I, IIa and IIx expression in gastrocnemius and biceps femoris muscles may explain the concomitant reduction in PGC 1 expression.

In epididymal fat, however, PGC 1β expression was specifically upregulated in high-fat fed Mstn null mice compared with all other treatments. Similarly, adipose triglyceride lipase expression was increased in high-fat fed Mstn null mice compared with all other treatments. With a role similar to that of hormone-sensitive lipase, adipose triglyceride lipase is responsible for the hydrolysis of tri- and diacylglycerols with a preference for the hydrolysis of primary ester bond (Zimmermann *et al.*, 2004). Thus, an increased expression of adipose triglyceride lipase would suggest increased lipolysis within the adipocyte. In epididymal fat, UCP 2 expression was increased by high-fat feeding but it did not differ between the two genotypes (Figure 3.4). This coupled with

increased PGC 1β and adipose triglyceride lipase expression in the same tissue may indicate an increase in thermogenesis. However, the main source of thermogenesis in rodents is brown adipose tissue. This type of adipose differs from the epididymal fat pad used in this experiment in that it has a larger capacity for thermogenesis. Therefore, determining the expression of UCP and other genes involved in thermogenesis in subscapular (brown) fat of Mstn null mice is warranted.

Furthermore, it is also possible that the apparent "energy-wasting" phenomenon observed in Mstn null mice (i.e. reflected by reduced fat accumulation while consuming excess calories) may be unrelated to thermogenesis. Protein turnover, which is required to maintain normal muscle function for example, is a large part of resting energy expenditure (Welle and Nair, 1990). Muscle weight of Mstn null mice is increased 75-100% compared with wild type mice; this would imply a greater amount of protein turnover is needed in Mstn null mice. In fact, Mstn null mice have been shown to have a higher metabolic rate (McPherron and Lee, 2002). However, this increase is attenuated if corrected for lean mass, which suggests the increased lean mass is responsible for the increased metabolic rate. There is also evidence that protein turnover, like adaptive thermogenesis, may be an adaptation to maintain body weight when presented with an excess of calories. Protein turnover was shown to be increased in obese individuals (Welle and Nair, 1990) and recently, mice lacking a gene responsible for branched-chain amino acid metabolism exhibited diet-induced obesity resistance with increased protein turnover in the absence of thermogenic alterations. Thus, increased protein turnover in Mstn null mice, occurring as a result of increased muscle mass or as a specific adaptation to the high-fat diet, may explain their partial resistance to obesity development. Though

Mstn expression has been implicated in muscle atrophy (Ma *et al.*, 2003), rates of protein turnover have not been established in Mstn null mice.

In conclusion, the Mstn null mutation resulted in a partial blunting of the effects of high-fat feeding. Though Mstn null mice fed high-fat diets did exhibit an increase in body fat content, this increase was less pronounced than in wild type mice. Furthermore, other markers of the metabolic syndrome often associated with high-fat diet-induced obesity, namely serum triglyceride and liver fat content, were also improved in Mstn null mice compared with wild type mice. Unlike other mutations or conditions which result in obesity resistance, however, the Mstn null mutation did not protect against the development of insulin resistance as evidence by increased blood glucose levels following glucose injection of mice fed high-fat diets compared with control-fed mice. Though some genes involved in adaptive thermogenesis were specifically upregulated in high-fat fed Mstn null mice, the mechanism by which Mstn null mice resist obesity is still unclear. A closer investigation of metabolic rate, specifically related to protein turnover given the increased lean mass in Mstn null mice, is warranted.

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Tables and Figures

Figure 3.1. Body weight of Mstn null (light circles) and wild type (dark diamonds) mice fed control (solid lines) and high fat (dashed lines) diets for 12 weeks (4-16 weeks of age). Effect of time, genotype, diet, and interactions were significant ($P < 0.05$) and are shown.

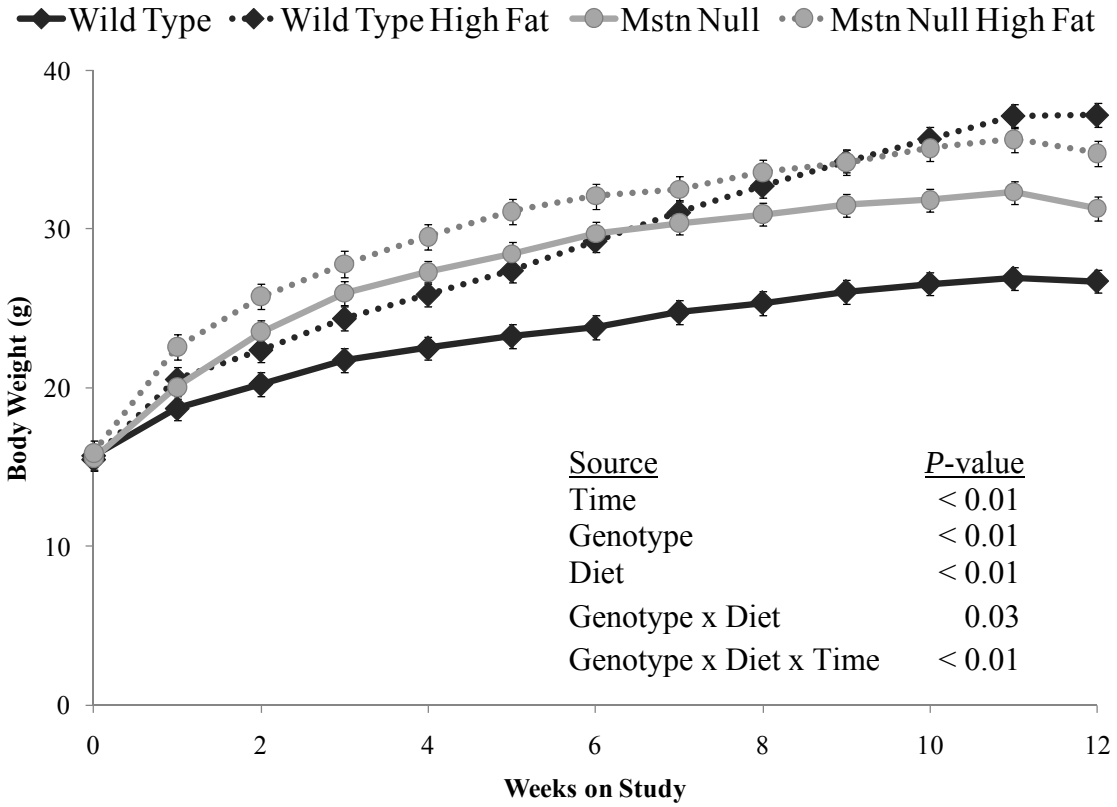

Figure 3.2. Fasting blood glucose (A) and total area under the curve for glucose values during glucose tolerance tests (C) of Mstn null and wild type mice prior to dietary treatment and at 4, 8, and 12 weeks on high fat and control diets. * indicates different than wild type control (P < 0.05). Data presented as least square means ± standard error. (B) Blood glucose values during glucose tolerance tests of myostatin (Mstn) null (light circles) and wild type (dark diamonds) mice after control (solid lines) or high fat (dashed lines) diets were fed for 12 weeks. Effect of diet on glucose values was significant (*P* < 0.01) while genotype effect and interaction were not significant (N.S.).

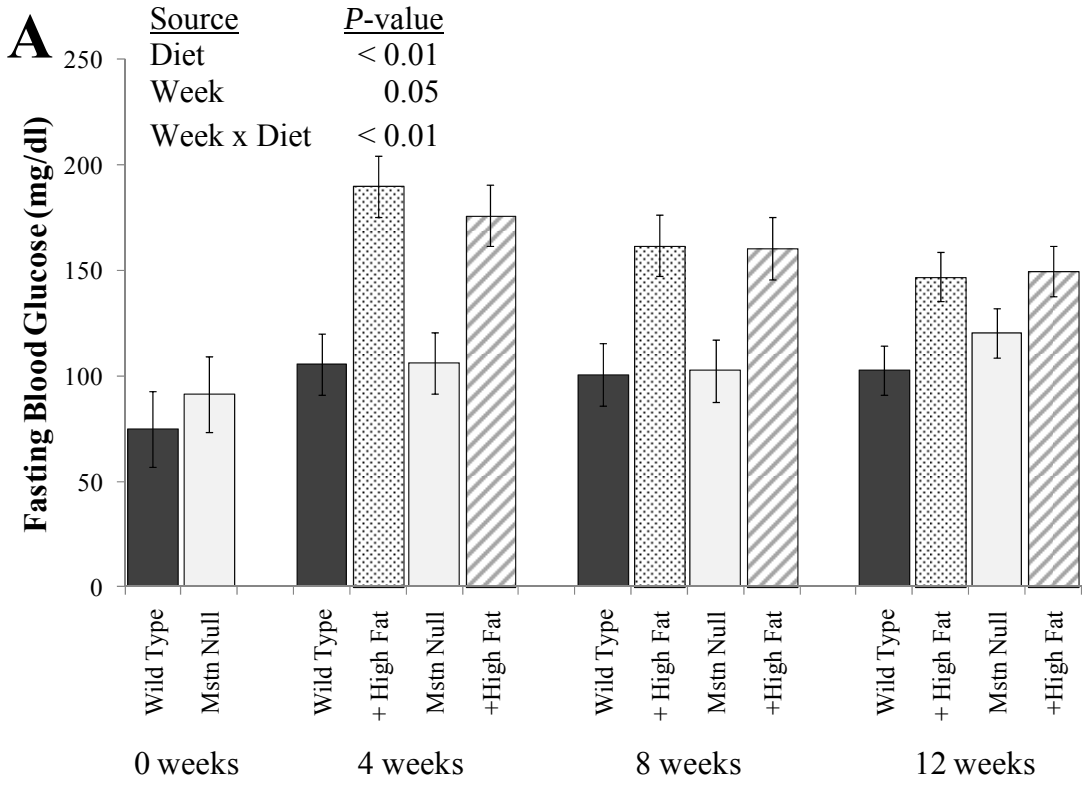

Figure 3.2 (cont.)

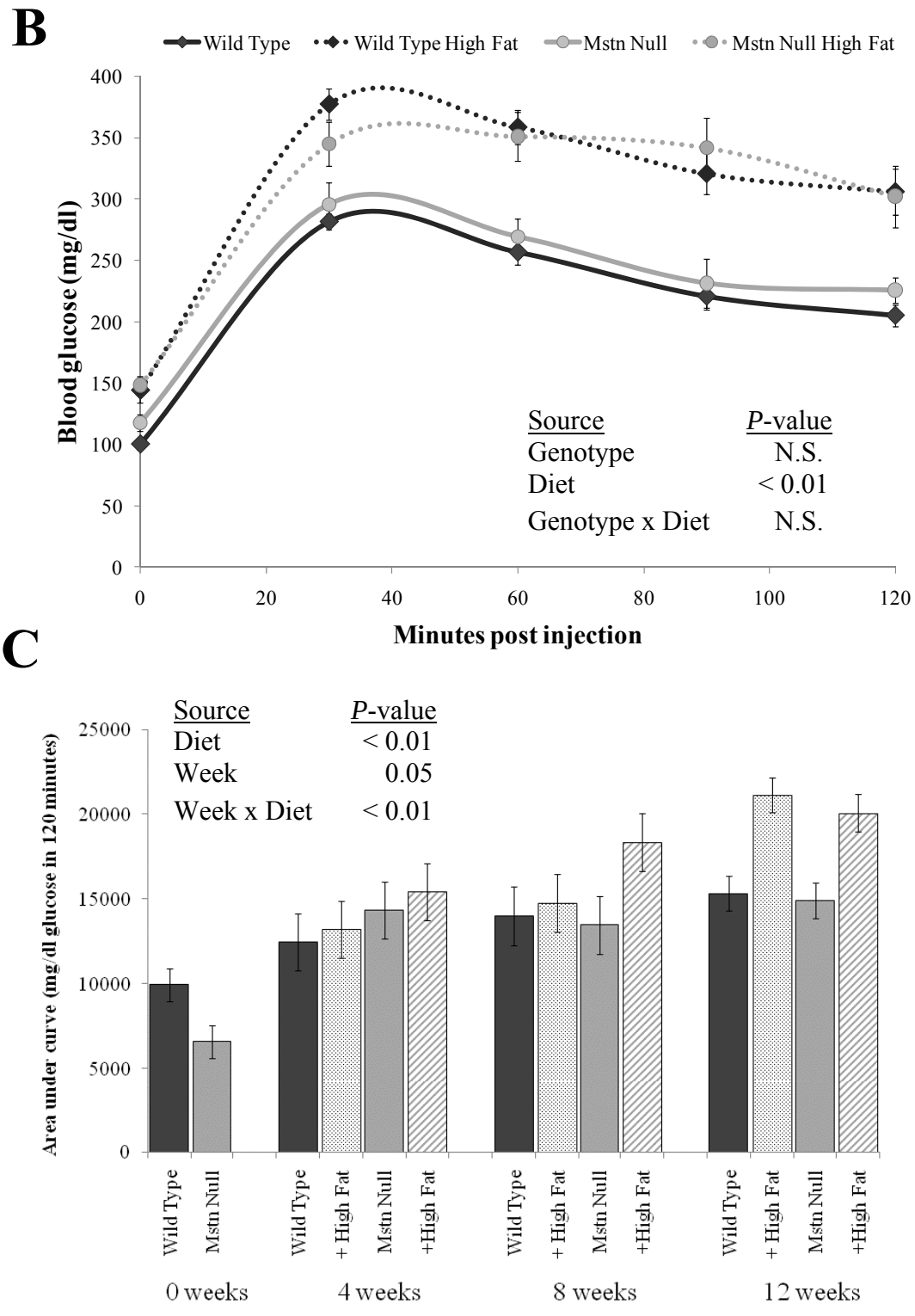

Figure 3.3. Serum triglycerides (A) and insulin (B) of wild type and Mstn null mice after control and high fat diets were fed for 12 weeks. Bars are least square means ± standard error. Bars with different letters differ ($P < 0.05$).

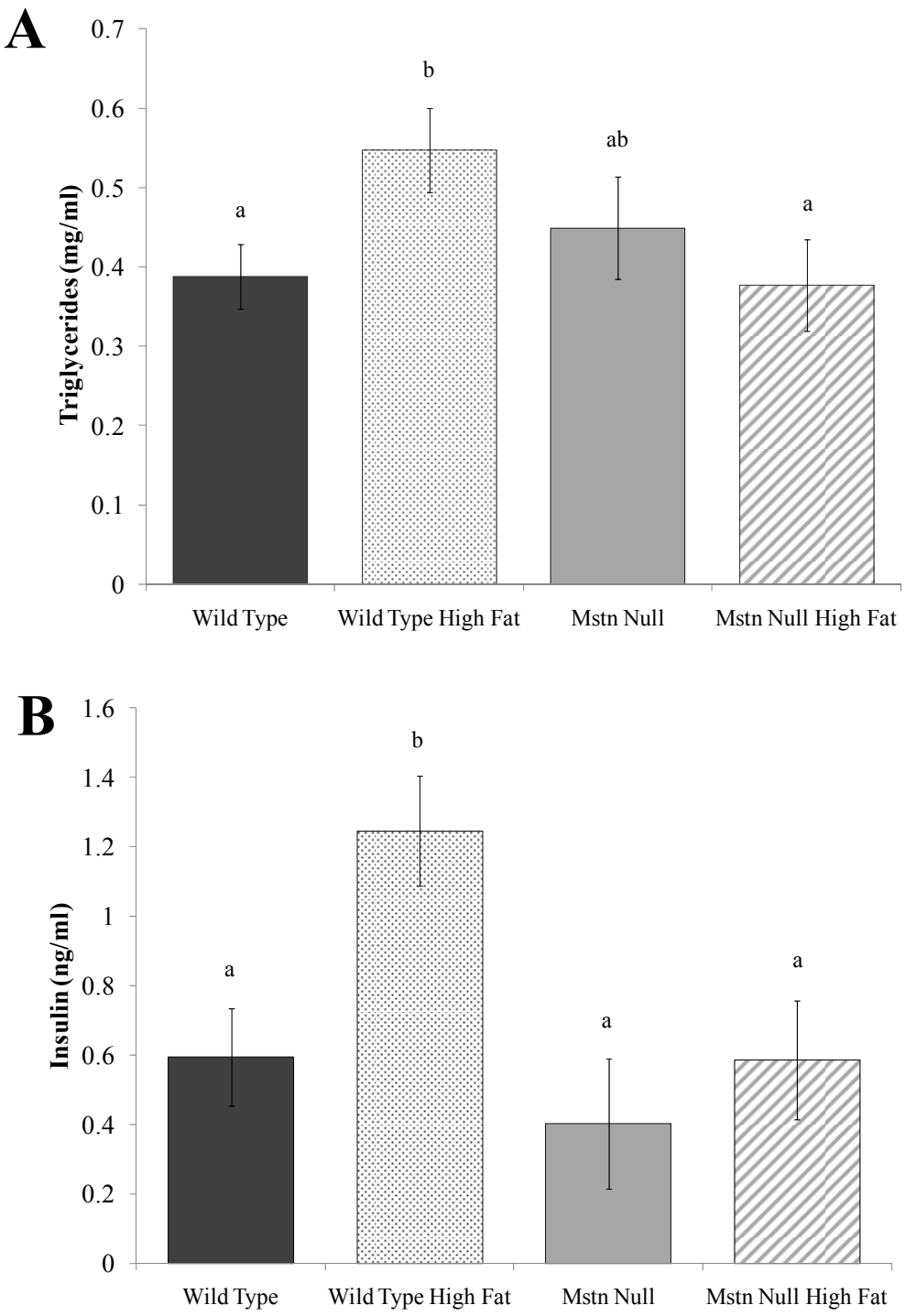

Figure 3.4. Weight and gene expression of epididymal fat in wild type and myostatin (Mstn) null mice after receiving control and high fat diets for 12 weeks. Mean fold change compared with wild type mice fed control diets ± standard error are displayed. Bars with different letters are different ($P < 0.05$). Abbreviations: PPARγ coactivator 1β (PGC 1β); uncoupling protein 3 (UCP 2)

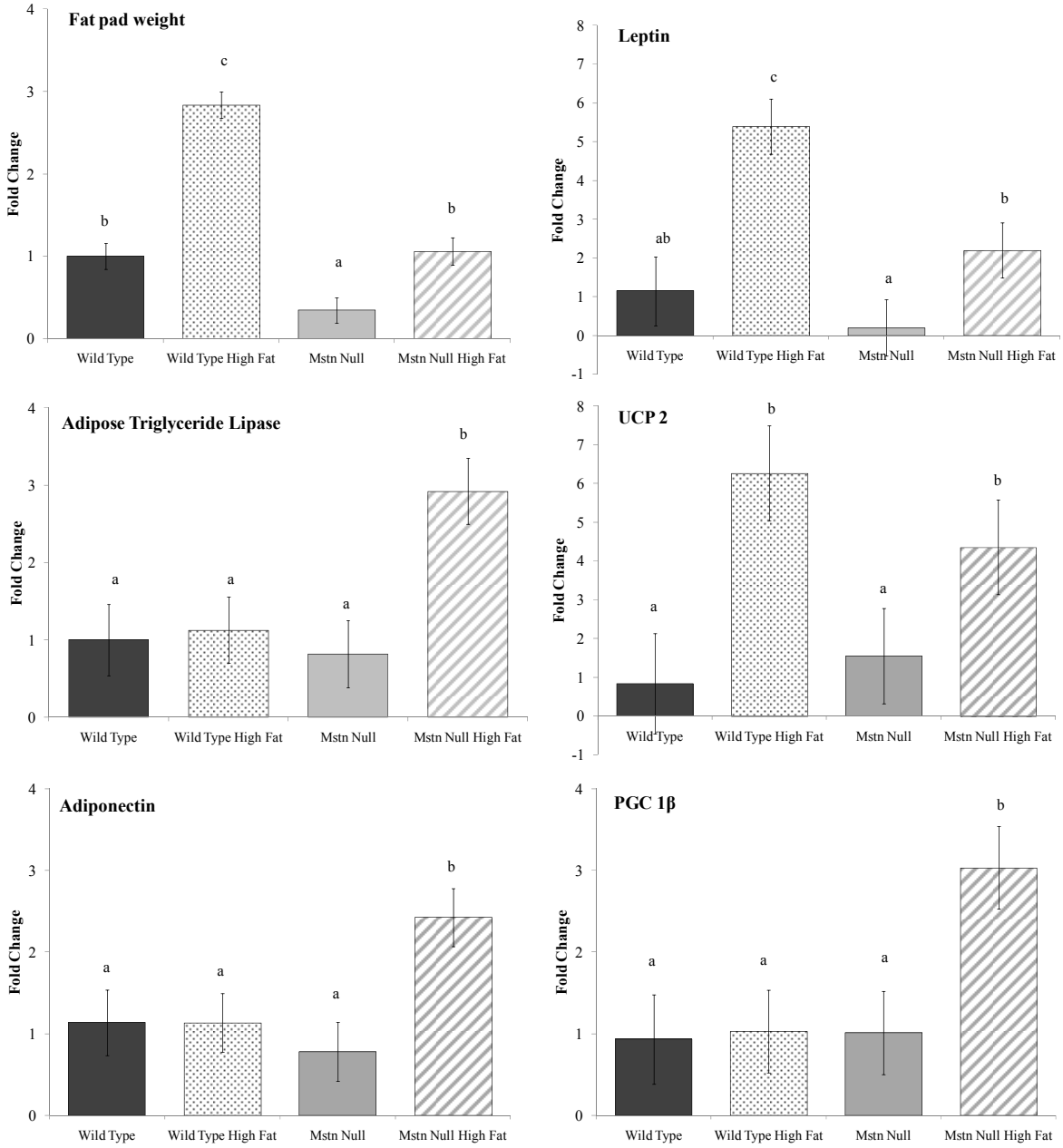

Table 3.1. Effects of genotype (G) and diet (D) and their interaction (GxD) on body and liver composition of wild type and myostatin null mice prior to (baseline) and after consuming control or high fat diets for 12 weeks[1]

	Wild Type		Myostatin Null			P-value		
	Control	High Fat	Control	High Fat	SEM	G	D	GxD
Baseline								
Number of mice	5	--	5	--			--	--
Body fat (%)[2]	6.1	--	4.8	--	0.31	0.02	--	--
Body protein (%)[2]	17.2	--	17.3	--	0.57	0.88	--	--
Body energy (kcal)[3]	20.2	--	18.8	--	1.53	0.56	--	--
Liver fat (%)[2]	7.2	--	7.7	--	0.67	0.64	--	--
12 weeks								
Number of mice	5	5	5	5				
Body fat (%)[2]	12.4	25.9	8.5	12.2	2.22	<0.01	<0.01	0.04
Body protein (%)[2]	18.3	15.2	19.5	19.3	0.59	<0.01	0.01	0.02
Body energy (kcal)[3]	45.6	103.5	48.2	67.1	9.94	0.11	<0.01	0.07
Liver fat (%)[2]	9.8	13.4	8.1	5.4	1.91	0.02	0.83	0.11

[1] Mice were provided ad libitum access to control (10% kcal from fat) or high fat (60% kcal from fat) diets. Values are lsmeans ± S.E.M.

[2] Body fat and protein are expressed as a percentage of body weight. Liver fat is expressed as a percentage of liver weight.

[3] Body energy was calculated from body fat and protein content assuming 8.79 kcal/g of fat and 4.27 kcal/g of protein.

Table 3.2. Effects of genotype (G) and diet (D) and their interaction (GxD) on food intake, caloric intake and efficiency of weight gain in wild type and myostatin null mice fed control or high fat diets for 12 weeks[1]

	Wild Type		Myostatin Null		P-value		
	Control	High Fat	Control	High Fat	G	D	GxD
Number of mice	11	11	12	9			
Weight gain (g)	12.2 ± 1.00	22.9 ± 1.00	17.4 ± 0.96	20.2 ± 1.10	0.23	<0.01	<0.01
Food intake (g)	245.2 ± 3.72	211.2 ± 3.72	265.2 ± 3.56	213.8 ± 4.11	<0.01	<0.01	0.03
Efficiency of gain (mg/g) [2]	50.1 ± 3.94	107.7 ± 3.94	65.7 ± 3.77	94.5 ± 4.35	0.76	<0.01	<0.01
Caloric intake (Kcal) [3]	944 ± 17.1	1107 ± 17.1	1021± 16.3	1120 ± 18.9	0.01	<0.01	0.08
Caloric Efficiency (%) [4]	2.7 ± 0.24	7.5 ± 0.24	2.9 ± 0.23	4.3 ± 0.26	<0.01	<0.01	<0.01

[1] Mice were provided ad libitum access to control (10% kcal from fat) or high fat (60% kcal from fat) diets. Values are lsmeans ± S.E.M.

[2] Efficiency of gain was calculated by dividing weight gain (mg) by food intake (g).

[3] Calculated from dietary caloric density: control, 3.85 kcal/g; high fat: 5.24 kcal/g.

[4] Caloric efficiency was calculated by dividing caloric gain, estimated from body composition data, by caloric intake x100.

Table 3.3. Effects of genotype (G) and diet (D) and their interaction (GxD) on fat pad and muscle weights of wild type and myostatin null mice fed control or high fat diets for 12 weeks[1]

	Wild Type		Myostatin Null		P-value		
	Control	High Fat	Control	High Fat	G	D	GxD
Number of mice	17	16	17	14			
Fat pad weight (mg)							
Epididymal	724 ± 115.2	2055 ± 115.2	249 ± 111.8	768 ± 123.2	<0.01	<0.01	<0.01
Inguinal	450 ± 115.6	1711 ± 115.6	192 ± 112.1	444 ± 123.5	<0.01	<0.01	<0.01
Retroperitoneal	186 ± 36.2	572 ± 26.2	35 ± 35.1	200 ± 38.7	<0.01	<0.01	<0.01
Subscapular	339 ± 78.6	1038 ± 78.6	87 ± 76.2	340 ± 84.0	<0.01	<0.01	<0.01
Muscle weight (mg)							
Tibalis Anterior	69 ± 3.0	72 ± 3.0	119 ± 2.9	125 ± 3.2	<0.01	0.14	0.52
Triceps Brachii	90 ± 6.4	97 ± 6.4	182 ± 6.2	194 ± 6.8	<0.01	0.16	0.69
Biceps Femoris	129 ± 6.5	133 ± 6.5	223 ± 6.3	234 ± 7.0	<0.01	0.04	0.66
Gastrocnemius	139 ± 4.9	150 ± 4.9	265 ± 4.8	274 ± 5.3	<0.01	0.25	0.66

[1] Mice were provided ad libitum access to control (10% kcal from fat) or high fat (60% kcal from fat) diets. Values are lsmeans ± S.E.M.

Table 3.4. Effects of genotype (G) on expression fold changes of genes in bicep femoris and gastrocnemius muscles of wild type and myostatin (Mstn) null mice fed control diets for 12 weeks [1]

	Biceps Femoris				Gastrocnemius			
	Wild Type	Mstn Null	SEM	P-value	Wild Type	Mstn Null	SEM	P-value
Number of mice	8	8			8	8		
MHC I	1.51	0.50	0.372	0.08	1.06	0.80	0.124	0.16
MHC IIa	1.06	0.13	0.087	<0.01	1.04	0.20	0.083	<0.01
MHC IIx	1.18	0.32	0.177	<0.01	1.10	0.06	0.129	<0.01
MHC IIb	1.02	1.20	0.088	0.17	1.03	1.14	0.091	0.40
PGC 1α	1.22	0.87	0.206	0.25	1.06	0.59	0.095	<0.01
PGC 1β	1.25	0.52	0.219	0.03	1.12	0.19	0.131	<0.01
UCP 3	1.03	1.05	0.180	0.96	1.02	1.33	0.132	0.12
LPL	1.19	0.58	0.211	0.07	1.09	1.77	0.292	0.13

[1] Values are least square means compared with a calibrator group. Abbreviations: myosin heavy chain (MHC), peroxisome-proliferator activated receptor γ coactivator (PGC), uncoupling protein (UCP), lipoprotein lipase (LPL).

GENERAL SUMMARY

Though the phenotype of myostatin (Mstn) mutant animals is very striking, the question remains whether the loss of Mstn function fundamentally changes growth potential, metabolism, or inherent regulation in tissues, or if it simply is an effect independent of other factors. To that end, we sought to increase muscle growth even further in Mstn null mice through the use of the β-adrenergic agonist (βAA) clenbuterol. We also aimed to alter the fat accumulation and metabolism of Mstn null mice by feeding diets high in fat.

Clenbuterol Treatment of Myostatin Null Mice

Overall, the individual effects of clenbuterol and ablation of Mstn function share some phenotypic similarities including increased protein accretion and reduced fat accumulation. Clenbuterol treatment increases expression of faster muscle fiber types (Oishi *et al.*, 2004), and Mstn null mice inherently possess more fast fibers, as well. Additionally, both treatments also involve the Akt/mTOR pathway, thereby stimulating translation of new proteins (Amirouche *et al.*, 2009; Kline *et al.*, 2007). There are, however, some differences in the two proposed mechanisms. Loss of Mstn function results in prenatal muscle hyperplasia (Thomas *et al.*, 2000) and increased satellite cell activity (McCroskery *et al.*, 2003). Clenbuterol treatment may alter satellite cell activation (Maltin and Delday, 1992), but cannot result in muscle cell hyperplasia as muscle cell number is largely set at birth. Thus, we hypothesized that the mechanisms of each effect were different enough so as to be additive.

The results of our study reveal that the effects of clenbuterol treatment and the lack of Mstn function were completely additive for body weight, carcass composition, and muscle and

fat pad weights. The interaction of clenbuterol treatment and genotype—indicative of a less than additive respsonse—was only significant for gastrocnemius muscle weight. In this case, the magnitude of the difference, less than 2 mg, was very small. For all other traits, the two effects were completely additive. These data suggest that the mechanism of increased muscle growth from βAA treatment and ablation of Mstn function are indeed independent.

Additionally, these data suggest that regulation of Akt/mTOR signaling may comprise only a small part of either the effect of clenbuterol or of Mstn deletion. Myostatin inhibits mTOR signaling while clenbuterol activates it. Thus, the loss of myostatin and the administration of clenbuterol may result in the same effect—increased mTOR signaling. The additive effects of Mstn deletion and clenbuterol indicate that mTOR is not the only pathway responsible for increased muscle protein accretion in this model or that each single treatment does not maximize mTOR signaling. It would therefore be interesting in future work to investigate mTOR signaling in Mstn null mice to determine if the loss of Mstn function in these mice does alter its' regulation.

With regard to fat accumulation, reductions due the loss of Mstn function and from clenbuterol treatment were fully realized in our experiment. Body fat composition and fat pad weights were reduced by clenbuterol treatment in both wild type and Mstn null mice. Clenbuterol directly impacts lipolysis and lipogenesis through activation and inhibition of enzymes. The impact of the Mstn null mutation on fat accumulation, however, may be more indirect.

Overall, the results of clenbuterol treatment in Mstn null mice suggest that β-adrenergic signaling is not altered by the loss of Mstn function. A microarray analysis of this model was performed and will be detailed in full in another work. Results from this analysis, however,

confirm that clenbuterol treatment in Mstn null and wild type mice is additive with regards to gene expression changes. Improvements to our study include a histological analysis of muscle cells to confirm muscle hypertrophy in both wild type and Mstn null mice treated with clenbuterol. Furthermore, measuring down-stream signals of β-adrenergic signaling in muscle cells and adipocytes would confirm that β-adrenergic signaling is not altered in Mstn null mice.

High-fat Diet-induced Obesity in Myostatin Null Mice

Given the larger muscle mass of Mstn null mice, we hypothesized that insulin-stimulated glucose uptake may be increased simply due to a greater amount of tissue available for activity. However, slow, oxidative fibers are more insulin responsive than fast, glycolytic fibers (Kriketos *et al.*, 1996) and an increased amount of slow, oxidative fibers has been shown to be associated with resistance to obesity in rats (Abou Mrad et al., 1992). Thus, given the increased fast, glycolytic phenotype of Mstn null mice, it is possible that they are less, and not more, resistant to obesity.

As previously detailed, there is some evidence to suggest that Mstn null mice are partially resistant to the effects of high-fat diets (Guo *et al.*, 2009; Hamrick *et al.*, 2006). However, one criticism of these studies is that they did not employ mouse strains with increased propensity for obesity caused by high-fat diets. Thus, strain-specific differences may serve as a confounding factor in this line of research.

Our study confirms, in an obesity-prone strain, that Mstn null mice are partially resistant to high-fat diet-induced obesity. Fat accumulation was increased in high-fat fed Mstn null mice compared with control-fed Mstn null mice. The increase in fat accumulation in high-fat fed Mstn null mice, however, was much less than that of high-fat fed wild type mice. Overall, high-fat

feeding of Mstn null mice resulted in body fat accumulation similar to control-fed wild type mice. As caloric and fat intake was increased in all high-fat fed mice, the lack of obesity in Mstn null mice would suggest adaptive energy wasting in this genotype. Expression of genes involved in white adipose tissue thermogenesis including PGC 1β and UCP 2 were increased in high-fat fed Mstn null mice compared with wild type mice. Lipid mobilization and thermogenic genes in skeletal muscle of Mstn null mice, however, were not altered in a way that would suggest improved thermogenesis in these animals. Though characterization of brown adipose tissue thermogenesis is needed in this model, it does not appear that altered thermogenesis explains the reduced fat accumulation in Mstn null mice. It is plausible that increased protein turnover due to the increase in muscle mass in Mstn null mice may be a source of "energy wastage" and account for the decreased fat accumulation in both control and high-fat fed Mstn null mice. Characterization of rates of protein turnover with and without the addition of high-fat diets in Mstn null mice may elucidate the mechanism of decreased fat accumulation in these mice.

In conclusion, altering muscle and fat mass in Mstn null mice is certainly possible. While the phenotype of Mstn null mice is extreme, this mutation alone does not result in maximal muscle growth or minimal fat accumulation. Muscle growth can be increased and fat growth decreased by clenbuterol treatment. High-fat feeding increases adipose growth in Mstn null animals.

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APPENDIX

Glucose Tolerance Tests

Figure A.1 Blood glucose values during glucose tolerance tests of myostatin (Mstn) null (light circles) and wild type (dark diamonds) mice at 4 weeks of age and prior to dietary treatments. Genotype effect was not significant.

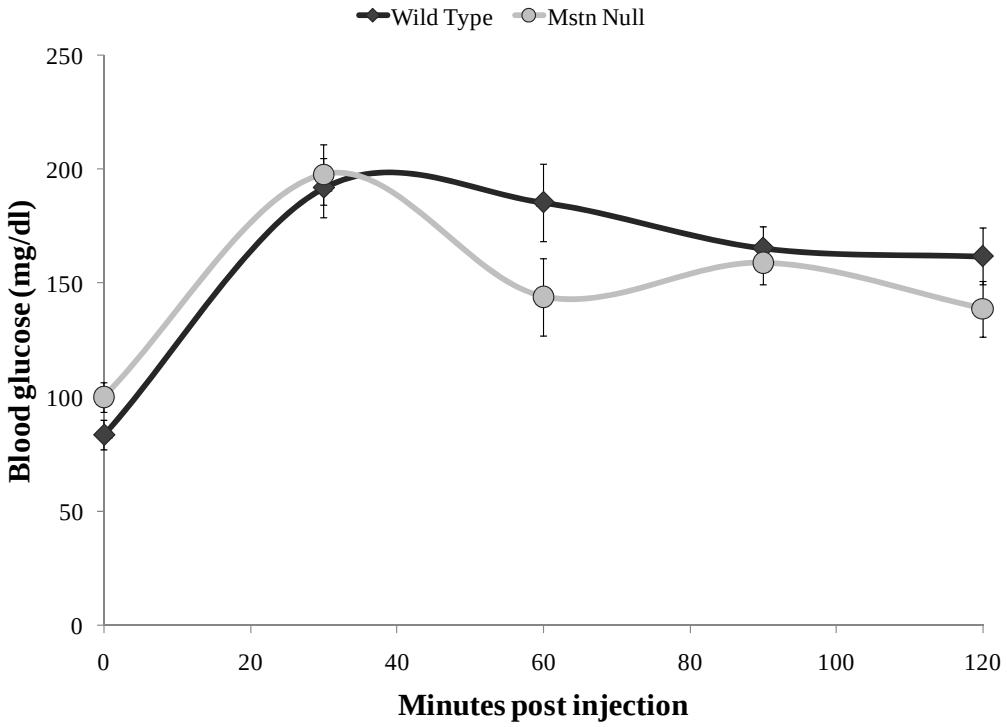


Figure A.2 Blood glucose values during glucose tolerance tests of myostatin (Mstn) null (light circles) and wild type (dark diamonds) mice after control (solid lines) or high fat (dashed lines) diets were fed for 4 weeks. Effect of diet on glucose values was significant ($P < 0.01$) while genotype effect and interaction were not significant (N.S.).

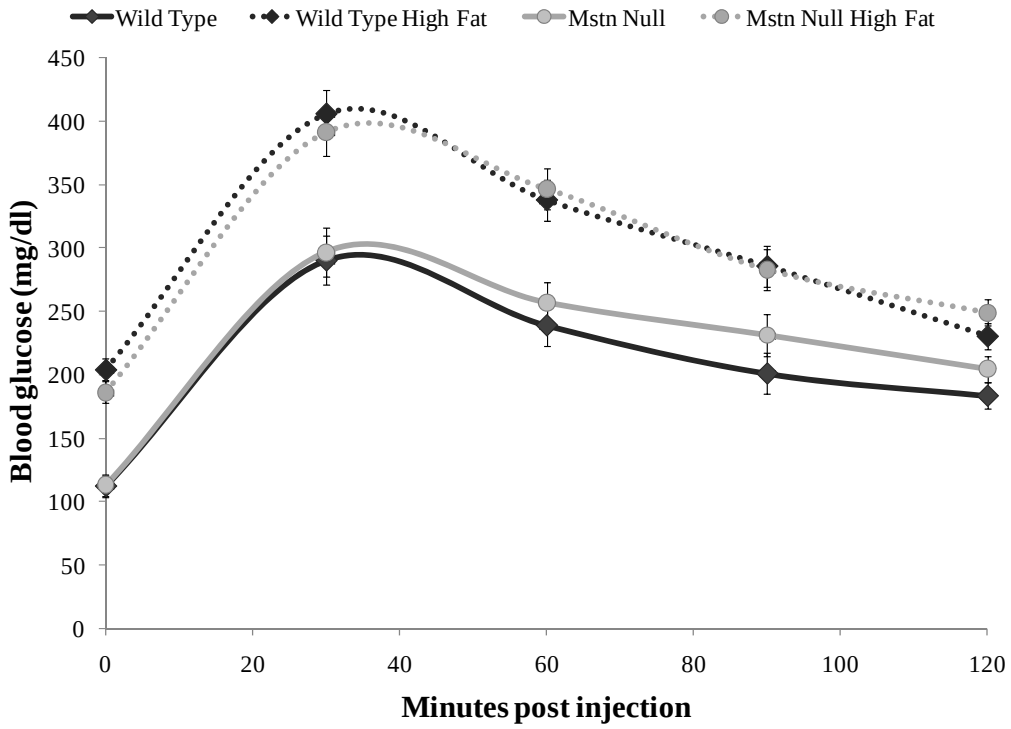
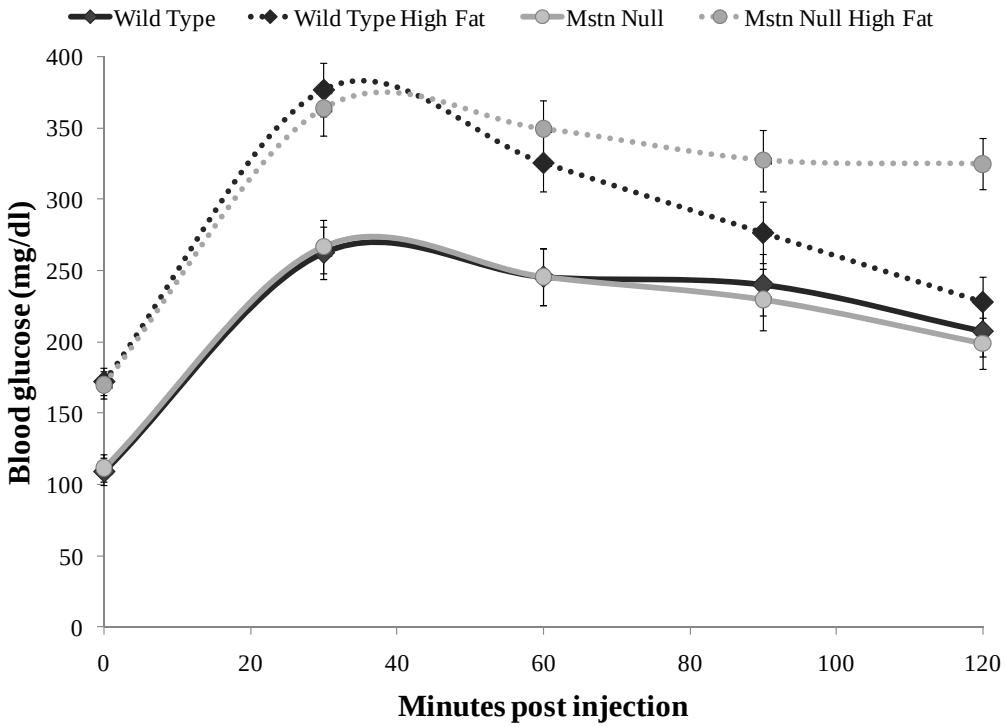


Figure A.3 Blood glucose values during glucose tolerance tests of myostatin (Mstn) null (light circles) and wild type (dark diamonds) mice after control (solid lines) or high fat (dashed lines) diets were fed for 8 weeks. Effect of diet on glucose values was significant ($P < 0.01$) while genotype effect and interaction were not significant (N.S.).

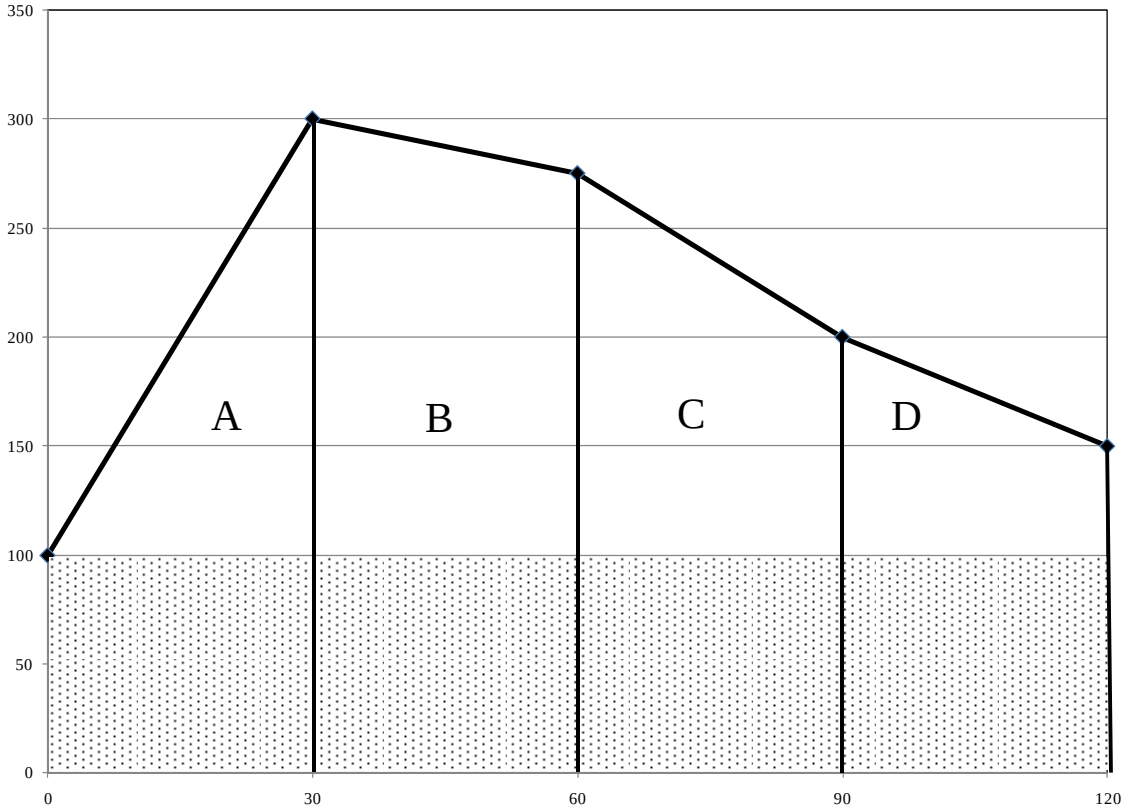


Diet Formulations

Diets from Chapter 3 obtained from Research Diets Incorporated, New Brunswick, NJ.

	Control Diet Product # D12450B		High Fat Diet Product # D12492	
	% of diet	% of kcal	% of diet	% of kcal
Protein	19.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20
Fat	4.3	10	34.9	60
	g/kg	% of kcal	g/kg	% of kcal
Casein, 80 Mesh	189.6	19.72	258.4	19.72
L-Cystine	2.8	0.30	3.9	0.30
Corn Starch	298.6	31.06	0	0
Maltodextrin 10	33.2	3.45	161.5	12.32
Sucrose	331.7	34.51	88.9	6.78
Cellulose, BW200	47.4	0	64.6	0
Soybean Oil	23.7	5.55	32.3	5.55
Lard	19.0	4.44	316.6	54.35
Mineral Mix, S10026	9.5	0	12.9	0
Dicalcium Phosphate	12.3	0	16.8	0
Calcium Carbonate	5.2	0	7.1	0
Potassium Citrate	15.6	0	21.3	0
Vitamin Mix, V1001	9.5	0.99	12.9	0.99
Choline Bitartrate	1.9	0	2.6	0
FD&C Blue Dye #1	0	0	0.06	0
FD&C Yellow Dye #5	0.05	0	0	0

Calculations for Area under the Curve



Area under the curve for glucose tolerance was calculated using a trapezoidal method. The data above are for demonstration purposes and correspond to glucose readings of 100 at 0 minutes, 300 at 30 minutes, 275 at 60 minutes, 200 at 90 minutes and 150 at 120 minutes post injection. Minutes are on the horizontal axis and glucose on the vertical axis.

First, the area of each outlined trapezoid is calculated and summed together.

Trapezoid A: (100+300)/2 x 30 = 6000

Trapezoid B: (300+275)/2 x 30 = 8625

Trapezoid C: (275+200)/2 x 30 = 7125

Trapezoid D: (200+150)/2 x 30 = 5250

6000 + 8625+7125+5250 = 27000

Then, the area below the baseline reading (shaded area) is calculated as follows and subtracted from the trapezoidal sums:

Baseline area: 100x120=12000 Area under the curve: 27000-12000=15000

Protocol for RNA Extraction, cDNA Synthesis and Gene Expression

RNA Extraction

1. Prepare tissue for extraction by placing 50-100 mg into an Eppendorf Safe-lock tube.
2. Add 1 ml Trizol solution and keep on ice.
3. Disrupt in the Tissuelyzer for 3 minutes on 30 Hz.
4. Centrifuge at 4ºC for 15 minutes at 14,000xg.
5. Remove supernatant and add to new 1.5 ml tubes containing 100 µl BCP (1-bromo-3-chloropropane).
6. Mix by vortexing 15 s. Incubate at room temperature for at least 10 minutes.
7. Centrifuge at 4ºC for 15 minutes at 12,000xg.
8. Transfer upper phase into new 1.5 ml tubes containing 500µl isopropanol. Place in -20ºC freezer overnight.
9. Centrifuge at 4ºC for 15 minutes at 12,000xg and RNA pellet will form.
10. Decant supernatant and add 1 ml 75% ethanol. Vortex sample to disturb but not dissolve pellet.
11. Centrifuge at 4ºC for 15 minutes at 12,000xg.
12. Decant supernatant and allow pellet to air dry.
13. Add 25 µl DNAase-free water and quantify RNA concentration and purity with Nanodrop.
14. Dilute RNA with DNAase-free water to concentration of 500 ng/ml.

cDNA Synthesis—Qiagen Quantitect Reverse Transcription Kit

1. Mix DNA-wipeout solution by adding 2 µl DNA Wipeout and 10µl water to 2µl of each RNA sample.
2. Incubate according to kit directions.

3. Mix Transcriptase solution by adding 1 µl transcriptase, 4 µl buffer and 1 µl primers to each sample.
4. Incubate according to kit directions.
5. Use cDNA immediately or store at -20ºC.

Gene Expression—Taqman Primer/Probe Kits

1. Dilute cDNA as appropriate based on validation tests. For muscle samples, a 1:100 dilution usually works well.
2. Add 2µl of Primer/Probe to 20 µl Taqman Universal PCR Master Mix for each reaction.
3. Add 18µl cDNA to master mix.
4. Pipette 10µl in triplicate into 384 well plate.

AUTHOR'S BIOGRAPHY

Anna Carol Day Dilger, born October 12, 1979, is the middle child of David and Brenda Day of Noblesville, Indiana. She has two brothers, Nathaniel and Eric. Anna earned a B.S. degree in Animal Sciences from Purdue University in 2002 and following graduation, married Ryan Neil Dilger of Mariah Hill, Indiana, on June 22, 2002. Anna and Ryan have one daughter, Allison Joy, born November 28, 2007. Anna was awarded a National Science Foundation Graduate Fellowship and continued her education in the area of muscle biology under the direction of David Gerrard. She earned her M.S. degree in Animal Sciences at Purdue University in December 2004. Anna was awarded a Jonathan Baldwin Turner Fellowship from the University of Illinois at Urbana-Champaign and completed her Ph.D. degree in September 2009 under the guidance of Dr. John Killefer.