Robert E. Gray. THE EFFECT OF WEIGHT LOSS ON INTRAMUSCULAR LIPID CONTENT AND MUSCLE FIBER TYPE DISTRIBUTION IN THE MORBIDLY OBESE. (Under the direction of Joseph A. Houmard, Ph.D.) Department of Exercise and Sports Science, June 2002.

In obesity, intramuscular lipid content is associated with insulin resistance. Weight loss has been shown to reduce the amount of lipid within skeletal muscle. The relationship between muscle fiber type and lipid content is unknown. The purpose of this study was to determine the relationship between intramuscular lipid content and muscle fiber type in morbidly obese individuals, before and after weight loss. Percutaneous muscle biopsies from the vastus lateralis were performed in 6 morbidly obese subjects prior to and approximately 1-year after gastric bypass surgery. Histochemistry was used to determine muscle fiber type (Mysosin ATPase), fiber area (alpha-glycerophospate dehydrogenase), lipid content (Oil Red-O), oxidative capacity (NADH tetrazolium reductase), and glycolytic capacity (alpha-glycerophospate dehydrogenase) in transverse, serial cryosections. Quantification was performed by digital image analysis. Weight loss resulted in a 47 % reduction of body mass index (52.2 ± 2.5 vs. 27.9 ± 0.8, kg/m^2). Weight loss was accompanied by a significant improvement in insulin sensitivity (HOMA, 7.0 ± 1.9 vs. 0.5 ± 0.1). Weight loss resulted in a 30 % reduction of total fat per fiber area within the muscle (LAI %, 30.8 ± 5.7 vs. 21.7 ± 3.8, %). Lipid content was significantly higher in type I compared to type II fibers before and after weight loss ($p < .01$). Lipid content relative to fiber area was highest in type I fibers, before and after weight loss, and was reduced similarly in both fiber types after weight loss ($p < .05$). No significant changes were found in oxidative or glycolytic enzyme capacity following

weight loss. In summary, lipid content is reduced similarly across fiber types and lipid content relative to fiber area is reduced following weight loss in morbidly obese individuals. These results provide more information regarding the relationship of obesity, insulin resistance, and intramuscular lipid content.

THE EFFECTS OF WEIGHT LOSS ON INTRAMUSCULAR LIPID CONTENT AND MUSCLE FIBER TYPE DISTRIBUTION IN THE MORBIDLY OBESE

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THE EFFECTS OF WEIGHT LOSS ON INTRAMUSCULAR LIPID CONTENT AND FIBER TYPE DISTRIBUTION IN THE MORBIDLY OBESE

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DEDICATIONS

This thesis is dedicated first and foremost to my parents. Their support throughout my undergraduate and graduate years has been immeasurable. I cannot imagine going through the past six years without their love and guidance. This thesis is also dedicated to Mike, Jason, Fred, and Bryan. I could not have asked for better friends.

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CHAPTER I

INTRODUCTION

Obesity is a growing health problem in the United States, contributing to an estimated 300,000 deaths annually (Pi-Sunyer, 1993). It is estimated that 31% of adult males and 35% of adult females are overweight when all race and ethnic groups are combined. From the time period between the NHANES II and NHANES III studies (1976-1980 and 1988-1991), the prevalence of overweight increased 8% (Kuczmarski et al. 1994). Of even greater concern is the fact that there is a 5% prevalence rate of morbid obesity in the United States (Matory et al. 1994; Atkinson et al. 1993; Hamilton, 1996). In this population, many weight loss interventions fail to provide long-term results however the surgical gastric bypass procedure has been shown to be an effective treatment of morbid obesity (Pories et al. 1992).

It has been hypothesized that obesity may be associated with impaired lipid oxidation resulting from defects in skeletal muscle (Kelley et al. 1999). Skeletal muscle has an important role in energy expenditure. At rest, skeletal muscle accounts for 20-30 % of the body's energy expenditure and up to 90 % of energy expenditure during exercise. Roughly 80 % of skeletal muscle oxidation at rest is fueled by fat oxidation (Astrup et al. 1997).

Positive fat balance and fat mass gains may be promoted by a reduction in skeletal muscle lipid oxidation (Kim et al. 2000). Kelley et al. (1999) found that despite similar fatty acid uptake between lean and obese individuals, obese subjects demonstrated a lower rate of fasting lipid oxidation, increasing the rate of net lipid storage. Additionally,

skeletal muscle fat oxidation did not improve after weight loss, suggesting that these defects may be primary to obesity.

The mechanisms responsible for defects in skeletal muscle lipid oxidation remain unknown, however a possible mechanism that has been explored is a reduction in the activity of CPT-1, which is the rate-limiting step in transport of long chain fatty acids into the mitochondria for oxidation. Kim et al. (2000) found a lower CPT-1 activity and lower key oxidative enzyme activities in obese skeletal muscle. With impaired ability to transport long chain fatty acids into the mitochondria for oxidation, a build up of muscle triglyceride may be expected. Additionally, lower oxidative enzyme capacity may also result in an increased concentration of skeletal muscle lipids (Ruderman et al. 1995).

Through histochemical use of Oil Red-O dye, which stains lipid within the muscle, intramuscular triglyceride content has been found to be increased in obesity and reduced by weight loss (Goodpaster et al., 2000; He et al. 2001). There is also evidence of increased intramuscular triglyceride concentrations in highly trained athletes; however, these increases must be appraised in context to other markers of metabolic capacity such as fiber type distribution (Kelley and Mandarino, 2000). Higher amounts of intramuscular triglyceride tend to be physiologically beneficial for highly trained athletes, possibly because of differences in fiber type distribution. Type I fibers have higher lipid concentrations but they also have a higher oxidative capacity due to increased mitochondrial volume and capillary density (Ingjer et al., 1979; Staron et al., 1984). It has been suggested that lean individuals may have a higher proportion of type I fibers, while obese individuals may have a higher proportion of type II fibers (Kriketos et al. 1997;

Wade et al. 1990; Lillioja et al. 1987; Hickey et al. 1995), however there is also evidence suggesting that there are no differences in fiber type distribution between lean and obese individuals (He et al. 2001; Raben et al. 1998).

Little is known about the fiber type distribution found in the morbidly obese population. Additionally, little is known regarding the intramuscular lipid profile of the morbidly obese before and after weight loss. Therefore, the purpose of this study was to determine intramuscular lipid content and fiber type distribution found in morbidly obese individuals before and after weight loss. The secondary purpose of this study was to determine the effects of weight loss upon the distribution of lipids among different fiber types within the muscle.

Statement of the Problem:

1. Is weight loss associated with decreased intramuscular lipid content?
2. Does weight loss affect muscle fiber type distribution?
3. Does weight loss cause a decrease of intramuscular lipid within a particular fiber type?
4. Is weight loss associated with a change in fiber size and oxidative capacity?

Limitations of the study:

1. The sample studied was all female.
2. Dietary practices of the subjects were not analyzed pre-surgery or controlled post-surgery.

Delimitations

1. The sample studied was primarily female.
2. The sample included skeletal muscle sections pre-weight loss (N=6) and post-weight loss (N=6)
3. The muscle samples were only extracted from the vastus lateralis.

Hypotheses:

1. Weight loss is associated with a reduction of lipid content in the vastus lateralis in morbidly obese subjects.
2. Weight loss is associated with a decrease in muscle fiber size.
3. Muscle fiber oxidative capacity is not changed due to weight loss.
4. Fiber type distribution is not changed due to weight loss.
5. Ratios of intramuscular triglyceride content between fiber types are not changed due to weight loss.

Definition of Terms:

1. Gastric Bypass: a surgical procedure that involves partitioning of the stomach to reduce the area available for digestion, therefore reducing caloric intake.
2. Intramuscular Triglyceride: triglyceride stored within the muscle fiber.
3. Morbidly Obese: physiological state of extreme obesity, usually characterized as BMI $\geq$37 kg/m^2 or $\geq$170% of ideal body weight (Pories et al. 1992).
4. Muscle Biopsy: invasive technique developed by Bergstrom; used to remove a sample of tissue from a muscle belly.

5. Obesity: physiological state accompanied by excess adipose tissue, usually defined as BMI $\geq$30 kg/m^2 (Pories et al. 1992).
6. Oil Red-O Staining: histochemical technique used to stain neutral lipids, mainly triglyceride, within the muscle fiber a reddish-orange color.
7. Type I Muscle Fibers: slow twitch, red muscle fibers characterized by high aerobic capacity.
8. Type IIa Muscle Fibers: fast twitch, red muscle fibers that act as an intermediate between type I and IIb muscle fibers, having aerobic and anaerobic capabilities.
9. Type IIb Muscle Fibers: fast twitch, white muscle fibers characterized by high glycolytic capacity.

CHAPTER II

REVIEW OF LITERATURE

Obesity is now beginning to be thought of as a disease, however the pathogenesis of this disease is unknown. A possible mechanism may be a reduction of skeletal muscle lipid oxidation capacity. A reduced capacity to utilize fatty acids may result in a build up of triglyceride within skeletal muscle. Intramuscular lipid depots may be increased in obesity and possibly reduced by weight loss. Additionally, intramuscular triglyceride distribution may be related to fiber type distribution. This chapter will review the relevant literature focused upon these issues. The chapter is divided into the following sections:

1. Obesity
2. Skeletal Muscle Fat Oxidation
3. Evidence for Increased Intramuscular Lipid Content with Obesity
4. Effects of Weight Loss on Intramuscular Lipid Content
5. Surgical Intervention and Weight Loss in the Morbidly Obese

Obesity

Obesity is a serious and common health problem. In the United States, obesity contributes to an estimated 300,000 deaths annually (Pi-Sunyer, 1993). At present, it is well established that obesity is related to an increased risk of mortality and morbidity, including coronary heart disease, type 1 and 2 diabetes, hypertension, hyperlipidemia, certain types of cancer, and other illnesses (NIH 1985).

Obesity has been defined as a physical state characterized by excessive adiposity that may be related to negative health outcomes (Besseman, 2000). The physiological

state at which excess adiposity constitutes an obese state is arbitrary and several variables have been used to characterize obesity . Body mass index (BMI) has been used to assess weight relative to height. Obesity related health problems tend to increase beyond a BMI of 25 kg/m^2. A BMI in the range of 25 to 29.9 kg/m^2 is considered overweight, while a BMI > 30 kg/m^2 is considered obese. Another index of obesity is the use of waist circumference measurements. A waist circumference greater than 100 cm is considered obese. Percent fat is also used to estimate obesity. A percent fat greater than 25% for males and greater than 30% for females is considered obese. Additionally, a body weight greater than 124% and 120% of ideal weight is considered obese for males and females, respectively (Kuczmarski et al. 1994).

The prevalence of obesity is increasing at an alarming rate in the United States. It is estimated that 31% of adult males and 35% of adult females are overweight when all race and ethnic groups are combined. Alarmingly, nearly 50% of all adult non-Hispanic black and Mexican American women are classified as overweight (Kuczmarski et al. 1994). From the time period between the NHANES II and NHANES III studies (1976-1980 and 1988-1991), the prevalence of overweight has increased 8%. Additionally, there has been an increase in the mean weight of adults aged 20 to 74 years by 3.6 kg. Over a similar time span, there has also been an increase in the prevalence of overweight children (Toriano et al. 1998). Massive obesity or morbid obesity occurs at 170% of ideal body weight or a BMI greater than 37 kg/m^2. Of great concern are the estimates showing that this excessive state of obesity has a 5% prevalence rate in the United States (Matory et al. 1994; Atkinson et al. 1993; Hamilton, 1996).

With an increasing prevalence of overweight and obese individuals in the United States, there will be an increase in obesity associated disease prevalence. Research must be continued to determine what mechanisms may be responsible for obesity. Additionally, effort must continue in order to determine appropriate weight loss interventions that may reverse the deleterious effects associated with obesity.

Skeletal Muscle Fat Oxidation

Causes for obesity include genetic, environmental, psychological, and physiological factors. Whether individuals are genetically predisposed, diseased, emotionally unstable, sedentary, or practice poor dietary habits, the excess accumulation of body fat results from a caloric intake above and beyond the energy need of the body. Excess caloric intake is stored in the body in the form of fat. While this is a simple concept, the exact mechanisms in the pathogenesis obesity remain unresolved. It is unknown as to why individuals of a similar weight, height, food intake, and energy expenditure can have wide variations in body fat content (Hamilton et al. 1996). Previously, obesity was thought to be a product of poor self-discipline, inactivity, and overeating. Now, because of distinct biochemical and physiological abnormalities, obesity is beginning to be recognized as a disease (Atkinson et al. 1991). Currently, research is providing insight into the mechanisms underlying the biochemical and physiological abnormalities associated with obesity.

It has been hypothesized that obesity may be associated with impaired lipid oxidation resulting from oxidation defects in skeletal muscle. Skeletal muscle has an important role in energy expenditure. At rest, skeletal muscle accounts for 20-30% of the

body's energy expenditure and up to 90% of energy expenditure during exercise. Roughly 80% of skeletal muscle oxidation at rest is fueled by fat oxidation (Astrup et al. 1997). With this in mind, skeletal muscle plays an important role in whole body lipid oxidation. Positive fat balance and fat mass gains may be promoted by a reduction in skeletal muscle lipid oxidation (Kim et al. 2000).

Simoneau et al. (1995) examined skeletal muscle oxidative enzyme capacity and muscle composition in obese women (N=17). Muscle composition was determined by CT scan. Briefly, fat and muscle display different attenuation values (measured in Hounsfield Units, HU). Fat attenuation values are negative, while muscle attenuation values are positive, with water serving as a reference (0 HU). Muscle enzyme activity was analyzed from vastus lateralis samples, obtained from a muscle biopsy. Each sample was analyzed for activity levels of creatine kinase (CK), hexokinase (HK), phosphofructokinase (PFK), glyceraldehydephosphate (GAPDH), citrate synthase (CS), cytochrome c oxidase (COX), and 3-hydroxyacle CoA dehydrogenase (HADH). There was a significant correlation between muscle enzyme activity and low attenuation values. In the subjects with low attenuation values (i.e. more fat within the muscle), there was a lower citrate synthase activity ($r = -.54$) and higher activities of creatine kinase ($r = .56$), phosphofructokinase ($r = .41$), and glyceraldehydephosphate ($r = .52$). Low attenuation values in skeletal muscle may be a result of increased fat content within the muscle. Results from this study suggest that there is a relationship between increased lipid within skeletal muscle and decreased oxidative capacity that may cause a predisposition for net storage of fat within skeletal muscle and possibly adipose tissue.

A possible mechanism responsible for lipid oxidation defects within skeletal muscle is a reduction in the activity of CPT-1, which is the rate-limiting step in transport of long chain fatty acids into the mitochondria for oxidation. Winder et al. (1995) reported that increased skeletal muscle glucose metabolism in rats led to increased malonyl-CoA concentration which inhibited CPT-1 and blocked free fatty acid entry into the mitochondria. Ruderman et al (1999) noted that in skeletal muscle, CPT-1 is regulated by malonyl-CoA. With impaired ability to transport long chain fatty acids into the mitochondria for oxidation, a build up of muscle triglyceride may be expected. This build up of lipid in the muscle may produce increases in long chain fatty acid Co-A, which could lead to increases of diaglycerol concentrations within the muscle. Ruderman et al. (1999) hypothesized that an increased concentration of malonyl-CoA, decreased CPT-1 activity, and increased long chain fatty acids within the cell may be associated with obesity.

Kim et al. (2000) examined the role of CPT-1 and post CPT-1 events in association with skeletal muscle lipid oxidation and obesity. Muscle samples from the vastus lateralis of lean (N=9) (BMI < 28 kg/m^2) and obese (N=9) (BMI > 28 kg/m^2) individuals were studied. Additionally, muscle samples from the rectus abdominus were studied in order to compare locomotive muscle groups and non-locomotive muscle groups. Palmitate oxidation was significantly lower in the obese group (6.8±2.2 nmol CO_2/g/h) when compared to the lean group (13.7±1.4 nmol CO_2/g/h). Additionally, palmitoyl carnitine oxidation was significantly lower in the obese group (21.1±3.3 nmol CO_2/g/h) when compared to the lean group (38.5±3.6 nmol CO_2/g/h). Octanoate

oxidation was also significantly lower in the obese group (4.4±1.3 nmol CO_2/g/h) than in the lean group (11.7±1.0 nmol CO_2/g/h). There was a significant correlation between BMI and palmitate (r = -.76), palmitoyl carnitine (r = -.82), and octanoate oxidation (r = -.82). Additionally, lipid oxidation was found to be depressed at a BMI > 30 kg/m^2, but was not further influenced by the degree of obesity. CPT-1 activity in the vastus lateralis was significantly lower in the obese group (18.2±2.4 nmol/g/h) when compared to the lean group (27.4±3.1 nmol/g/h) and CPT-1 activity correlated negatively with BMI (r = -.48). The obese group demonstrated a decreased rate of citrate synthase activity in the vastus lateralis (34.5±3.7 nmol/g/h) when compared to the lean group (53.8±4.5 nmol/g/h), and citrate activity correlated negatively with BMI (r = -.65). Data from the rectus abdominus muscle tended to show a decreased oxidative capacity in association with obesity. Citrate activity and β-HAD activity were reduced in the obese group compared to the lean group. PFK activity was found to be increased in obese individuals. Additionally, the obese group demonstrated a significantly higher PFK/citrate synthase ratio, which is an index of muscle glycolytic capacity. Results from this study suggest that long chain fatty acid oxidation in human skeletal muscle is reduced, possibly because of a decrement in CPT-1 activity. Limited oxidation of long chain fatty acids may result in storage of lipids within the muscle. Additionally, results from this study suggest that post CPT-1 events may also diminish fat oxidation in relationship to obesity. Key enzymes in the oxidation pathway were found to be lower in obese individuals. Together, reduced oxidative enzyme capacity and reduced CPT-1 activity may play a role in pathogenesis of obesity. Of great interest is the fact that both palmitate and palmitoyl

carnitine oxidation was found to be lower in obese individuals. Palmitate is CPT-1 dependent, while palmitoyl carnitine, a medium chain fatty acid, is not dependent upon CPT-1 for oxidation. This suggests that there is evidence of a post CPT-1 defect in skeletal muscle of the obese, which is associated with diminished oxidative enzyme activity and higher glycolytic capacity.

There is a consensus that skeletal muscle in lean, healthy individuals tends to rely on lipid oxidation for the majority of energy expenditure at rest during fasting conditions. Additionally, under postabsorptive conditions, skeletal muscle is an important site for systematic utilization of free fatty acids because of the high fractional extraction characteristics of skeletal muscle (Kelly et al. 1999). The relationship between fatty acid metabolism, obesity, and weight loss was examined by Kelley et al. (1999). Metabolic assessments were performed on lean (N=16) and obese (N=40) subjects. Metabolism was measured across the leg via the limb balance technique. With this technique, catheters are inserted into the radial artery and the femoral vein. To measure free fatty acid uptake, oleate was infused into the radial artery and arterial and femoral venous blood samples were obtained at 5-minute intervals and measured for oxygen and carbon dioxide content. At 10 minute intervals, blood samples were obtained in order to measure free fatty acid specific activity, arterial insulin, plasma free fatty acid concentration, and glucose. Resting blood flow across the leg was measured by venous occlusion strain-gauge plethysmography. After resting measurements were obtained, a muscle biopsy was obtained from the vastus lateralis. Post biopsy, a 3-hour continuous infusion of insulin was started. Blood glucose was measured every 5-minutes and arterial and venous blood

sampling repeated during the final 40 minutes of insulin infusion. Weight loss intervention included caloric restriction with no increase in physical activity. The goal of the weight loss program was a weight loss of 10-15 kg over a four-month period. Results show that during fasting conditions, the obese group displayed higher arterial concentrations of free fatty acids (obese, 541±25 μmol/l; lean, 486±37μmol/l) but the group differences were not statistically significant. In both groups, femoral venous plasma free fatty acid concentrations were higher than arterial concentrations (obese, 626±32μmol/l; lean, 575±41μmol/l), however there were no significant differences between groups. Additionally, there were no significant differences in free fatty acid uptake between the lean and obese groups (obese 342±28 nmol/min/leg dl; lean, 346±47 nmol/min/leg dl). Of interest, rates of lipid oxidation across the leg were significantly lower in the obese group (obese, 95±14 nmol/min/leg dl; lean, 158±17 nmol/min/leg dl). Also, net FFA storage was found to be significantly higher in the obese subjects (247±26 nmol/min/leg dl) when compared to the lean subjects (188±48 nmol/min/leg dl). In regards to enzyme activity, obese subjects were found to have a lower activity of cytochrome c oxidase (oxidation capacity marker enzyme) and a lower CPT activity. During fasting conditions, the RQ across the leg was higher in the obese group (.90) when compared to the lean group (.83), while basal rates of energy expenditure between the groups were not statistically different. After insulin infusion, rates of free fatty acid uptake remained similar between the groups. Rates of fat oxidation in the lean subjects were lower after insulin infusion, while fat oxidation rates in the obese group were higher than that of the lean subjects and unchanged from resting rates. Leg RQ values in the lean

group (.99) increased significantly after insulin infusion, however the RQ in the obese group failed to increase significantly (.91).

Similar measurements were obtained on 32 obese subjects post weight loss. Mean weight loss was 14kg and there were no significant changes in maximal oxygen consumption. After weight loss, resting energy expenditure across the leg was decreased. Leg RQ values remained unchanged after weight loss during fasting conditions (.90). Rates of free fatty acid uptake across the leg were lower after weight loss, however these rates continued to exceed rates of fatty acid oxidation. Fatty acid oxidation rates were lower when compared to pre-weight loss values (Pre-WL 110 ± 25 nmol/min/leg dl, Post-WL 84 ± 22 nmol/min/leg dl), however this was not statistically different. In response to insulin stimulation, leg RQ was higher after weight loss (.96) when compared to pre-weight loss values (.91). Results from this study suggest that obese and lean individuals have similar rates of free fatty acid uptake in skeletal muscle. Despite similar uptake, obese subjects demonstrate a lower rate of fasting lipid oxidation, increasing the rate of fatty acid net storage. Additionally, resting leg RQ values suggest that obese individuals have a tendency to rely less on fat oxidation during resting conditions, where only one third of energy production was accounted for by lipid oxidation. Strikingly, after weight loss intervention, rates of skeletal muscle lipid oxidation were lower than pre-weight loss values and there was no change in resting leg RQ values. These results suggest that there are defects in lipid oxidation within skeletal muscle of obese individuals and these defects may be primary to obesity.

In an effort to determine whether a decrement in fat oxidation may be primary to obesity, a novel method of study is to examine post-obese or formerly obese individuals. Formerly obese individuals have previously been obese, and after weight loss intervention, are within their normal ideal weight range. The advantage of studying a cohort of this nature is that these subjects can be considered obesity-prone, normal weight subjects. Additionally, when formerly obese individuals are compared to never obese individuals of a similar body stature, it is easier to generalize results surrounding the question of whether decreased skeletal muscle fat oxidation may be a primary impairment of obesity.

Raben et al. (1998) compared the activity of skeletal muscle oxidative enzymes between post-obese (N=9) and never-obese women (N=9). The post-obese group (PO) had a family history of obesity, had been >20% overweight, and had also been weight stable for at least two months. The groups were matched on age, weight, height, blood pressure, waist-to-hip ratio, BMI, and fat free mass. Muscle enzyme activity was measured from the vastus lateralis obtained from muscle biopsy samples. β-hydroxyacyl-CoA dehydrogenase (HADH), citrate synthase (CS), lactate dehydrogenase (LDH), and lipoprotein lipase (LPL) activities were measured. HADH, CS, and LDH activity was determined by fluorometric determination of the NAD-NADP coupled reaction. BMR and RER were determined by indirect calorimetry. Additional measurements included aerobic capacity and present activity status. The enzyme activity of HADH and CS was found to be 20% lower in the post obese group ($P < .05$). LDH activity was found to be 30% lower in the post obese group, but results did not attain statistical significance. The

ratio of HADH-to-CS was not different between groups. CS, LPL, and HADH activity correlated negatively with %FM ($r = -.53$, $r = -.42$, $r = -.44$, respectively). BMR, RER, and aerobic capacity measures were not statistically different between groups. Additionally, estimated physical activity factor was not statistically different between groups. These findings suggest that decrements in oxidative enzyme activity may be a primary mechanism in obesity. HADH, a β-oxidation enzyme, was found to be reduced in PO individuals. Additionally, CS, the rate-limiting step in the Kreb's Cycle, was reduced in PO individuals. Further supporting this evidence are the negative correlations between fat mass with CS activity and HADH activity.

Skeletal muscle fiber type distribution may be another factor associated with impaired skeletal muscle oxidative capacity. Type I (oxidative, slow twitch) fibers have a higher capacity for substrate oxidation than type II (glycolytic, fast twitch) fibers. It has been hypothesized that a lower distribution of type I to type II fibers may result in lower oxidative capacity of skeletal muscle and therefore, fiber type distribution may be a primary risk factor for obesity (Astrup et al. 1997).

Kriketos et al. (1996) examined the relationships between fiber type, oxidative capacity, and adiposity in a cohort of male Pima Indians (N=48). Muscle samples obtained from the vastus lateralis via muscle biopsy where stained for fiber type using the myosin adenosinetriphosphase (ATPase) method. Muscle fiber oxidative capacity was determined using the NADH tetrazolium reductase technique where fibers that are stained intensely (dark) were subjectively considered to be more oxidative. Muscle oxidative capacity was also determined by measuring the activities of citrate synthase

(CS) and hexokinase (HK). The mean BMI for this cohort was 32±1 kg/m^2 and the mean percent fat was 28±1%. Mean values for type I, type IIa, and type IIb fiber types were 39.4±1.4%, 29.6±1.7%, and 30.4±2.0%, respectively. CS and HK activity was determined to be 12.7±0.6 units/g and 1.25±0.1 units/g, respectively. Significant relationships were found between percent fat and the percentage of type I and IIb fibers. Percent fat correlated positively with type IIb fiber percent ($r = .40$, $P = .005$) and correlated negatively with type I percent ($r = -0.44$, $P = .002$). Additionally, waist circumference was also found to correlate significantly with fiber type (type I, $r = -0.30$; type IIb, $r = 0.30$). CS activity was significantly correlated with percent fat ($r = -0.43$) and waist circumference ($r = -.037$). Oxidative capacity of the muscle was found to be significantly related to the percentage of type I fibers ($r = 0.95$) and percent fat ($r = -0.47$). Direct comparisons could not be made between lean and obese individuals because of the subject stratification, however, results from this study suggest that fiber type percentage may be related to measures of adiposity. Adiposity measures were associated with a lower percentage of type I fibers and a higher percentage of type IIb fibers. Furthermore, there is evidence of a relationship between fiber type and oxidative capacity. Oxidative capacity correlated positively with type I fiber percent. Of interest, decreased citrate synthase activity, a marker of oxidative capacity, was negatively associated with increased body fat percentage and waist circumference. Additionally, there was a negative correlation between type IIb fiber percent and citrate synthase ($r = -0.40$) and a positive correlation between type I fiber percent and citrate synthase activity ($r = .35$). These findings are in agreement with work done by Lillioja et al. (1987). Fiber

type was examined in 23 Caucasian and 41 Pima Indian males. Percent fat significantly correlated with fiber type I ($r = -0.32$) and fiber type IIb ($r = 0.32$). Waist to thigh measures were also significantly correlated with fiber type ($r = -0.39$, $r = 0.40$ for type I and type IIb, respectively).

He et al. (2001) recently examined fiber type in relation to oxidative enzyme activity in lean (N=22), obese (N=20), and obese with NIDDM (N=20) individuals. ATPase staining was performed to determine the fiber type of vastus lateralis muscle samples obtained via muscle biopsy. Serial sections of the muscle samples were analyzed for oxidative (SDH) and glycolytic (α-GPDH) activity. A quantitative histochemical microphotometric procedure was used to determine enzyme activity in a single muscle fiber. No significant group differences were found between groups in regards to fiber type distribution, however there was a trend for both obese groups to have a lower percentage of type I fibers ($P = 0.054$). Type IIb fibers had the lowest oxidative activity while type I muscle fibers were associated with the highest oxidative activity. A significant group effect was also found in regards to oxidative capacity. Oxidative enzyme capacity was found to be 25% higher in muscle, in all fiber types, from the lean group when compared to the obese or NIDDM group. No differences were found between the two obese groups. SDH activity was significantly higher in the lean group when compared to the obese groups (lean, 43±2; obese, 32±3; obese NIDDM 32±3). Type I muscle fibers demonstrated lower glycolytic activity (αGPDH) in all groups. Glycolytic activity was similar between the IIa and IIb fiber types in all groups. There was not a group effect in regards to glycolytic activity in any fiber type. Additionally, a

glycolytic-to-oxidative enzyme activity ratio was calculated for each fiber type. Type I muscle fibers demonstrated the lowest ratio, while type IIb fibers were found to have the highest glycolytic-to-oxidative ratio. A significant group effect was found in the glycolytic-to-oxidative ratio for the IIa and IIb fiber types, with skeletal muscle from obese individuals displaying a higher glycolytic-to-oxidative ratio than muscle from the lean individuals. No group effect was found for type I fibers and the glycolytic-to-oxidative ratio. Single fiber oxidative enzyme activity was found to correlate significantly with BMI (Type 1, r = -0.42; Type IIa, r = -.40; Type IIb, r = -0.39). BMI did not significantly correlate with single fiber glycolytic activity.

The fiber type distribution between lean and obese individuals from He et al. (2001) were somewhat similar to the findings in the study performed by Raben et al. (1998). Raben et al. (1998) measured muscle morphology and enzyme activity in skeletal muscle of post-obese and never-obese individuals. Results on enzyme activity have been discussed previously. Briefly, oxidative enzyme activity was found to be 20% lower in the post obese group when compared to the never obese group. Additionally, oxidative enzyme activity negatively correlated with percent fat mass (CS, r = -.53; LPL, r = -.42; HADH, r = -.44). In regards to fiber type, the fiber type percentage was similar in both groups and fat free mass correlated positively with type I fibers (r = 0.66) and negatively with type IIb fibers (r = -0.53). Taken together, these results suggest that fiber type distribution may not be a predisposing factor in obesity. He et al. (2001) found no differences in fiber type distribution between lean and obese individuals and Raben et al. (1998) found no differences in distribution between post-obese and never-obese

individuals. If fiber type distribution was a primary mechanism, then a trend for obese individuals to display a different fiber type distribution including a higher proportion of type II fibers to type I fibers would be expected. Research has been performed that shows differences in fiber type distribution between lean and obese individuals (Kriketos et al. 1997; Wade et al. 1990; Lillioja et al. 1987; Hickey et al. 1995). Confounding evidence may result from the fact that human fiber type distribution is not homogenious, as it tends to be in animals. The proportion of fiber types may vary widely within each individual and within each muscle (Basset et al. 1994).

At best, research surrounding the area of fiber type distribution and obesity is equivocal. Additionally, fiber type may not be as important as once thought in regards to development of obesity. Instead, a stronger case may be developed surrounding the idea that detrimental effects on skeletal muscle may develop from obesity or may be primary to obesity. Impaired skeletal muscle fat oxidation is associated with obesity (Kelley et al. 1999)(Kim et al. 2000). There is a trend for fat oxidation to be lower in obese individuals at rest, before and after weight loss (Kelly et al. 1999). Additionally, obesity is associated with lower activity of key oxidative enzymes in skeletal muscle (Raben et al. 1998; He et al. 2001; Kim et al. 2000; Simoneau et al. 1995). With this in mind, skeletal muscle lipid oxidation capacity may play a more important role in association with obesity than previously researched variables such as fiber type, however fiber type may be associated with defects in obese skeletal muscle.

Evidence for Increased Intramuscular Lipid Content with Obesity

Attempts have been made to examine the composition of skeletal muscle by use of computed tomography (CT). Goodpaster et al. (1997) reviewed the use of CT imaging for evaluating skeletal muscle composition. A CT image is composed of a two-dimensional pixel map that corresponds to a three-dimensional section within the patient. Each pixel has a numerical value, called Hounsfield Units, which correspond to the level of gray color within the image, which is determined by the physical properties of the tissue. By using CT imaging, it is possible to differentiate tissues within the body based on their attenuation characteristics, which are based on the tissue density and electron per unit mass. Muscle and fat can be differentiated because they have widely different attenuation values. Muscle has a positive attenuation value (0-100 HU) while fat displays a negative attenuation value (-190 to –30 HU).

By use of computed tomography, Kelley et al. (1991) examined the effects of obesity on skeletal muscle density by determining the thigh composition of middle-aged males of various weights. The subjects were divided into two groups, a non-diabetic group and a NIDDM group. The non-diabetic group had a BMI of 27.9±4.5 kg/m^2 while the BMI of the NIDDM group was 29.4±4.8 kg/m^2. Adipose tissue was defined as a range of attenuation values from –200 to –1 HU while lean tissue was defined as 0-100 HU. Lean tissue was further subdivided into normal density muscle (35-100 HU) and low-density lean tissue (0-34 HU). The volume of low-density lean tissue varied among subjects and had a positive correlation with BMI ($r = 0.63$, $P = 0.003$). Within each subject, efforts were made to assess the relationships between adipose, normal density

muscle, and low-density lean tissue. Between low-density lean tissue and adipose volume, there was a strong, positive correlation ($r = 0.80$, $P < 0.0001$). There was a trend for lean tissue in the thigh to increase with obesity, and this increase was associated with an increase in low-density lean tissue with no change in normal density muscle, suggesting that obesity is associated with an increase in low-density skeletal muscle. Low-density skeletal muscle may be a result of increased lipid within or around the muscle.

Goodpaster et al. (2000) also used CT imaging to explore the association between obesity and skeletal muscle fat content. Subjects were placed in three groups of lean (N=15), obese (N=40), and obese NIDDM individuals (N=11). Whole body fat mass and fat free mass were determined by use of dual energy X-ray absorptiometry (DEXA). CT was used to characterize adipose tissue amount and location within the thigh. Manual tracings of adipose thigh tissue were drawn and separated in order to divide adipose tissue into three distinct compartments: intermuscular adipose tissue (IMAT), subcutaneous adipose tissue (SCAT), and subfascial adipose tissue (SFAT). Skeletal muscle attenuation values were determined by averaging the attenuation values from all pixels ranging from 0-100 HU. Additionally, distribution of attenuation values were classified as either normal muscle density, 31-100HU (NDM) or low-density muscle, 0-30HU (LDM). Intermuscular adipose tissue (cm^2) in both obese groups (144±8, obese; 134±15, obese NIDDM) was significantly higher than intermuscular adipose tissue in the lean group, 86±13. Subfascial adipose tissue and subcutaneous adipose tissue was also significantly higher in the obese groups when compared to the lean group. While

intermuscular, subfascial, and subcutaneous adipose tissue all increased with BMI, intermuscular adipose tissue increased the most, increasing 5-fold in the obese group (33.6±0.6 kg/m^2) and 9-fold in the obese NIDDM group (BMI, 36.0±1.1 kg.m^2). Cross-sectional areas of normal density muscle were similar in all three groups (181±6cm^2 lean, 180±12cm^2 obese, 175±15cm^2 obese NIDDM); however, area of low density muscle was greater in the obese groups (81±13cm^2, lean; 137±8cm^2, obese; obese NIDDM, 142±15cm^2). This is an interesting finding because lower attenuation values suggest a greater lipid content within the muscle because of the negative attenuation characteristics of lipid. Additionally, these results suggest that there is a relationship between BMI and intermuscular adipose tissue, with intermuscular adipose tissue increasing with BMI at a higher rate than the other measures of adipose tissue.

In an attempt to discover more about the relationship between obesity and increased lipid content within skeletal muscle, Goodpaster et al. (2000) again used computed tomography to compare skeletal muscle attenuation values with skeletal muscle lipid content. Subjects for this study included 45 men and women, 10 of which were NIDDM patients. BMI ranged from 18.5 to 35.9 kg/m^2. Subjects were sedentary and were weight stable for at least 3 months. Attenuation values were determined for lipid emulsion phantoms stored in a plastic container. The phantoms contained 0, 2, 4, or 6 g/100ml lipid emulsion and each were scanned four times to determine test reliability. Attenuation values from the human subjects were determined to be the mean attenuation value from all pixels with a range of 0-100 HU in order to exclude triglyceride levels. To test the hypothesis that skeletal muscle lipid content is reflected by CT attenuation of

skeletal muscle, the authors used a combination of CT scans of the mid-thigh and vastus lateralis muscle biopsies for each subject. Triglycerides were extracted from the biopsy muscle samples by the chloroform-ethanol method and content was determined spectrophotometrically. Additionally, Oil Red O staining was performed on the biopsy muscle samples to objectively quantify lipid content of the muscle. The skeletal muscle lipid content from Oil Red O staining was measured as the area occupied by lipid droplets. Additionally, a lipid accumulation index (LAI) was calculated (LAI= total cross-sectional area occupied by lipid droplets of the muscle fiber X 100/total cross-sectional area of the fiber). Linear regression was used to determine the association of lipid concentration between lipid-emulsion phantoms and muscle mean attenuation. One-way analysis of variance was used to determine between-group differences in mean muscle attenuation. Interestingly, results show that there was a near perfect linear relationship between lipid content of the phantoms and CT attenuation values. The authors note that there was a low standard deviation and coefficient of variation between the four repeated attenuation values on the same phantom solution, indicating that reliable measures of attenuation can be determined from lipid emulsion phantoms. Results from between-groups muscle attenuation variance tests show that the lean subjects displayed significantly higher values of attenuation than the obese or obese NIDDM subjects. Attenuation values did not differ between the obese group and the obese NIDDM group. Results also showed an association between skeletal muscle lipid content determined in muscle biopsy samples and CT attenuation values. Lipid content determined by Oil Red O staining was found to negatively associated with muscle

attenuation ($r = -.43$, $P < 0.01$). Triglyceride content determined by extraction was also found to be negatively associated with attenuation values ($r = -.58$, $P < 0.019$). These results suggest that skeletal muscle attenuation values determined by CT imaging will vary depending upon lipid content within the muscle. With this in mind, it is now clear that an increased amount of skeletal muscle lipid content may have caused the differences found in the skeletal muscle attenuation values of obese individuals as reported by Goodpaster et al. (2000) and Kelley et al. (1991). Additionally, the increased amount of intermuscular adipose tissue (IMAT) reported by Goodpaster et al. (2000) may be responsible for the increased intramuscular triglyceride content of skeletal muscle found in obese individuals. While these results seem intuitive, the limitation of CT imaging is the inability to directly measure and locate lipid content in relation to muscle fiber type.

Another method used to examine skeletal muscle triglyceride is biochemical extraction of lipid from muscle biopsies. This method involves extraction of lipids from a muscle tissue sample by use of chloroform and methanol. Pan et al. (1997) and Manco et al. (2000) both used this technique to determine the association between skeletal muscle triglyceride and insulin resistance. Pan et al. (1997) studied Pima Indians, a population known for high rates of diabetes. This cohort included 38, non-diabetic, male Pima Indians. Mean BMI for this group was 32.7 ± 1.1 kg/m^2 (range, 19.0-52 kg/m^2) and mean body fat percentage was 29 ± 1 % (range, 18-44%). Skeletal muscle triglyceride (mTG) analysis was performed on biopsy samples removed from the vastus lateralis. The mean value for mTG was 5.4 ± 0.3 μmol/g wet wt (range, 1.3-9.8 μmol/g wet wt). Of interest, no significant relationships were found between any measure of adiposity and skeletal

muscle triglyceride content. Additionally, skeletal muscle triglyceride content was not related to measures of carbohydrate or lipid oxidation. Manco et al. (2000) evaluated the content of lipid within skeletal muscle in 30 non-diabetic subjects. The BMI of this cohort was 27.0±7.0 kg/m^2 (range, 13.25-38.13 kg/m^2). Subjects were divided into lean and obese groups. Lipid extraction was performed on muscle biopsies taken from the rectus abdominis muscle. In contrast to Pan et al. (1997), the obese group was found to have a 2-fold higher level of mTG (Obese, 11.6±2.2 μmol/g wet wt; Lean, 6.2±1.4 μmol/g wet wt). Also in contrast to Pan et al. (1997), mTG concentration was correlated to BMI ($r = .81$, $P < .0001$). Possible reasons for the conflicting results may be due to the different muscle groups studied by each author. Additionally, different subject populations were used in each study, making it difficult to generalize these findings. It must also be noted lipid extraction may not result in a true measure of skeletal muscle triglyceride. Manco et al. (2000) and Pan et al. (1997) reported similar measures of mTG, 6.2-11.6 μmol/g wet wt and 1.3-9.8 μmol/g wet wt, respectively. However, Phillips et al. (1996) reported a range of 2.6-404.4 μmol/g wet wt with a mean of 33.9 μmol/g wet wt. A possible limitation of the lipid extraction method used in these studies is the variability between samples and it is possible that samples can be contaminated from other adipose depots. Another limitation of this method, like CT imaging, is the inability to locate the lipid stores in relation to fiber type.

Attempts have been made using Oil Red-O staining to quantify lipid within skeletal muscle fibers (Phillips et al. 1996; Goodpaster et al. 2000). Oil Red-O is a soluble dye that stains neutral lipid with an orange red tint. While this histological

method has been successful, the limitation of this method is the type of quantification used to determine skeletal muscle triglyceride concentration from the lipid stained muscle samples. Phillips et al. (1996) performed Oil Red-O staining on rectus abdominis biopsy samples from 27 women (Mean BMI, 24.8 kg/m^2; Range, 19.2-31.9 kg/m^2). Results from this study show that intracellular lipid measured by Oil Red-O staining was significantly correlated with muscle triglyceride content determined by biochemical extraction ($r = .43$, $P = .02$). Additionally, intracellular lipid content correlated with waist to hip ratio ($r = .47$, $P = .01$) but did not correlate with BMI. The limitation of this study, as stated previously, is the method of lipid quantification from Oil Red-O staining. Phillips et al. (1996) used a subjective histological scoring system in order to quantify the lipid concentrations. With this method, a blinded observer graded each biopsy into one of five categories in reference to a series of photograph standards, and then a histological lipid score was calculated.

In an effort to develop a more objective quantification method of skeletal muscle lipid staining, Goodpaster et al. (2000) examined intramuscular lipid content by use of Oil Red-O staining in conjunction with digital image analysis. Oil Red-O staining was performed on muscle biopsy samples taken from the vastus lateralis. After staining, the images were examined with an Olympus light microscope using a 40X oil immersion objective. Eight contiguous fields of view within the biopsy section where digitally captured using a CCD camera and saved as grayscale images (Tagged Image File Format). The saved images were then analyzed with NIH software. After intensity thresholds were set, quantification was performed using the NIH software. Data were

expressed as the area and number of staining per area of the image field. The neutral lipid content per unit area of muscle fiber was calculated from the sum of the stained sections for each of the 8 fields of view. A lipid accumulation index (LAI) was then calculated for each of the 8 fields of view within the stained section and a mean was calculated for each subject. LAI was calculated as total area occupied by lipid droplets of muscle fiber X 100 / total CSA of muscle fiber. Intramuscular lipid content was examined in 34 subjects separated into groups of lean (N=9), obese (N=15), obese NIDDM (N=10). The BMI of the obese (33.6±0.8 kg/m^2) and obese NIDDM (36.0±0.9 kg/m^2) groups were significantly higher than that of the lean group (23.4±1.1 kg/m^2) as was percent body fat (lean, 14.9±1.8%; obese, 35.3±1.9%; obese NIDDM, 40.2±2.2%). Aerobic capacity did not differ significantly between groups. Significant group differences were found between groups for the number of lipid stained spots, LAI (percent of fiber area occupied by lipid staining), and total area of lipid staining. The obese groups (obese, 1985±216; obese NIDDM, 2445±309) had a significantly higher score for total number of lipid stained spots when compared to the lean group (1278±205). There were also significant group differences in total fat (μm^2). The obese group had 1641±228μm^2 total fat and the obese NIDDM group had 2431±438μm^2, which is significantly higher than the total fat of the lean group, 964±182 μm^2. Significant differences were also found between the obese NIDDM group and the lean group for LAI%, 3.62±0.65% and 1.42±0.28% respectively. LAI% between the lean and obese groups did not reach statistical significance (P = .07), but the obese group did display a higher LAI, 2.53±0.41% (obese) and 1.42±0.28%

(lean). No statistical differences were found between the obese and obese NIDDM groups ($P = .2$). Of interest, LAI was positively correlated with BMI ($r = .42$, $P < .01$) and was not correlated with age or aerobic capacity. A limitation in this study, as mentioned previously with Phillips et al. (1996), is the ability to quantify the histological staining results. In an attempt to demonstrate that the novel concept of digital examination is a reliable method of quantifying intramuscular lipid, Goodpaster et al. (2000) performed a replication reliability study. In this study, the Oil Red-O staining procedure was performed in the same manner as previously mentioned and the LAI % was measured in five subjects by separate technicians. The variation of LAI for the mean of two measures averaged 0.48%, thus the interobserver variance was less than the group differences in LAI.

High concentrations of intramuscular triglyceride are not restricted to obesity. Research has been performed that shows increased levels in trained athletes as well. Kelley and Mandarino (2000) note that increased muscle triglyceride levels must be appraised in context to other markers of metabolic capacity within the muscle. Such markers of metabolic capacity within skeletal muscle are oxidative capacity and distribution of the different skeletal muscle fiber types.

There are two predominant fiber types found within skeletal muscle, fast twitch (type II) and slow twitch (type I), which are distinguishable from each other by their metabolic and contractile properties. Type II fibers have the ability to generate energy quickly, primarily because of a well-developed glycolytic energy systems. Fast twitch fibers are further subdivided into two subgroups; Type IIa and Type IIb. Type IIa fibers

have intermediate mitochondrial volume and capillary density enabling these fibers to have an intermediate capacity for aerobic and anaerobic energy production. Type IIb fibers, having low mitochondrial volume and capillary density, display low capacity to generate energy aerobically but great capacity for anaerobic energy production. Slow twitch fibers have high mitochondrial volume and capillary density. These fibers have an increased ability to produce energy primarily through aerobic pathways because of increased blood flow and oxidative capacity (Bassett et al. 1994).

In a study performed by Staron et al. (1984), the mean percent fiber type distribution found in trained runners was 68.2% type I fibers and 27.8% type II fibers, while the distribution for the untrained group was 23.1% type I fibers and 76.9% type II fibers (43.2% type IIb). Mitochondrial volume percent of type I fibers in the untrained group was found to be 3.04% and 4.43% in the trained group. This variable also increased in the type II fibers of the trained group when compared to the untrained group. Lipid volume % in type I fibers was .29% in the untrained group and .82% in the trained group. These results are in agreement with Prince et al. (1987), who found greater mitochondrial volume (5.94% highly trained endurance athletes, 4.49% active males) and greater lipid volume (.99% highly trained endurance athletes, .66% active males) in type I fibers. Also noted was the increased lipid volume percent difference between type I fibers (.99%) and type II fibers (.50%) in highly trained endurance athletes.

Ingjer et al. (1979) examined the mitochondrial content and capillary supply of different skeletal muscle fiber types in untrained and endurance trained men. Fiber type distribution in endurance trained males was 68.6% type I, 19.2% type IIa, and 6.6% type

IIb. The distribution found in untrained males was 39.2%, 39.6%, and 12.8%. Average capillary number around type I fibers was 4.76 in untrained men and 7.79 in trained men. These results were similar in type II fibers as well. Mitochondrial volume was seen to increase in the fiber types relative to increased capillary density.

Results from these studies provide evidence for an increased distribution of type I fibers in endurance athletes. Additionally, mitochondrial volume and capillary density is associated with endurance training state in skeletal muscle fibers types. Lipid volume is increased in type I fibers when compared to type II fibers in the trained vs. untrained states, while lipid volume in highly trained endurance athletes is significantly increased in type I fibers. Highly trained endurance athletes have an increased ability to oxidize the increased lipid stores within their skeletal muscle because of the increased oxidative capacity of their skeletal muscle fibers.

In summary, intramuscular fat has been measured through use of computed tomography, biochemical extraction, and Oil Red O staining. Increased muscle triglyceride levels have been found in obese and highly trained states. These increases are physiologically beneficial for highly trained athletes but are not beneficial in obese states possibly because of differences in skeletal muscle fiber type distribution and oxidative capacity. Little is known about the fiber type distribution found in the morbidly obese population. Additionally, little is known regarding the intramuscular lipid profile of the morbidly obese in relation to fiber type and weight loss.

The Effects of Weight Loss on Intramuscular Lipid Content

Weight loss is common intervention used to combat obesity and is prescribed to obese individuals with hopes to improve impaired metabolic functioning and to reduce risk factors for disease (Goodpaster et al. 2000). Although the effects of weight loss have been researched extensively, very little is known about the effects of weight loss on skeletal muscle lipid concentrations.

Goodpaster et al. (2000) examined the effects of weight loss on skeletal muscle composition and thigh adipose tissue distribution. 15 lean, 40 obese, and 11 obese NIDDM subjects were examined in this study. Thigh adipose tissue distribution and skeletal muscle composition were analyzed using CT imaging. Methods and results concerning the between groups comparisons (lean, obese, and obese NIDDM) have been discussed previously. Briefly, intermuscular adipose tissue (cm^2) in both obese groups (144±8, obese; 134±15, obese NIDDM) was significantly higher than intermuscular adipose tissue in the lean group, 86±13. Subfascial adipose tissue and subcutaneous adipose tissue was also significantly higher in the obese groups when compared to the lean group. While intermuscular, subfascial, and subcutaneous adipose tissue were all related to BMI, intermuscular adipose tissue increased the most with BMI, increasing 5-fold in the obese group (BMI, 33.6±0.6 kg/m^2) and 9-fold in the obese NIDDM group (BMI, 36.0±1.1 $kg.m^2$). Cross-sectional areas of normal density muscle were similar in all three groups (181±6cm^2 lean, 180±12cm^2 obese, 175±15cm^2 obese NIDDM); however, the area of low-density muscle was greater in the obese groups (81±13cm^2 lean, 137±8cm^2 obese, 142±15cm^2). This is an important finding because lower attenuation

values suggest a greater lipid content within the muscle. These results suggest that obesity is associated with an increased amount of intermuscular adipose tissue and low-density skeletal muscle determined by CT imaging.

The same variables were analyzed after weight loss in a subgroup of the obese subjects (N=38; obese, 28; obese NIDDM, 10) that had a BMI > 30 kg/m^2. The weight loss intervention was designed to achieve a 15kg weight loss by dietary restriction for 12 weeks. After 4 weeks of weight stabilization, post weight loss procedures were performed. Average weight loss was 15 kg of body weight (11.2 kg fat mass) for both obese groups. BMI decreased in the obese group (34.3±0.6 kg/m^2 to 29.3±0.6 kg/m^2) and the obese NIDDM group (36.2±1.2 kg/m^2 to 31.7±1.2 kg/m^2). Subcutaneous and subfascial adipose tissue were reduced in both groups in a similar fashion, 22% in the obese group and 18% in the obese NIDDM group. Of interest, there was a higher proportion of intermuscular adipose tissue lost after weight loss (36%) in both groups when compared to subcutaneous and subfascial adipose tissue ($P < .01$). Additionally, the mean value for muscle attenuation was significantly increased in both groups after weight loss ($P < .01$). Of greater interest, the increased value for muscle attenuation in both obese groups was accounted for by a significant decrease in low-density muscle (obese, 134±10 to 110±8 HU; obese NIDDM, 150±22 to 102±15 HU). There were no significant changes in normal density muscle in either group. Results from this study are noteworthy because of the changes in lean tissue composition associated with weight loss. Cross sectional area of skeletal muscle was found to be higher in the obese groups when compared to the lean group before weight loss with the differences attributed to increased

amounts of low density muscle. As previously noted, low density muscle could be attributed to increased skeletal muscle lipid concentrations. Additionally, muscle cross sectional area was reduced primarily because of the reduction in low density muscle with no concurrent changes in normal density muscle. This suggests that weight loss is associated with a decrement in skeletal muscle lipid concentrations. The limitation of this study is the inability to measure and locate intramuscular lipid stores in relation to muscle fiber type.

Goodpaster et al. (2000) examined the relationship between obesity and intramuscular fat before and after weight loss. In this study, where CT imaging was previously used, intramuscular fat was analyzed using Oil Red-O staining and digital image analysis. These methods give a more precise location of lipid stores within the muscle. The methodology and pre-weight loss results have been discussed previously. Briefly, intramuscular lipid content was examined in 34 subjects separated into groups of lean (N=9), obese (N=15), obese NIDDM (N=10). The BMI of the obese (33.6±0.8 kg/m^2) and obese NIDDM (36.0±0.9 kg/m^2) groups were significantly higher than that of the lean group (23.4±1.1 kg/m^2) as was percent body fat (lean, 14.9±1.8%; obese, 35.3±1.9%; obese NIDDM, 40.2±2.2%). The obese groups (obese, 1985±216; obese NIDDM, 2445±309) had a significantly higher score for total number of lipid stained spots when compared to the lean group (1278±205). Significant differences were also found between the obese NIDDM group and the lean group for LAI, 3.62±0.65% and 1.42±0.28% respectively. LAI between the lean and obese groups did not reach statistical significance (P = .07), but the obese group did display a higher LAI, 2.53±0.41% (obese)

and 1.42±0.28% (lean). Additionally, no statistical differences were found between the obese and obese NIDDM groups (P = .2). Of interest, LAI was positively correlated with BMI (r = .42, P < .01) and was not correlated with age or aerobic capacity. 15 subjects were selected for weight loss intervention (8 obese, 7 obese NIDDM). Each subject underwent a 16-week dietary restriction protocol designed to result in a 10 kg weight loss. Intramuscular fat was analyzed after week 17. Mean body weight decreased significantly in both groups (obese, 15.0±1.5 kg; obese NIDDM, 14.2±1.9 kg). No changes were found in aerobic capacity in either group. The number of Oil Red-O stained spots, total staining area, and LAI was reduced after weight loss in both groups however the reductions for the obese group did not reach statistical significance (P = .07). Lipid content within the muscle fibers was reduced by a mean value of 31%, 23% in the obese group and 41% in the obese NIDDM group. LAI was significantly reduced from 3.43±0.53% to 2.35±0.31% in both groups combined post weight loss. Additionally, the number of lipid stained spots in both groups combined decreased to 1908±169 from 2321±232. Goodpaster et al. (2000) has shown that obesity is associated with increased intramuscular triglyceride. Furthermore, weight loss induced by caloric restriction is related to a decrease in fat mass, total body weight, and more importantly, intramuscular lipid concentrations. Additionally, it has now been demonstrated that quantified Oil Red-O staining is a useful method for characterizing intramuscular lipid before and after weight loss.

There is a lack of research concerning weight loss and intramuscular triglyceride concentrations. Another area of interest that has not been researched is the effects of

weight loss on skeletal muscle lipid content in morbidly obese states. Goodpaster et al. (2000) demonstrated that intramuscular triglyceride was significantly decreased after weight loss in obese NIDDM subjects, and although intramuscular triglyceride was decreased in obese non-diabetic subjects after weight loss, results did not reach statistical significance. This may be attributed to the fact that the obese NIDDM subjects had a higher BMI before weight loss (obese NIDDM, 36.0±0.9 kg/m^2; obese 33.6±0.8 kg/m^2). There may be a relationship between the degree of obesity and the extent of weight loss effects. This could be further explored by studying the effects of weight loss on intramuscular triglyceride in morbidly obese individuals. Another possibility is the fact that the groups were separated by disease status with one obese group having type II diabetes and the other obese group being non-diabetic. Intramuscular triglyceride has been shown to be a high correlate of insulin resistance (Phillips et al. 1996; Pan et al. 1997; Goodpaster 1997; Kelley and Mandarino 2000). Furthermore, weight loss has been shown to be associated with improved insulin sensitivity (Goodpaster 1999; Kelley 1993). It is possible that a connection between weight loss, improved insulin sensitivity, and decreased intramuscular fat may have resulted in a greater weight loss effect in the obese NIDDM group. Also, very little is known regarding the effects of weight loss on intramuscular lipid distribution between fiber types. There is literature that has shown that type I muscle fibers have an increased amount of lipid stores when compared to type II muscle fibers (Injer et al. 1979; Prince et al. 1987; Staron et al. 1984), however the effects of weight loss on this distribution is not known.

Surgical Intervention and Weight Loss in the Morbidly Obese

There are a limited number of treatment options for morbidly obese individuals. Many weight loss programs, such as low calorie diets, report a decent success rate for the first 6 months to 1 year, however there is a 95% probability for a rebound weight gain. Weight loss needs to be accompanied with an individualized program consisting of improving dietary habits, behavioral modification, and exercise in order to lessen the probability of weight regain (Atkinson, 1991).

Morbidly obese patients who cannot sustain weight loss and cannot lose weight successfully with medical treatment, may be eligible for surgical intervention. A common surgical procedure involves the use of gastric stapling with a vertical banded gastroplasty. This procedure results in a reduced functional area of the stomach, 10-30 ml, with a fixed outlet from the upper gastric pouch to the distal stomach. This method usually results in an initial weight loss of 40kg, however the long term effects of this procedure are not known.

Another common surgical procedure is the gastric bypass. In this more complicated procedure, the stomach is divided into a small upper pouch and a large lower pouch. The jejunum is divided, with the distal end connected to the lower pouch of the stomach. The proximal end of the jejunum is then attached to the distal jejunum, creating a Roux-en-Y arrangement. This arrangement allows the distal gastric pouch and the duodenum to drain. Possible long-term effects of this procedure include osteoporosis due to calcium malabosorption and vitamin B_{12} deficiency (Atkinson, 1991).

Long-term results of a similar gastric bypass procedure, the Greenville gastric bypass, have been reported by Pories et al. (1992). Based on follow-up data on 479 patients over a 10-year span, the Greenville gastric bypass procedure has resulted in long-term weight loss, with a maximal weight loss occurring 1-year post-operation (~70kg) and only a mean 10% weight regain. Additionally, results from this follow-up show significant improvements in hyperglycemia, hyperinsulinemia, and insulin action.

The gastric bypass surgery has been shown to be an effective weight loss intervention in the morbidly obese population (Atkinson et al. 1991; Pories et al. 1992). This procedure results in a significant weight reduction and maintenance while also improving metabolic control (Pories et al. 1992). Therefore, the gastric bypass procedure provides an appropriate model for comparison of weight loss and muscle triglyceride content.

CHAPTER III

METHODOLOGY

Subjects

Vastus lateralis muscle samples (N=10) were obtained from otherwise healthy, morbidly obese patients that were referred for elective gastric bypass surgery. Due to freeze fracture of the muscle, the sample was reduced (N=6) and all data is representative of this sample. This procedure, performed at Pitt County Memorial Hospital by the Department of Surgery at East Carolina University, reduces caloric intake by reducing the functional stomach to approximately 30ml and by delaying gastric emptying with a gastric outlet. The majority of weight loss occurs within one year of the operation and then body weight tends to stabilize (Pories et al. 1993). Subjects included in the study had a BMI $\geq$37 kg/m^2 or were greater than 170% of ideal body weight prior to the surgery. Exclusion criteria are listed below:

1. Subjects who were on medication known to affect carbohydrate metabolism.
2. Subjects with septicemia, peritonitis, bleeding, thyroid dysfunction, alcoholism, or malnourishment.
3. Children, pregnant women, prisoners, or mentally disabled persons were not studied.

Muscle Biopsy Procedures

Subjects studied were instructed to fast for 12 hours prior to the morning of the muscle biopsy procedure. Muscle samples were obtained from the vastus lateralis by the percutaneous needle biopsy technique. The biopsy site was shaved and then cleaned with an iodine swab. The incision site was numbed with ethyl chloride and 5.0cc of lidocaine.

A 1cm incision was cut through the skin and muscle fascia at the distal end of the vastus lateralis. A 5mm Bergstrom needle was inserted perpendicular to the length of the muscle fiber. Once the needle was in the proper position, the needle was opened while suction was applied with an attached 50cc syringe. The notch of the Bergstrom needle was closed in order to cut the muscle. The needle was removed and pressure combined with iced compression was applied to the incision site for 15-20 minutes. A Steri-strip was used to close the incision and a pressure bandage was applied around the thigh. Subjects were instructed to not remove the pressure bandage for 3-8 hours and to not remove the Steri-strip for at least 3 days. The muscle biopsy procedure was performed before gastric bypass surgery and again, approximately 1-year post surgery.

Histochemical Preparation

After removal from the biopsy needle, the muscle sample was mounted and frozen in isopentane cooled over liquid nitrogen. Tragacanth gum was mixed with OCT until a workable paste formed. The muscle sample was cut and placed vertically into the mounting medium. As the isopentane began to freeze, the muscle sample was immersed until thoroughly frozen. The muscle sample was placed in foil and stored in liquid nitrogen for subsequent analysis.

Plasma Analysis

Prior to the biopsy, subjects were in a 12 hour fasted state and a venous blood sample was taken for the analysis of plasma insulin and glucose concentrations to determine an indication of in vivo insulin activity. Samples were collected in 7 ml Vacutainers (Becton Dickinson & Company, Franklin Lakes, NJ) containing EDTA for

preventing coagulation. Samples were centrifuged for 10 minutes at 2000 x g. Plasma was aliquoted to two 1.7 ml micro centrifuge tubes. Samples were frozen at - 80 degrees Celsius until analyzed. Glucose and lactate values were determined using the YSI 2300 STAT Plus Glucose Analyzer (Yellow Springs, OH). Insulin levels were determined in 200 μL of plasma by automated Microparticle Enzyme Immunoassay (MEIA) utilizing an Imx Analyzer (Abbot Laboratories Diagnostics Division, Abbot Park, IL).

Fasting insulin and glucose plasma values were used to determine HOMA values [insulin (μU/mL) x glucose (mg/dl x .05551) X 22.5^{-1}]. These values provide a measure of the insulin sensitivity of the participants (Matthews et al. 1985). This method of determining in vivo insulin sensitivity is less invasive than other techniques and requires only one blood sample. Although not as sensitive as the hyperinsulemic-euglycemic clamp technique for determining insulin sensitivity, a strong positive correlation ($r=0.72$, $P < .05$) has been observed between HOMA and glucose disposal during a hyperinsulemic clamp (Matthews et al. 1985).

Histochemical Procedures

Prior to histochemical staining, frozen muscle samples were removed from liquid nitrogen and placed in a cryostat/microtome (Tissue-Tek, Miles Inc., Elkhart IN) at -20 degrees Celsius. Serial, transverse sections, 12μm thick, were cut from each muscle sample inside the cryostat, using the microtome. Sections were then placed on glass slides. Each slide was coded, depending on the histochemical procedure to be used. For each subject, pre and post weight loss sections were placed on the same slides. Each slide

was labeled to indicate which sections were pre or post weight loss. The sections were allowed to air dry for approximately 30 minutes prior to staining.

The histochemical stains used in this study included: Myosin ATPase, Oil Red-O, NADH Tetrazolium Reductase, and Alpha-glycerophospate Deyhyrogenase. The myosin ATPase procedure was used to determine muscle fiber type. Oil Red-O (ORO) staining was used to stain neutral lipids within the muscle fiber, mainly triglyceride. Muscle oxidative capacity and fiber area was assessed using the NADH tetrazolium reductase (NADH) and alpha-glycerophospate dehydrogenase (α-GPDH) stains, respectively. Additionally, α-GPDH was also used to determine muscle glycolytic capacity.

Intramuscular lipid staining. The ORO staining procedure was modified from Goodpaster et al. (2000). An ORO stock solution was prepared before the experimental procedure. This solution was composed of 300 mg of ORO and 100 ml of 2-propanol, (99%), mixed thoroughly and stored at room temperature. The ORO working solution was prepared immediately before the staining procedure. This solution was comprised of 24 ml ORO stock solution mixed with 16ml distilled water. After a 10-minute standing period, the solution was filtered by aspiration, with #42 Whatman paper, to rid the solution of any undissolved ORO. The previously cut sections were placed in the Columbia jar containing the ORO working solution. After a 12 minute incubation period at room temperature, the sections were rinsed twice with distilled water (2 x 20 seconds). The sections were then rinsed in cold, running tap water for 10 minutes. The sections were allowed to dry at room temperature and a cover slip was applied over the sections

using glycerol jelly. Stained skeletal muscle triglyceride appeared as red speckles when viewed under light microscopy.

Fiber typing. The Myosin ATPase staining method was used to stain for fiber types I, IIa, and IIb. Before the procedure, an acid preincubation solution and an alkaline stock solution were prepared. The acid preincubation solution was prepared by mixing 1.94 g sodium acetate and 2.94 g sodium barbital in 100 ml distilled water. This solution was then aliquoted into usable portions (~25ml) and stored at –20 degrees Celsius. The alkaline stock solution was prepared by mixing 2.25 g glycine, 2.40 g calcium chloride, 1.76 g sodium chloride, and 1.10 g sodium hydroxide in 300 ml of distilled water. The stock solution was brought to a pH of 9.4. and the final volume was increased with distilled water to 400 ml and stored at 2-8 degrees Celsius.

The previously cut sections were incubated in the acid preincubation solution (~25ml / 10 sections, 4.54 pH) for 5 minutes at room temperature. The acid preincubation solution was poured out of the Columbia jar and the sections were gently rinsed 3x with distilled water. The sections were then immediately taken out of the acid preincubation jar and placed in an alkaline working solution (alkaline stock + 40 mg ATP, 9.4 pH at 37 degrees Celsius). The sections remained in the alkaline working solution for 45 minutes at 37 degrees Celsius. After the incubation period, the alkaline working solution was poured off and the sections were gently rinsed 3x with distilled water. A 1% calcium chloride solution (wt/vol) was added to the Columbia jar and the sections were incubated in this solution for 3 minutes at room temperature. Following another rinse (3x with distilled water), the sections were incubated in a 2% cobalt chloride solution (wt/vol) for

3 minutes. During this incubation, a 1% solution of ammonium sulfide (vol/vol) was prepared. After the 2% cobalt chloride incubation, the sections were rinsed 3x and incubated in the ammonium sulfide solution at room temperature for 1 minute. After a final rinse, the sections were removed from the Columbia jar and placed out of the light and allowed to air dry. After drying, a cover slip was placed on the slide using permount and the edges of the cover slip were sealed with an acrylic fingernail polish. Under light microscopy, stained fibers appeared as dark (type I), moderately dark (type IIb), or no stain (type IIa).

Oxidative capacity. Individual muscle fiber oxidative capacity was assessed using the NADH tetrazolium reductase (NADH) stain. A working solution was prepared by mixing6 mg Nitro blue tetrazolium and 24 mg NADH in 30 ml of Trizma buffer solution (pH 7.4 at 25 degrees Celsius). After mixing, the solution was placed in a 37 degree Celsius water bath and allowed to warm for 20-30 minutes. The sections were incubated in the working solution for 30 minutes at 37 degrees Celsius. The working solution was poured off and the sections were gently rinsed 3x, (15 seconds for each rinse) with distilled water. The sections were allowed to air dry at room temperature. A cover slip was applied to the slide using permount. Under light microscopy, stained fibers appeared as dark blue, indicating high oxidative capacity or light blue, indicating low oxidative capacity.

Glycolytic capacity and fiber area. Alpha-glycerophospate deyhyrogenase (α-GPDH) staining was used to determine glycolytic capacity and fiber size. A working solution was prepared by mixing6 mg NBT, 120 mg glycerol-3-phosphate, and 6 mg

menadione in 30 ml of Trizma buffer solution (pH 7.4 at 25 degrees Celsius). The solution was poured into a Columbia jar and allowed to warm in a 37 degree Celsius water bath for 20-30 minutes. The sections were incubated in the working solution for 45 minutes. The solution was poured off and the sections were gently rinsed 3x with distilled water. The sections were allowed to air dry. A cover slip was applied to the slide using permount and the edges of the cover slip were sealed with an acrylic fingernail polish. Under light microscopy, highly glycolytic fibers stained dark blue and low glycolytic fibers stained light blue.

Microscopy and Imaging

Stained sections were viewed under light microscopy using a Nikon Microphot FX microscope (Diagnostic Instruments, Sterling Heights, Michigan) at a total magnification of 10X. When the desired field of view was obtained, software (Spot Advanced 3.2.4, Diagnostic Instruments, Sterling Heights, Michigan) was used to generate a live image of the field of view. After focusing the image as needed, the live image was digitally captured. Each image was stamped with a calibration mark and saved as a 32 bit tagged image format file (TIFF).

Image Analysis

Sigma Scan Pro 5.0 (SPSS Science, Chicago, Ill.) software was used to perform image analysis and the technician (Rob Gray) was blinded to the subject and pre/post weight loss condition.

A lipid accumulation index (LAI) was calculated for each subject, pre and post weight loss, in order to determine the area occupied by lipid droplets within each muscle

section (Goodpaster et al. 2000). The pre and post weight loss ORO images were analyzed simultaneously. The images were initially converted to grayscale, 8-bit images. Using the image analysis software, at least 3 fields were selected from each image that were free from staining artifact. Each field was then saved as a new image. The original image calibrations were then copied to the new images. The width and height of the new image was measured by the software and multiplied by the image calibrations to determine the area of new image.

Intensity thresholding was performed to analyze the number of lipid droplets and lipid droplet area within each new image. For each new image, a histogram was plotted on a intensity scale of 0-255 AU. The peak of the histogram was recorded for each image. The image with the lowest peak on the 0-255 scale was used to determine the high end of the intensity threshold. The low end of the intensity threshold was the peak minus 10 units. This was evaluated for each subject and held constant for pre and post analysis of the images. The number of lipid droplets and lipid droplet area was calculated by the software for each new image. LAI was calculated for each new image as LAI = (Number of Lipid Droplets X Area of Lipid Droplet) 100 / Area of the measured field. Mean LAI was then calculated for each muscle section. A high LAI is an indication of more lipid within the muscle fiber.

To determine the relationships between fiber type, intramuscular lipid content, aerobic capacity, glycolytic capacity, and fiber area, four images representing each histochemical stain were viewed simultaneously. First, from the fiber type image, muscle fibers were classified as either type I or type II fibers. Every fiber from the image was

counted and fiber type percentage was calculated. Then a subset of fibers from the fiber type image were identified and matched on the ORO, NADH, and α-GPDH images. Using the image analysis software, individual muscle fibers were selected and analyzed for fiber area and average intensity of staining per fiber. Muscle fibers were only analyzed if they could be identified on each of the four images. Mean fiber area was calculated for type I and type II muscle fibers from the α-GPDH image. Optical transmittance was determined as the mean light intensity on a 0-255 scale (0 representing completely dark and 255 representing completely light). Optical transmittance was determined for type I and type II fibers from the NADH and α-GPDH images to quantify oxidative and glycolytic capacity, where a low optical transmittance is an indication of increased capacity. Respectively, a glycolytic to oxidative capacity ratio was calculated for type I and type II fibers. To assess lipid content relative to fiber type, optical transmittance was determined for type I and type II fibers from the ORO image on a 0-1000 intensity scale with 0 representing completely dark (i.e. maximum amount of lipid) and 1000 representing completely light (i.e. no visibly stained lipid). Additionally, lipid content was corrected for fiber area and was calculated for type I and type II fibers by the ratio of ORO to fiber area. Lipid content was measured on the optical transmittance scale; therefore a lower ratio would represent more fat per fiber area. A composite value of lipid content, weighted by fiber type proportion, was calculated for pre and post weight loss samples. Composite fat was calculated as CF = (type I ORO optical transmittance X fractional type I fiber proportion) + (type II ORO optical transmittance X fractional type II fiber proportion). Again, a lower composite fat score is an indication of increased lipid

Statistical Analysis

Means, standard error, and range were calculated for all data. A paired T-test was used to compare pre and post weight loss means for body weight, BMI, fasting insulin, fasting glucose, HOMA, and the lipid accumulation index. A 2x2 repeated measures ANOVA was used to determine significance between fiber type, lipid staining, oxidative capacity, glycolytic capacity, and fiber area, before and after weight loss. Statistical significance was accepted at the $p < .05$ level.

CHAPTER IV

RESULTS

Subject Characteristics

Subject characteristics, before and after weight loss, are presented in Table 1. Body mass (Figure 1) and body mass index (BMI, Figure 2) significantly decreased after weight loss by approximately 47%. There was a significant decrease (-89%) in fasting insulin values following weight loss (25.4 ± 7.4 vs. 2.8 ± 0.3 μU/ml, Figure 3). Fasting glucose was lower after weight loss, however this did not reach statistical significance (114 ± 18 vs. 76.8 ± 2.8 mg/dl, Figure 4). Accordingly, there was a significant, 92% improvement in insulin sensitivity as measured by the HOMA index (7.0 ± 1.9 vs. .5 ± 0.1, Figure 5).

Fiber Type Distribution and Fiber Area

Vastus lateralis fiber type distributions and fiber are results are shown in Table 2. No significant differences were found in fiber type distribution following weight loss (Figure 6).

No significant differences in fiber area were found between fiber types after weight loss. The area of type I fibers did not differ from type II (Figure 7, Table 2). There was a trend for reduction in fiber area following weight loss in both fiber types. Type 1 fiber area was reduced by 13.9% and type II fiber area was reduced by 25.1%, however these results did not reach statistical significance ($p = .09$).

Effects of Weight Loss on Lipid Staining Parameters

Weight loss resulted in significant changes of all measures pertaining to intramuscular lipid content (Table 3). To assess lipid content relative to fiber type, optical transmittance (arbitrary units, AU), was determined on a 0-1000 scale, with 0 representing no light transmittance and 1000 representing the maximum light transmittance through the muscle fiber. Therefore, a muscle fiber containing more lipid would be represented by a lower optical transmittance than a muscle fiber containing less lipid. Type I fibers were found to have significantly lower optical transmittance, and thus more lipid than type II fibers before and after weight loss (-36.2 % and -14.5 %, respectively, $p < .01$). Weight loss resulted in a significant increase of optical transmittance, indicating a reduction of lipid with the intervention, in both type I and type II fibers (273 ± 60 vs. 495 ± 71 AU, 421 ± 55 vs. 579 ± 71 AU, respectively, $p < .05$). Lipid content decreased by approximately 45% in type I fibers with weight loss, compared to a 27% reduction in type II fibers, however there was no significant interaction between fiber type and weight loss, indicating both fiber types responded similarly to the intervention (Figure 8).

The lipid accumulation index (LAI, %) and fat composite were calculated in order to determine total lipid content within the muscle section. Both measures resulted in a ~30% reduction of lipid after weight loss. Weight loss resulted in a significant reduction in LAI, lipid area per muscle section area, 30.8 ± 5.7 % to 21.7 ± 3.8 % ($p < .05$,

Figure 9). Fat composite, which was lipid content relative to fiber type (optical transmittance x fractional fiber type proportion) was calculated for each muscle section, before and after weight loss. As illustrated in Figure 10, weight loss resulted in a significant increase in optical transmittance, indicating less total fat (366 ± 58 vs. 544 ± 70 AU, $p < .05$).

The fat to fiber area ratio (optical transmittance / fiber area) was significantly lower in type I compared to type II fibers, before and after weight loss, indicating more lipid per area in type I fibers ($p < .01$, Figure 11). Weight loss resulted in significant increase of this ratio in both fiber types, indicating a reduction in fat content relative to fiber area (Table 3).

Effects of Weight Loss on Oxidative and Glycolytic Staining Parameters

The effects of weight loss on oxidative (NADH) and glycolytic (α-GPDH) staining parameters are presented in Table 4. Oxidative and glycolytic capacity, relative to fiber type, was measured by optical transmittance (i.e. a lower light transmittance would indicate a higher capacity vs. a higher light transmittance). As determined by NADH staining, type I fibers were found to have a significantly lower optical transmittance than type II fibers, before and after weight loss (Pre-WL, 94.4 ± 5.3 vs. 154 ± 6.0 AU, Post-WL, 88.5 ± 8.0 vs. 139 ± 9.1 AU, $p < .01$). This is an indication that type I fibers demonstrate a higher oxidative capacity than type II fibers. Weight loss did not result in any significant changes in oxidative capacity for either fiber type (Figure 12).

As determined by α-GPDH staining, significant differences in glycolytic capacity were found between fiber types before and after weight loss ($p < .01$, Figure 13). When

compared to type I fibers, type II fibers had a significantly lower optical transmittance, indicating a higher glycolytic capacity prior to weight loss (125 ± 10.2 vs. 167 ± 6.5 AU, respectively) and this trend was similar following weight loss (125 ± 2.7 vs. 169 ± 6.2, respectively). Weight loss did not result in significant changes in the glycolytic capacity of either type I or type II fibers.

Glycolytic to oxidative ratios were calculated for each fiber type (glycolytic optical transmittance / oxidative optical transmittance) before and after weight loss. The glycolytic to oxidative ratio was significantly lower in type II compared to type I fibers, indicating a higher glycolytic to oxidative capacity in type II fibers ($p < .01$). Weight loss did not significantly alter this ratio in either fiber type (Figure 14).

Table 1

Descriptive Data Before and After Weight Loss

Parameter	Pre-Weight Loss	Post-Weight Loss	% Change
Age (years)	36.8 ± 4.6		
Height (m)	1.7 ± 0.1	1.7 ± 0.1	0
Weight (kg)	148 ± 9.9	79.1 ± 4.4 †	-47
BMI (kg/m^2)	52.2 ± 2.5	27.9 ± 0.8 †	-47
Fasting Insulin ($\mu U/ml$)	25.4 ± 7.4	2.75 ± 0.3 *	-89
Fasting Glucose (mg/dl)	114 ± 18.0	76.8 ± 2.8	-33
HOMA	7.0 ± 1.9	0.5 ± 0.1 *	-92

NOTE. Values are the mean ± SE.
Age, Height, Weight, BMI (n = 6).
Fasting Insulin, Fasting Glucose, HOMA (n = 5)
HOMA, Homeostasis Model Assessment
HOMA = [insulin ($\mu U/mL$) x glucose (mg/dl x .05551)] X 22.5^{-1}
* $p < .05$ v Pre-WL
† $p < .01$ v Pre-WL

Table 2

Fiber Type Distribution and Fiber Area

Parameter	Pre-Weight Loss	Post-Weight Loss	% Change
Type I Fibers (%)	38.3 ± 5.2	38.9 ± 3.5	1.6
Type II Fibers (%)	61.7 ± 5.2	61.6 ± 3.5	-0.2
Type I Fiber Area (μm^2)	4163 ± 340	3583 ± 307	-14
Type II Fiber Area (μm^2)	4155 ± 393	3111 ± 296	-25

NOTE. Values are the mean ± SE.
n = 6

Table 3

Effect of Weight Loss on Intramuscular Lipid Staining Parameters

Parameter	Pre-WL	Post-WL	% Change
ORO Optical Transmittance (AU)			
Type I Fibers	273 ± 60	495 ± 71 *	45
Type II Fibers	421 ± 55 †	579 ± 71 * †	27
Fat to Fiber Area Ratio (AU)			
Type I Fibers	.07 ± .02	.15 ± .03 *	48
Type II Fibers	.11 ± .02 †	.19 ± .03 * †	46
Fat Composite (AU)	366 ± 58	544 ± 70 *	33
Lipid Accumulation Index (%)	30.8 ± 5.7	21.7 ± 3.8 *	-30

NOTE. Values are the mean ± SE.
n = 6
Fat to Fiber Area Ratio = Oil Red-O Optical Density X Fiber Area^{-1}
AU, Arbitrary Units
* $P < .05$ v Pre-WL
† $P < .01$ v Type I

Table 4

Effect of Weight Loss on Oxidative and Glycolytic Capacity Staining Parameters

Parameter	Pre-WL	Post-WL	% Change
NADH Optical Transmittance (AU)			
Type I Fibers	94.4 ± 5.3	88.5 ± 8.0	-6.3
Type II Fibers	154 ± 6.0 †	139 ± 9.1 †	-10
α-GPDH Optical Transmittance (AU)			
Type I Fibers	167 ± 6.5	169 ± 6.2	1.2
Type II Fibers	125 ± 10.2 †	125 ± 2.7 †	0
Glycolytic to Oxidative Ratio (AU)			
Type I Fibers	1.8 ± 0.1	1.9 ± 0.2	12
Type II Fibers	.81 ± 0.6 †	.92 ± .01 †	14

NOTE. Values are the mean ± SE.
n = 6
Glycolytic to Oxidative Ratio = α-GPDH Optical Density X NADH Optical Density^{-1}
AU, Arbitrary Units
† $p < .01$ v Type I

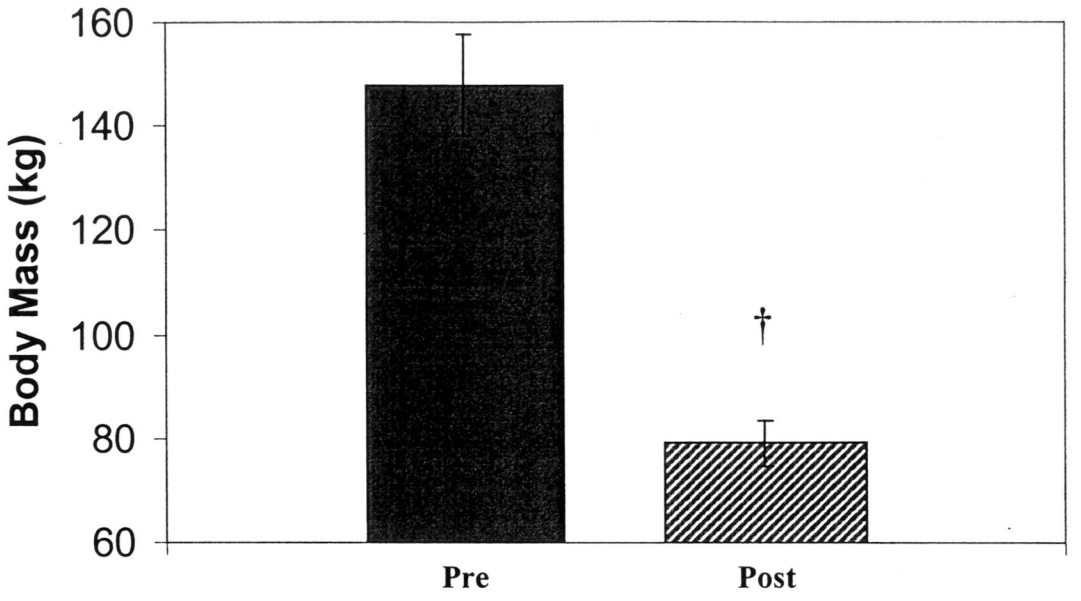

Figure 1

The Effects of Weight Loss on Body Mass in Morbidly Obese Individuals (n=6)

† $p < .01$ v Pre Weight Loss

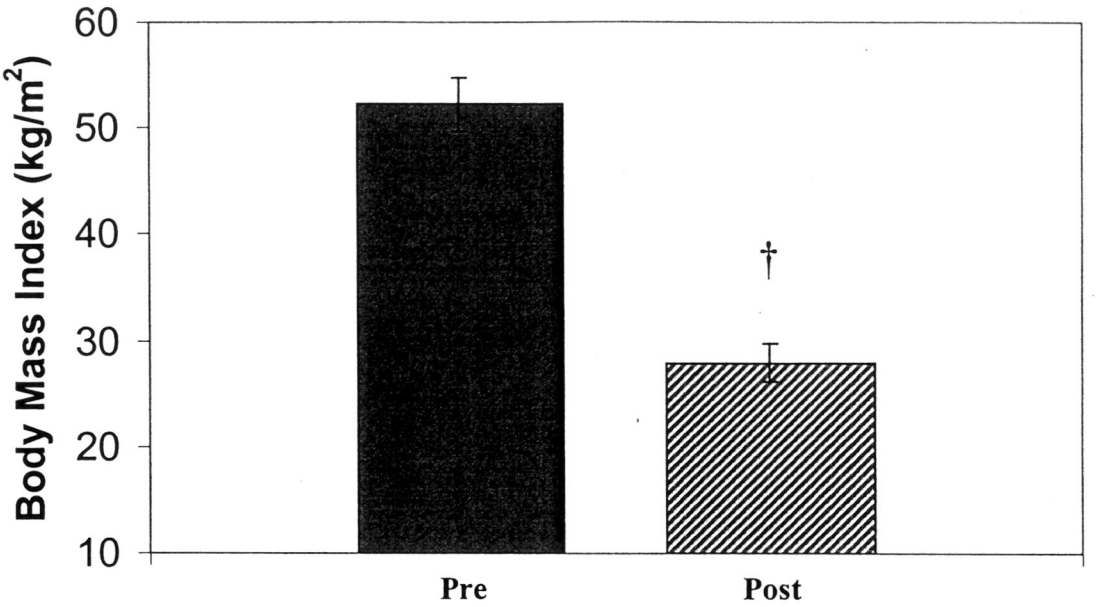

Figure 2

Effects of Weight Loss on Body Mass Index in Morbidly Obese Individuals (n=6)

† $p < .01$ v Pre Weight Loss

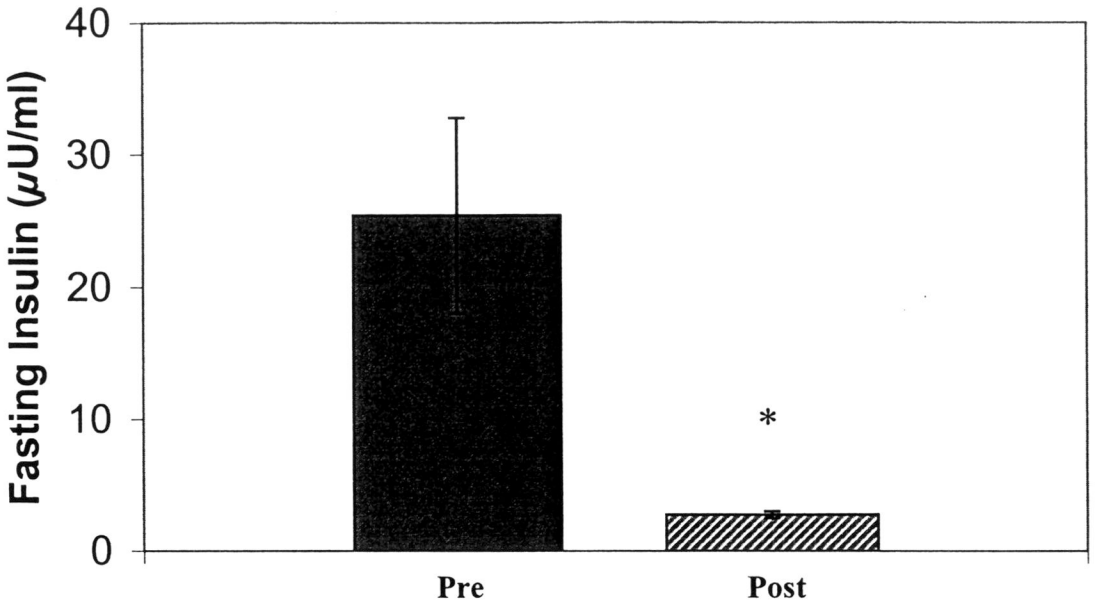

Figure 3

Effects of Weight Loss on Fasting Plasma Insulin in Morbidly Obese Individuals (n=5)

* $p < .05$ v Pre Weight Loss

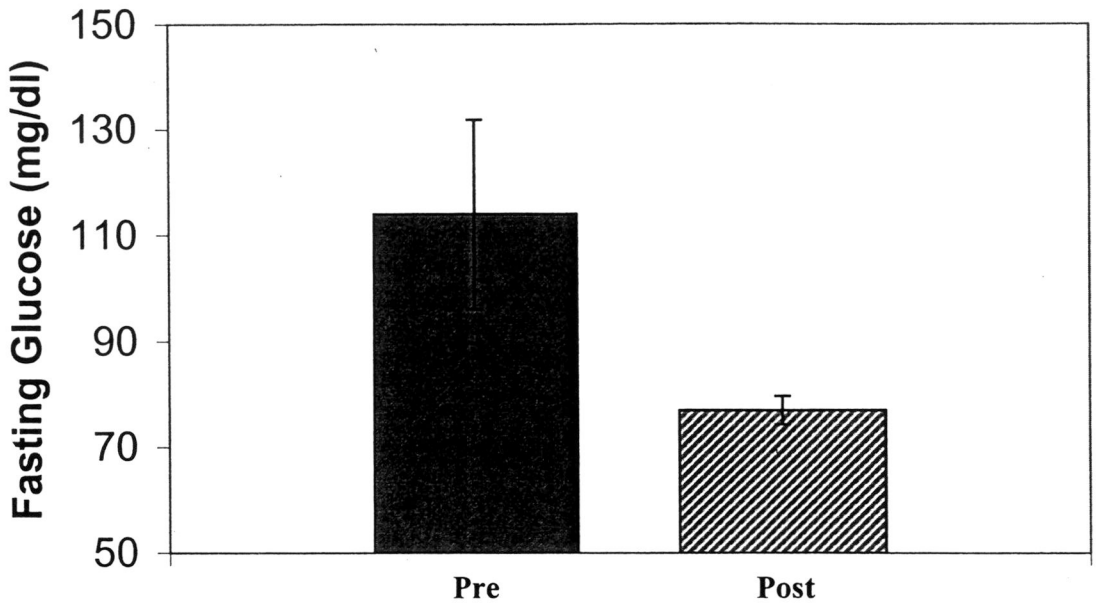

Figure 4

Effects of Weight Loss on Fasting Plasma Glucose in Morbidly Obese Individuals (n=5)

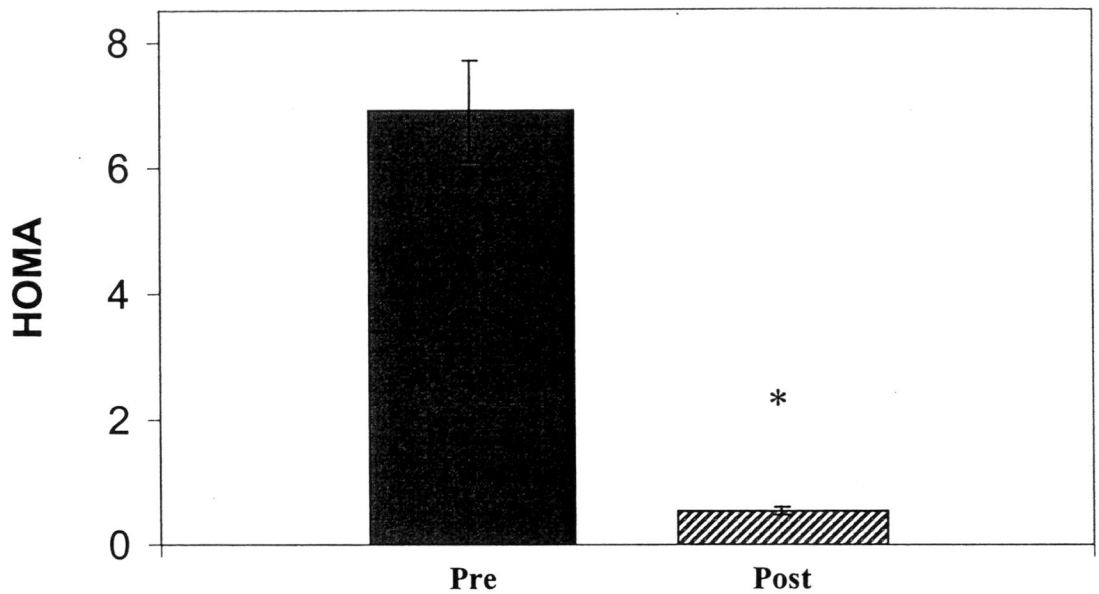

Figure 5

Insulin Sensitivity Before and After Weight Loss in Morbidly Obese Individuals (n=5)

HOMA, Homeostasis Model Assessment
HOMA = [insulin (μU/mL) x glucose (mg/dl x .05551)] X 22.5^{-1}
* $p < .05$ v Pre Weight Loss

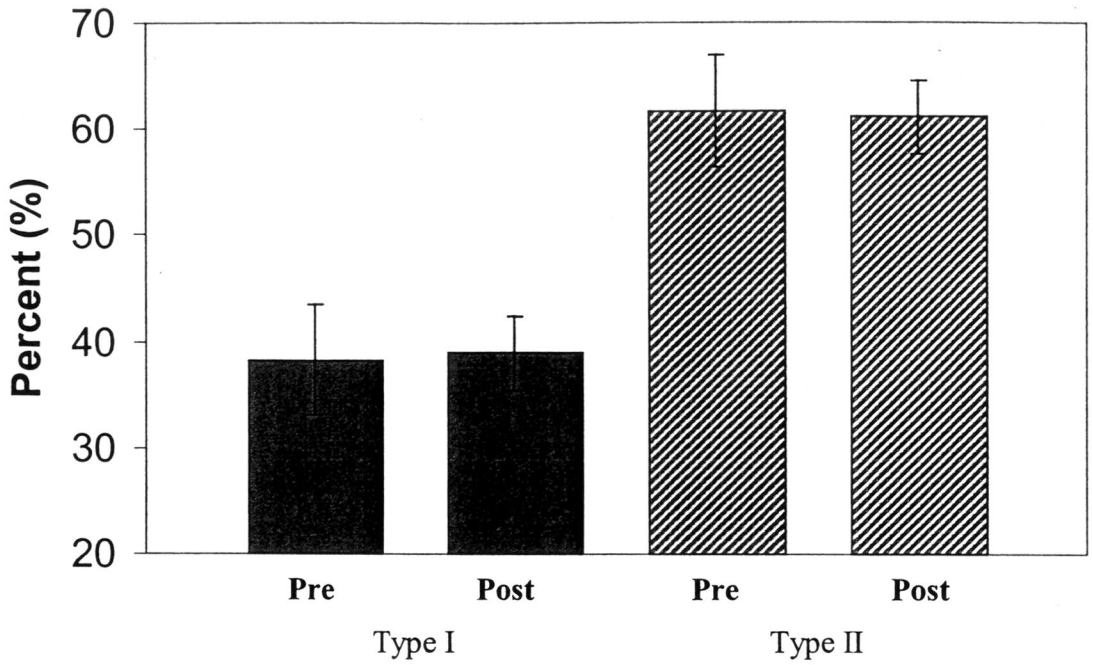

Figure 6

The Effects of Weight Loss on Vastus Lateralis Fiber Type Distribution in Morbidly Obese Individuals (n=6)

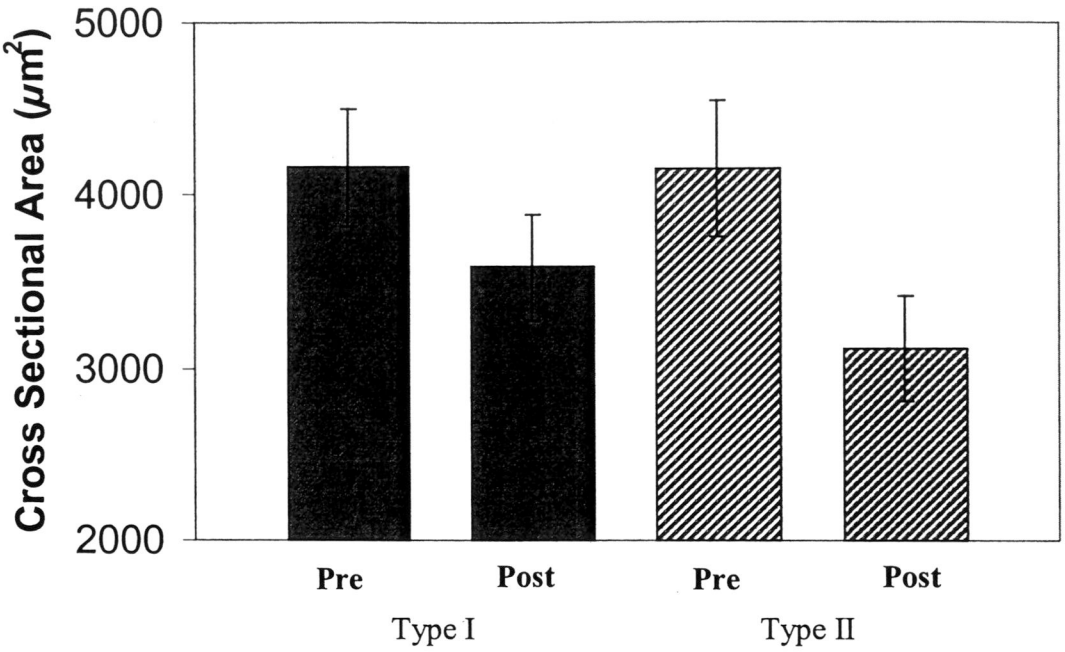

Figure 7

The Effects of Weight Loss on Vastus Lateralis Muscle Fiber Cross Sectional Area in Morbidly Obese Individuals (n=6)

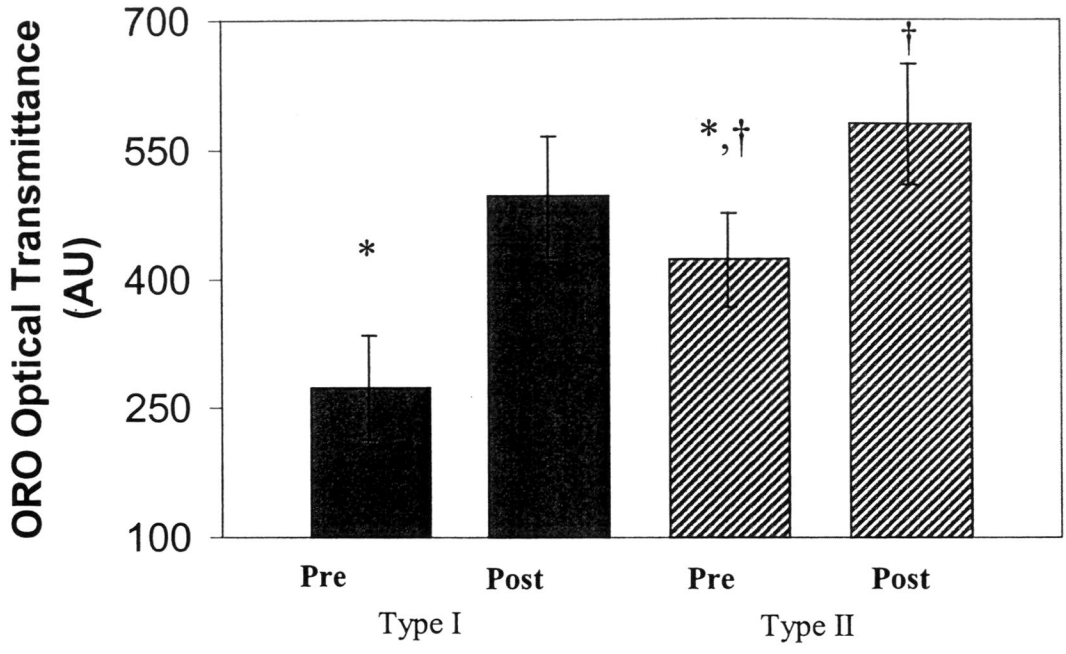

Figure 8

Oil Red-O Lipid Staining Relative to Vastus Lateralis Fiber Type Before and After Weight Loss in Morbidly Obese Individuals (n=6)

* $p < .05$ v Post Weight Loss
† $p < .01$ v Type I

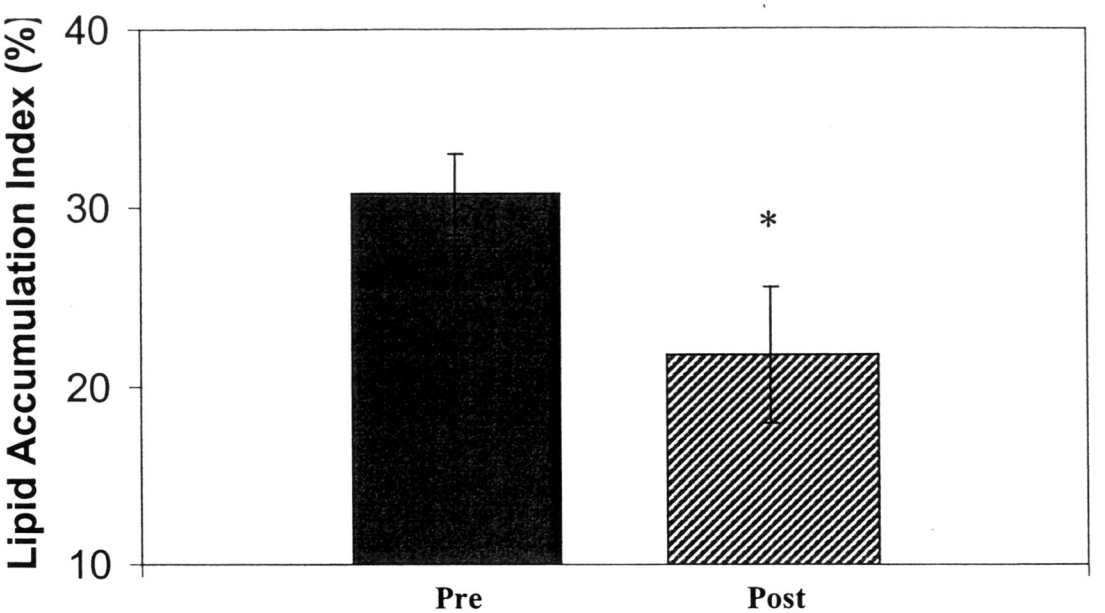

Figure 9

Effects of Weight Loss on the Lipid Accumulation Index of the Vastus Lateralis in Morbidly Obese Individuals (n=6)

* $p < .05$ v Pre Weight Loss

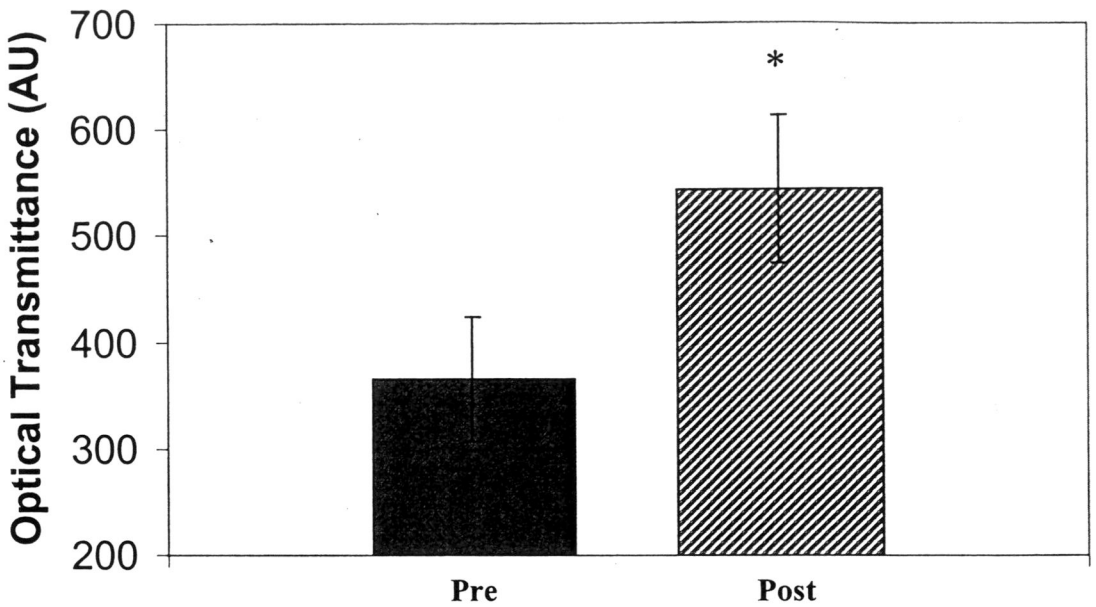

Figure 10

Fat Composite in the Vastus Lateralis Before and After Weight Loss in Morbidly Obese Individuals (n=6)

* $p < .05$ v Pre Weight Loss

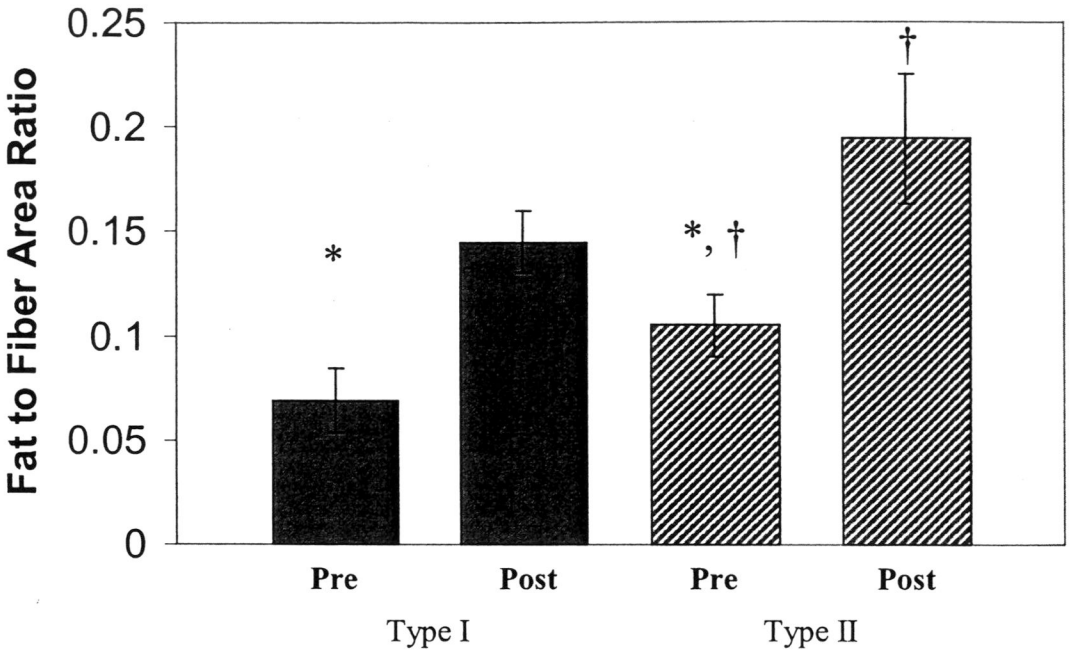

Figure 11

The Effect of Weight Loss on Vastus Lateralis Fat to Fiber Area Ratio in Morbidly Obese Subjects (n=6)

Fat to Fiber Area Ratio = ORO Optical Transmittance X Fiber Area $^{-1}$
* $p < .05$ v Post Weight Loss
† $p < .01$ v Type I

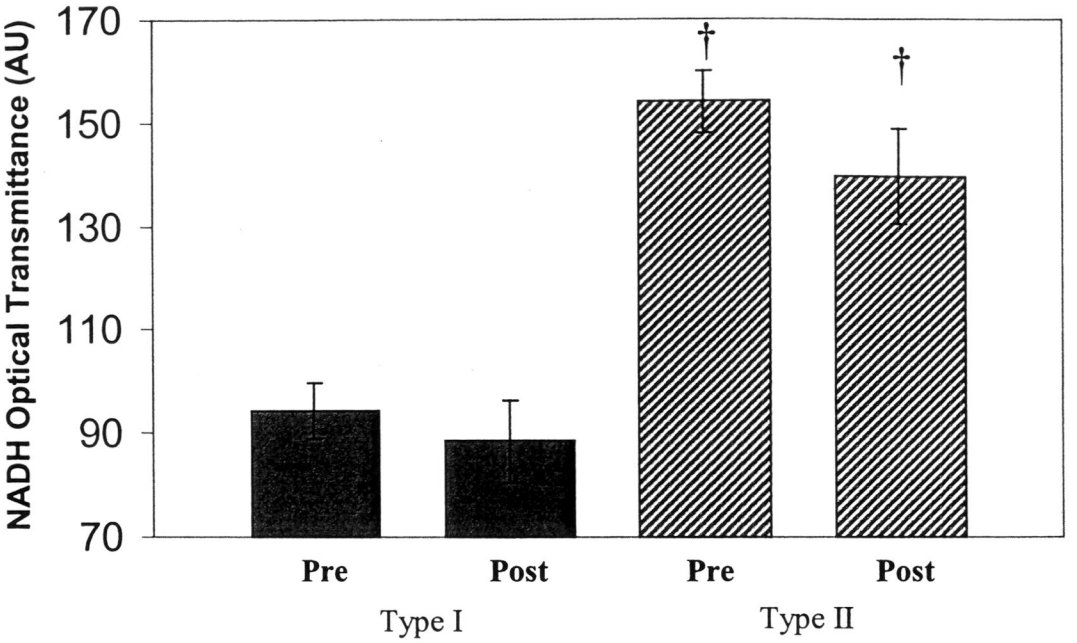

Figure 12

The Effect of Weight Loss on Vastus Lateralis Oxidative Staining (NADH) Parameters in Morbidly Obese Individuals (n=6)

† $p < .01$ v Type I

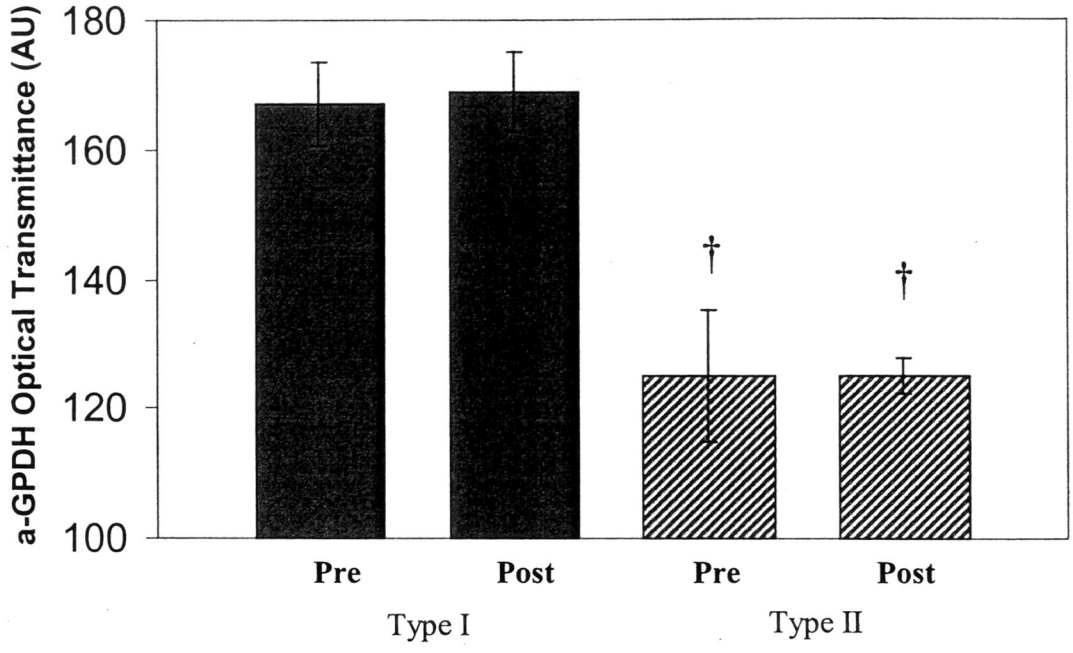

Figure 13

The Effect of Weight Loss on Vastus Lateralis Glycolytic Staining (α-GPDH) Parameters in the Morbidly Obese (n=6)

† $p < .01$ v Type I

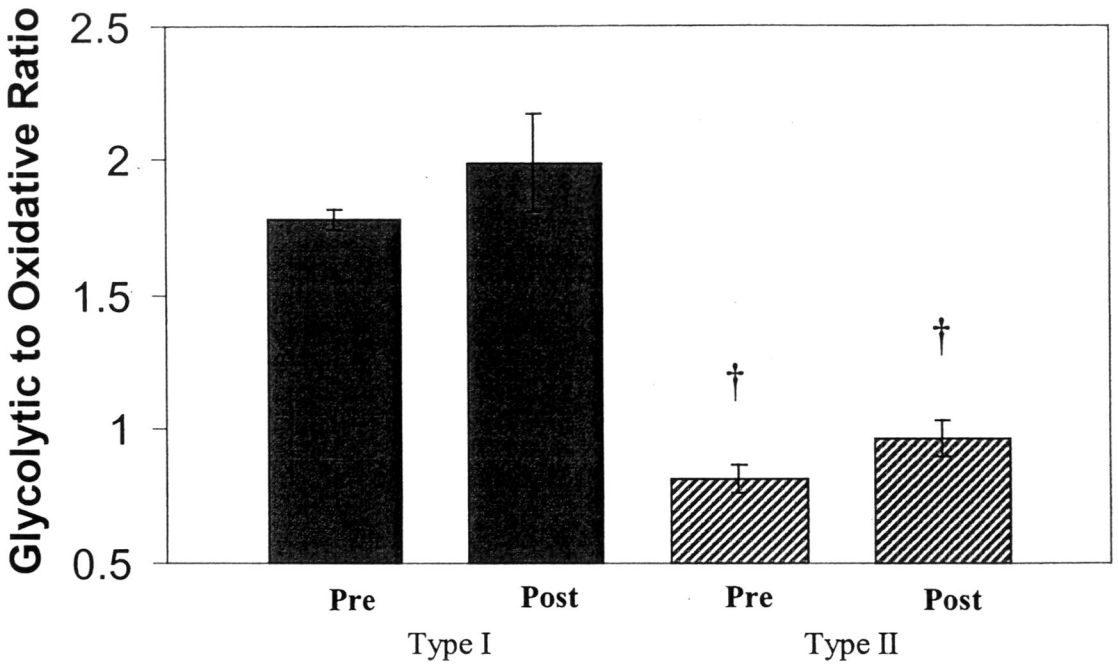

Figure 14

The Effect of Weight Loss on the Vastus Lateralis Glycolytic to Oxidative Ratio in Morbidly Obese Individuals (n=6)

Glycolytic to Oxidative Ratio = Glycolytic Optical Transmittance X Oxidative Optical Transmittance $^{-1}$
† $p < .01$ v Type I

CHAPTER V

DISCUSSION

Subjects

Skeletal muscle from morbidly obese individuals was analyzed before and approximately 1 year after gastric bypass surgery. Gastric bypass intervention resulted in mean weight loss of 69 kg and a 50% reduction in body mass index. Additionally, a 92% improvement in insulin sensitivity was observed due, in part, to vast improvements in fasting insulin (-89 %) and glucose (-33 %) measurements after weight loss. Considering the data presented by Pories et al. (1992), these results were expected, although the present sample size was small (n=6). Pories et al. (1992) reported that gastric bypass surgery, in the morbidly obese, resulted in a mean weight loss of 70kg, 1-year post surgery. Weight loss was accompanied by a 45% decrease in fasting blood glucose and a 75% reduction in fasting insulin (Pories et al. 1992). Weight loss induced via gastric bypass in morbidly obese individuals results in not only significant reductions of body weight and body mass index, but also increased control over hyperinsulinemia, hyperglycemia, and insulin action. Thus, the gastric bypass procedure serves as a useful weight loss intervention in this population by improving metabolic function and providing long-term weight reduction.

Association between Intramuscular Lipid Content in Obesity and Weight Loss

Attempts have been made to study intramuscular triglyceride content using various methodologies, such as lipid extraction and computerized tomography, both of which have their limitations. The limitations of these methods are similar in that neither

method can distinguish lipid stores location in relation to fiber type. Recently oil red-o histochemistry, combined with digital image analysis, has allowed investigators to quantify intramuscular lipid from muscle biopsy samples (Phillips et al. 1996, Goodpaster et al. 2000, He et al. 2001). As measured by oil red-o lipid staining, it is becoming clear that intramuscular lipid content is increased in obesity and obesity related insulin resistance (Goodpaster et al. 2000; He et al. 2001; Goodpaster et al. 2001). He et al. (2001) found a significant, 25-50% increase in muscle lipid content in obese individuals compared to lean individuals when using oil red-o methodology. In one of the first studies to digitally quantify oil red-o staining, Goodpaster et al. (2000), found a significant increase of intramuscular lipid content in obese and obese insulin resistant subjects compared to lean and has demonstrated that muscle lipid content is associated with BMI ($r = .42$, $p < .01$).

Only one study has compared intramuscular lipid content in obese subjects before and after weight loss (Goodpaster et al. 2000). In this study, a 16-week diet intervention resulted in a 16% reduction of body mass. Weight loss was accompanied by an 18% decrease in the number of lipid droplets and a 29% decrease in lipid area. The lipid accumulation index, was significantly decreased by 32% (3.43 ± 0.5 vs. 2.35 ± 0.3 %, $p < .01$).

The current study is the first to use oil red-o methodology to examine intramuscular lipid content in the morbidly obese population, before and after weight loss. Similar to Goodpaster et al. (2000), weight loss resulted in a 30% reduction of intramuscular lipid, as measured by the lipid accumulation index (30.8 ± 5.7 vs. $21.7 \pm$

3.8, %, $p < .05$). LAI values in the current study are higher than that of Goodpaster et al (2000). LAI % was calculated using the same formula, however our images were analyzed at a 10X magnification compared to 40X in Goodpaster et al. (2000). The advantage of using a lower magnification is the ability to include a higher number of muscle fibers within each image analyzed, therefore increasing the ability to measure a higher lipid area. This methodological difference may account for the higher LAI values in our study.

Weight loss intervention is associated with a reduction in muscle lipid content, however the extent and direct mechanisms for this reduction is unclear. Gastric bypass surgery reduces the functional area of the stomach, severely limiting food intake and thus, limiting fat consumption. This may be a simple, but reasonable hypothesis for the reduction of intramuscular triglyceride after weight loss in this population. Goodpaster (2000) hypothesized that intramuscular lipid is reduced to a similar extent to that of systematic fat loss during negative energy balance, however our results do not follow this trend. In Goodpaster et al (2000), both systematic fat and intramuscular lipid content were reduced by ~30 % with a 15 kg weight loss. In the current study, intramuscular lipid content was reduced by ~30 % after a ~70 kg weight loss. With such a massive weight loss (~47%), a larger decrease of intramuscular lipid was expected. It is unclear as to what mechanisms may regulate the extent of this reduction in response to weight loss.

Insulin resistance is associated with an increase of intramuscular lipid content in obesity (Phillips et al. 1996, Pan et al. 1997, Goodpaster et al. 2001). Goodpaster et al. (2001) has demonstrated that in lean, obese, and obese NIDDM individuals,

intramuscular lipid content is inversely associated with insulin sensitivity ($r = -.57$, $p < .05$). The present study is one of the first to show both a decrease in intramuscular lipid and an improvement in insulin sensitivity after weight loss, however no study has provided evidence of a specific relationship between improvements in insulin sensitivity and reduction of intramuscular lipid content.

To date, the mechanisms contributing to the negative impact of increased intramuscular lipid content on insulin sensitivity remain unclear. The current hypothesis suggests that intramuscular lipid serves as a surrogate for other lipid metabolites within the muscle that are known to affect insulin stimulated glucose metabolism (Kelley et al. 2001). Increased intramuscular lipid may result in an increase of long chain fatty acyl CoA and diacylglycerol within the muscle, both of which may interfere in the insulin signaling pathway, thus promoting insulin resistance (Ruderman et al. 1999, Kelley and Mandarino 2000). Intramuscular lipid content is also increased in endurance trained individuals compared to lean; however endurance trained individuals are insulin sensitive (Goodpaster et al. 2001). These data have led to the current hypothesis suggesting that the oxidative capacity of the muscle may serve to mediate the negative impact of increased intramuscular lipid on insulin stimulated glucose metabolism (Kelley and Mandarino 2000, Goodpaster et al. 2001). Additionally, intramuscular triglyceride serves as a readily available fuel source during exercise (van der Vusse et al. 1996) and this periodic depletion may serve as a protective mechanism that likely does not occur in obese, sedentary individuals (Goodpaster et al. 2001).

Fiber Type and Lipid Content

It has been suggested that muscle fiber type is a predisposing risk factor for obesity (Raben et al. 1998). The rationale behind this suggestion lies in the assumption that obesity may be related to a lower proportion of the oxidative fiber types, primarily type I fibers. Wade et al. (1990) has demonstrated that an inverse relationship may exist between type I fiber proportion and percent body fat. There is data that refutes this hypothesis, revealing no differences in fiber type distribution between lean and obese skeletal muscle. (He et al. 2001; Raben et al. 1998). Data from our lab (Hickey et al. 1995) has shown that type I fiber proportion is consistently lower in obese and obese, type II diabetics compared to lean individuals (40.4 % vs. 32.2 % vs. 50 %, respectively). In agreement with our previous findings, results from the current study show that type I fibers accounted for only 38% of the fiber type distribution. Weight loss had no effect on fiber type distribution, with type I fibers still accounting for only 38% of the total distribution. Few studies have measured fiber type distribution in this population, before and after weight loss. Our data is in agreement with Wadstrom et al. (1991), as gastroplasty induced weight loss did not result in a significant change of fiber type distribution in morbidly obese subjects (Type I Pre-WL, 52.8 ± 11.0 %; Post-WL, 48.2 ± 15.0 %, n=13). Our results suggest that in the morbidly obese population, a low proportion of oxidative fibers may indeed limit the oxidative capacity of the muscle and this impairment was not improved by weight loss.

Little is known regarding lipid content in relation to fiber type and even less is known regarding the relationship between fiber type, lipid content and the effects of

weight loss. In agreement with He et al. (2001) and Injer et al. (1979), our results indicate that more triglyceride is found within type I compared to type II fibers. This may be due, in part, to the higher oxidative capacity of type I fibers, with lipid preferentially being stored where it can be used more efficiently (He et al. 2001).

No study has determined the effects of weight loss on intramuscular lipid content in specific fiber types. A purpose of the current study was to determine if weight loss results in a selective reduction of lipid from a particular fiber type. After weight loss, lipid content was significantly reduced in type I and type II fibers ($p < .05$), however type I fibers still contained more lipid compared to type II fibers ($p < .01$). In agreement with our hypothesis, weight loss reduced lipid content similarly across fiber types, in type I fibers by –44% and in type II fibers by –27%. The current results provide new evidence, demonstrating that lipid content is reduced in a similar fashion across fiber types in response to weight loss.

The Effects of Weight Loss on Lipid Content Relative to Fiber Area

A nonsignificant trend for a reduction in fiber area following weight loss was evident. Type I fiber area tended to decrease by ~14% and a ~25% reduction of fiber area was found in type II fibers. A reduction is fiber area following weight loss has been noted previously in the morbidly obese population by Russell et al. (1984). Russell et al. (1984) found a selective type II fiber atrophy (-35%) after only 2-weeks of hypocaloric dieting. In the present findings, this trend was present for both fiber types. Wadstrom et al (1991) has also provided evidence for atrophy of both fiber types following weight loss in a morbidly obese population (n=16 females). In their study, fiber area decreased by 15%

in both fiber types after a 10% reduction in body mass. Obesity can induce muscle overloading, increasing fiber size via hypertrophy (Wadstrom et al 1991). Our current hypothesis is that fiber atrophy may result from an unloading effect produced by such a dramatic weight loss, similar to the effects of detraining on muscle fibers from a resistance trained athlete (Staron et al. 1981), however this effect was not robust enough to observe in our small group of subjects.

The lipid accumulation index (LAI) was calculated, in the current study and previously by Goodpaster et al. (2000), in order to compare total lipid area to fiber area. The limitation of the LAI is the inability to compare lipid area to fiber area between fiber types. The current study provides new information on the topic of lipid content relative to fiber area across fiber types, before and after weight loss.

Lipid content relative to fiber area was determined to be higher in type I fibers compared to type II fibers, before and after weight loss. More importantly, weight loss intervention resulted in significant mean reduction (-46%) in the lipid to fiber area ratio across both fiber types. These results suggest that less fiber area is occupied by lipid after weight loss in both type I and type II fibers, even with a reduction in fiber area. This is an important concept, given the relationship between intramuscular lipid content and insulin resistance. At the present time, no direct effect of intramuscular lipid on insulin-stimulated glucose metabolism has been discovered and Goodpaster et al. (2001) has hypothesized that intramuscular lipid may serve only as a surrogate for other factors in the muscle that may directly affect insulin-stimulated glucose metabolism, such as long chain fatty acyl CoA. If the extent of lipid reduction due to weight loss did not surpass

the magnitude of fiber atrophy, a change would not be expected in the lipid to fiber area ratio. Thus, lipid content relative to fiber area would remain similar after weight loss, and the unknown negative effect on insulin stimulated glucose metabolism may still be present. The present results indicate that the reduction of lipid due to weight loss exceeds the reduction of fiber area and thereby reducing the area of muscle occupied by lipid.

Muscle Oxidative and Glycolytic Capacity

Oxidative and glycolytic capacity was compared between fiber types and before and after weight loss. As expected, type I fibers displayed a higher oxidative capacity than type II fibers. These results are in agreement with He et al. (2001), where type I fibers were determined to have a significantly higher oxidative capacity than type II fibers as determined by SDH staining in lean, obese, and obese insulin resistant subjects. Glycolytic capacity and glycolytic to oxidative ratios were higher in type II fibers. In relation to fiber type, these results are similar to the glycolytic staining patterns found in previous studies (He et al. 2001; Simoneau et al. 1989). In agreement with our hypothesis, weight loss did not have an effect on oxidative or glycolytic capacity in either fiber type. Exercise was not controlled in the current study, which is one reason why oxidative and glycolytic staining was performed for pre and post weight loss samples. These results are in agreement with Kelley et al. (1999) who found no improvements in skeletal muscle oxidative enzyme capacity after weight loss in obese and obese NIDDM subjects when measured via the limb balance technique. In addition, oxidative enzyme capacity remained lower than that of lean individuals. Considering the weight loss intervention used in the current study and in Kelley et al. (1999), these results were

expected. In the current study, the gastric bypass procedure promoted weight loss by drastically reducing caloric intake and should have no effect on the respiratory capacity of the muscle. It is possible that an increase in skeletal muscle oxidative capacity may only be found in response to weight loss interventions incorporating aerobic exercise. The effects of exercise have not been extensively examined in this area; this is an important consideration because exercise training has been shown to increase muscle oxidative enzyme capacity in lean individuals (Gollnick et al. 1982).

Summary

The current findings demonstrate that weight loss intervention is associated with significant reductions in intramuscular lipid content and improved insulin action in a morbidly obese population. The present results provide new information, indicating intramuscular lipid content is reduced in a similar fashion across fiber types and less fiber area is occupied by lipid after weight loss. In addition, the current study provides more evidence that skeletal muscle from obese individuals may be characterized by defects primary to obesity. Skeletal muscle from morbidly obese individuals in the current study was characterized by a low proportion of type I fibers and weight loss had no effect upon this distribution, nor does it improve oxidative enzyme capacity. Future research should be conducted to determine the mechanisms attributed to the association between intramuscular lipid content and insulin resistance. Furthermore, future research should be conducted to examine the role of exercise, with or without weight loss, on intramuscular lipid content in relation to insulin resistance.

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APPENDIX. UMCIRB APPROVAL

University and Medical Center Institutional Review Board
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TO: Joseph A. Houmard, PhD, Human Performance Laboratory, ECU, 371 Sports Medical Bldg.

FROM: Paul Cunningham, M.D., Chair, UMCIRB

DATE: January 9, 2002

RE: Full Committee Approval for Continuous Review of a Research Study

TITLE: "Lipid Metabolism in Obesity, Weight Loss, and Exercise"

UMCIRB #95-248

The above referenced research study was initially reviewed and approved by the convened University and Medical Center Institutional Review Board (UMCIRB) on **01/30/96**. This research study has undergone a subsequent continuous review for approval on **01/09/02**by the convened UMCIRB. The UMCIRB deemed this National Institutes of Health sponsored study **more than minimal risk** requiring a continuous review in **12 months**.

The above referenced research study has been given approval for the period of **01/09/02** to **01/08/03**. The following are the most currently approved items as they have been previously submitted:

- protocol (version 12/21/95)
- informed consent documents
  - (control groups, version 2/12/01; weight loss, weight loss and con., and effect of exercise training, versions 12/12/01).

The following revisions to the above referenced research study have also been submitted and approved with this continuous review application for the period of 01/09/02 to 01/08/03:

- informed consent documents
  - (control groups, version 2/12/01; weight loss, weight loss and con., and effect of exercise training, versions 12/12/01)
  -

The following UMCIRB members abstained for reasons of potential for Conflict of Interest on this research study:
Dr. P. Cunningham
Dr. S. McCammon

The UMCIRB complies with 45 CFR 46, 21 CFR 50, 21 CFR 56, ICH Guidelines, UMCIRB standard operating procedures, institutional policies and other applicable federal regulations.

The UMCIRB recognizes the investigator and research team's commitment to comply with 45 CFR 46, the UMCIRB standard operating procedures and institutional policies in the conduct of all research. Investigator also must comply with 21 CFR 50, 21 CFR 56, ICH Guidelines, and all other applicable federal regulations in their research endeavors.