Alterations in insulin action and contraction-mediated metabolism in myotubes derived from Roux-en-Y gastric bypass patients

by

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Roux-en-Y gastric bypass (RYGB) surgery induces various metabolic benefits in severely obese (BMI > 40 kg/m^2) individuals, including improved insulin action in peripheral tissues, most notably skeletal muscle, and remission of type 2 diabetes. Despite these improvements, the mechanism in which RYGB improves metabolism is unclear. To examine this, primary human skeletal muscle cells were isolated from muscle biopsies obtained from individuals prior to, 1-month, and 7-months following RYGB. Insulin-stimulated glycogen synthesis, an index of insulin action, improved in myotubes derived from subjects at 1-month following RYGB, which was sustained at 7-months post-surgery. The cellular mechanisms involved appear to consist of distinct acute and chronic components, with the acute response consisting of reduced muscle glycogen content and increased phosphorylation of ACC, and the chronic response associated with a physiological increase in PGC1α protein abundance.

To further examine the combined role of RYGB surgery and muscle contraction on skeletal muscle metabolism, fully differentiated myotubes from RYGB patients were electrically stimulated to contract for 24-hours. Prior to surgery, myotubes were

unresponsive to the benefits of muscle contraction to subsequently increase insulin action, suggesting exercise resistance in these cells. However, only 1-month following RYGB surgery, myotubes became responsive to muscle contraction, as indicated by enhanced insulin-stimulated glycogen synthesis and AS160 phosphorylation, as well as increased basal glucose oxidation.

To explore whether the improvements in insulin action were due to an improved ability of cells to switch fuel preference, we examined in vitro metabolic flexibility in cells derived from RYGB patients. Utilizing several methods to examine in vitro metabolic flexibility, we were unable to detect major differences amongst the groups, suggesting that alterations in metabolic flexibility at the whole-body level following RYGB surgery are not retained in primary myotubes.

The results from this study suggest RYGB surgery alters the inherent characteristics of skeletal muscle that invoke improved insulin action and exercise-responsiveness. While previous research suggest insulin action in skeletal muscle is improved once substantial weight loss is achieved, we show that, when utilizing a muscle-specific model, improvements in insulin action occur as early as 1-month following RYGB surgery. Furthermore, it appears that acute adaptations in skeletal muscle following RYGB surgery invoke a cellular environment that is more responsive to the additional benefits of muscle contraction. Collectively, the results of this study provide valuable mechanisms in which RYGB surgery and muscle contraction can improve insulin action in skeletal muscle.

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ACC,	acetyl CoA carboxylase
AICAR,	5-Aminoimidazole-4-carboxamide ribonucleotide
AMPK,	adenosine monophosphate-activated protein kinase
ANOVA,	analysis of variance
AS160,	Akt substrate at 160 kDa
β-actin,	beta actin
BMI,	body mass index
CPT1,	carnitine palmitoyltransferase
FAO,	fatty acid oxidation
G6P,	glucose-6-phosphate
GLUT1,	glucose transporter 1
GLUT4,	glucose transporter 4
GS,	glycogen synthase
GSK3,	glycogen synthase kinase 3
IRS-1,	insulin receptor substrate 1
kDa,	kilodalton

MFN2, mitofusin 2

MHCI, myosin heavy chain I (slow) isoform

RYGB, Roux-en-Y gastric bypass

PDK, phosphoinositide-dependent kinase

PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha

PI3K, phosphatidylinositol-3 kinase

Ser, serine

Thr, threonine

TCA, tricarboxylic acid

TBC1D4, Tre-2/BUB2/cdc 1 domain family member 4

CHAPTER ONE

INTRODUCTION

The prevalence of obesity has dramatically increased over the last 20 years, with nearly one-third of the U.S. population being obese (101). Unfortunately, it does not appear that this trend is slowing, as recent estimates indicate that by 2030 over half of the U.S. population will be obese, with 11% of the population classified as severely obese (BMI $\geq$ 40 kg/m^2) (49). The concurrent increased risk of heart disease, stroke, type 2 diabetes, and cancer with obesity has led to tremendous increases in health care costs. A recent report has indicated that medical costs for obese individuals were ~$1,500 higher than normal weight individuals, with an estimated cost of ~$190 billion to treat the condition (50). These statistics indicate a critical importance of studying obesity to decipher potential mechanisms to reduce the incidences of this condition. Severe obesity and type 2 diabetes are both characterized by an impaired ability of insulin to regulate glucose homeostasis, resulting in hyperglycemia. The major culprit of impaired glucose homeostasis is insulin resistance in peripheral tissues, most notably liver, adipose tissue, and skeletal muscle (87). Therefore, understanding the mechanisms of insulin resistance in these tissues is crucial in treating various metabolic disorders.

Skeletal muscle accounts for ~70-90% of glucose disposal under insulin-stimulated conditions (43); thus, targeting skeletal muscle metabolism can have significant benefits in treating whole-body defects associated with severe obesity. Severely obese individuals present several defects in glucose metabolism, as indicated by reduced rates of insulin-stimulated glucose uptake, glycogen synthesis, and glucose

oxidation (16, 53, 59). Reductions in insulin-mediated glucose metabolism in skeletal muscle can have great implications on whole-body metabolism. Along with reductions in glycogen synthesis and glucose oxidation, Friedman et al. also observed increased production of lactate in severely obese muscle (53). An increase in lactate, a gluconeogenic precursor, can be utilized by the liver to increase blood glucose levels; thus inducing a hyperglycemic environment associated with severe obesity and type 2 diabetes (105). Insulin therapy may help reduce blood glucose levels; however, the clinical implications (i.e., hypoglycemia, tumor growth, etc.) make this therapy not ideal. Thus, targeting sites responsible for glucose metabolism is crucial for treatment of whole-body derangements in glucose homeostasis (8).

Insulin Action on Intracellular Metabolism

As glucose is impermeable to the cell membrane, the substrate requires a transporter (e.g., GLUTs) to enter the cell to be metabolized. While there are 12 known glucose transporters throughout the body (129), GLUT1 and GLUT4 appear to be the most critical for skeletal muscle glucose uptake. GLUT1 resides exclusively in the cell membrane, and mediates basal transport of glucose into the cell. GLUT4, the most abundant glucose transporter in skeletal muscle (129), is stored in intracellular storage vesicles under basal conditions. However, when stimulated by various factors (i.e., insulin and muscle contraction), the vesicles translocate to the plasma membrane and t-tubules, after which they are imbedded into the membrane to allow glucose to enter the cell. Studies utilizing skeletal muscle strips from rectus abdominus of severely obese patients have shown reduced rates of glucose uptake, suggesting an integral role of defective glucose transport in mediating insulin resistance (16, 59).

While GLUT4 translocation has received the most attention as the mechanism for reduced glucose entry into the cell, intracellular metabolism also plays a pivotal role. As glucose can freely exit the cell upon entry, mechanisms are required to trap glucose to be further metabolized. This occurs through hexokinase, which provides a phosphate group to glucose at the sixth carbon position via ATP hydrolysis. The addition of the phosphate groups provides a charge that prevents the glucose molecule from exiting the cell. The hexokinase reaction is crucial for further increases in glucose uptake, as it creates a lower concentration of free glucose in the cell, thus facilitating greater diffusion of extracellular glucose into the cell. Along with abundance and location of the enzyme, hexokinase activity is also dictated allosterically by its metabolic byproduct glucose-6-phosphate (G6P). A buildup of G6P inhibits the activity of hexokinase; therefore, facilitating the cellular fate of G6P not only impacts the activity of hexokinase, but also the ability of glucose to enter the cell (137).

Once entered into the cell and phosphorylated by hexokinase, G6P has many potential fates. Shulman et al. have shown that 80-90% of glucose that has entered skeletal muscle under insulin-stimulated conditions is shuttled towards glycogen synthesis, and accounts for the majority of reduced glucose disposal in type 2 diabetics, suggesting an integral role of insulin-mediated glycogen synthesis in the etiology of type 2 diabetes (8, 123). Glycogen synthesis is first initiated by transfer of the phosphate group of G6P to the 1-carbon position of glucose via phosphoglucomutase, after which UDP is added to the glucose molecule by UDP-glucose pyrophosphorylase forming UDP-glucose, which is then incorporated into the growing glycogen particle by alpha 1,4 linkages via glycogen synthase (GS) (8). The steps involved in glycogen synthesis are

controlled by both covalent and allosteric mechanisms. Covalently, upon insulin stimulation, active Akt phosphorylates and inactivates glycogen synthase kinase (GSK3). Under non-insulin stimulated conditions, active GSK3 phosphorylates glycogen synthase (GS) at several sites (site 4, 3c, 3b, and 3a), rendering GS inactive. Inactivation of GSK3 with insulin stimulation, along with increased phosphatase activity, results in dephosphorylation and activation of GS. Allosterically, glycogen synthase is controlled by intracellular G6P levels (84). An increase glucose entry following insulin stimulation, along with increased phosphorylation of glucose by hexokinase, increases G6P levels in skeletal muscle, which is then partitioned towards glycogen synthesis. While covalent modifications have been considered a major regulator of insulin-stimulated glycogen synthesis, Bouskila et al. have shown that insulin-stimulated glycogen synthesis is normal in knock-in mice with constitutively active GSK3 (25), suggesting a greater importance of allosteric activation of glycogen synthesis under insulin-stimulated conditions. In an elegant study to follow up their previous work, Bouskila and colleagues mutated various sites of GS in mouse skeletal muscle to eliminate the allosteric regulation of G6P on glycogen synthesis, and observed a decrease in insulin-stimulated glycogen synthesis (26). These data suggest that, while both are involved in insulin-stimulated glycogen synthesis, allosteric activation via increased G6P levels appear to play a bigger role in these cellular processes.

Insulin Signaling in Skeletal Muscle

The proximal signaling effects of insulin that lead to enhanced glucose metabolism in skeletal muscle have been well characterized, and involve insulin binding to the insulin receptor, invoking receptor kinase activity and autophosphorylation at tyrosine residues.

This induces the key proximal signaling events within the insulin signaling cascade, which include tyrosine phosphorylation of the insulin receptor substrate (IRS-1), activation of the phosphatidylinositol 3-kinase (PI-3 kinase), and activation of phosphoinositide-dependent kinase (PDK1) (60). PDK1 subsequently activates the kinase Akt, a master regulator of intracellular glucose metabolism which stimulates glucose entry into the cell via translocation of insulin-responsive glucose transporter GLUT4 to the cell membrane, as well glycogen synthesis via enhanced glycogen synthase activity. In muscle strips derived from severely obese patients, Goodyear et al. have observed reductions in the proximal insulin signaling cascade, as indicated by reduced tyrosine phosphorylation of the insulin receptor and IRS-1, along with reduced PI-3 kinase activity (59).

In comparison to the proximal signaling events, the distal events following Akt activation that enhance glucose metabolism are not as well defined. However, glucose entry appears to be reliant on the phosphorylation of the Akt-substrate at 160 kDa (AS160, also known as TBC1D4) to elicit GLUT4 trafficking to the cell membrane (119). Initially discovered in adipocytes by Kane et al., AS160 was found to have a Rab-GTPase domain at the carboxyl terminal (74), which leads to sequestering of GLUT4 storage vesicles intracellularly due to greater bound GDP (119). Once phosphorylated by Akt, the Rab-GTPase activity of AS160 is inhibited, thus increasing the amount of GTP bound to the GLUT4 storage vesicles which elicit GLUT4 translocation. AS160 contains 9 phosphorylation sites that are responsible for the reduction in Rab-GTPase activity (34); however, evidence has suggested a subset of these sites are critical for insulin-mediated glucose metabolism. Kramer et al. have shown that transient expression of mutated AS160 at 4 of the critical phosphorylation sites (Ser318, Ser588, Thr642, and Ser751; 4P

mutant) to prevent phosphorylation reduced muscle glucose uptake in mice (82). Furthermore, Chen et al. suggest the Thr642 sites is most critical for insulin action, as mice with whole-body mutation of this site (knock-in Thr642→Ala642) reduced skeletal muscle glucose uptake, which consequently led to whole-body insulin resistance (35). Human-based data has also shown an integral role of AS160 phosphorylation on insulin-action, as type 2 diabetics have reduced insulin-stimulated AS160 phosphorylation in skeletal muscle (75, 91).

The Effects of Exercise on Insulin Action

Obesity and type 2 diabetes are considered “lifestyle-related diseases” (48), as these diseases can be prevented by altering the lifestyle habits of the individual (22). Decreases in physical activity, along with increased caloric intake, are the main attributes that have led to an increased rate of obesity, and consequently insulin resistance and type 2 diabetes, in the United States. In order to reduce the risk of these diseases, clinical interventions are required to reduce the obesity epidemic. Exercise induces a wide range of benefits that can improve whole-body metabolism, and has been considered a cornerstone target to treat various metabolic disorders (41, 48, 65). At the level of skeletal muscle, exercise improves insulin sensitivity, fuel oxidation, and mitochondrial function (38, 48). It is important to understand how exercise induces these metabolic benefits for the following reasons: 1) to determine whether other interventions that improve insulin sensitivity (i.e., bariatric surgery, described later in this chapter) implement their action through similar mechanisms as exercise, and 2) to identify the underlining mechanisms and markers for potential pharmacological treatment for individuals who cannot exercise. The benefits of exercise can be broken down into the acute effects following a single bout

of exercise, as well as the chronic effect of consecutive bouts of exercise (e.g., exercise training).

Acute Exercise

Each training bout induces a consequential post-exercise situation in skeletal muscle which induces various metabolic benefits (146), most notably an increased sensitivity to insulin to induce glucose uptake. First discovered by Richter and colleagues in perfused hindlimb muscles of rats (115), the insulin-sensitizing effects of prior exercise have also been shown in humans, with the majority of glucose being shuttled towards glycogen synthesis (116). In data extrapolated from Richter et al. (116), Wojtaszewski et al. have shown a leftward shift the in the dose-response curve of insulin concentration and changes in glucose uptake following exercise, indicating that, at similar insulin concentrations, muscle that has been exercised can transport more glucose than a non-exercised leg (145).

Hansen et al. discovered that enhanced insulin-stimulated glucose transport following an acute bout of exercise may be attributed to GLUT4 translocation to the cell membrane (63); thus, understanding how GLUT4 translocation is enhanced is critical to understanding the insulin-sensitizing effects of exercise. It was hypothesized that improvements in insulin action were due to changes in the proximal insulin signaling cascade, most notably improved phosphorylation of the insulin receptor, IRS, Akt, and GSK3. However, Wojtaszewski et al. have shown that prior exercise in humans does not alter proximal insulin signaling (142, 143); thus, other targets must be involved in the insulin sensitizing effects of exercise. Utilizing a rodent model, the Cartee lab was the

first to show that insulin-stimulated phosphorylation of AS160 was enhanced following muscle contraction, which appears to be sustained up to 27-hours following contraction (4, 55). This phenomena appears to translate in humans, as prior exercise has also been shown to enhance insulin-stimulated phosphorylation of AS160 at multiple sites, suggesting an integral role of AS160 phosphorylation on the insulin-sensitizing effects of exercise (31, 33, 34, 102, 132, 134).

Role of Glycogen and Energetic Signals on Post-Exercise Insulin Action

Alterations in the energetic state of the cell may mediate the changes in insulin action post-exercise. During intense exercise, glycogen levels greatly diminish to meet the metabolic demands of muscle contraction (73, 98). Therefore, replenishment of muscle glycogen levels following exercise is of critical importance for subsequent bouts of exercise. Several studies have shown a relationship between reduced muscle glycogen levels and insulin action post-exercise in humans (19, 20, 116). Furthermore, refeeding of rats post-exercise, which would rapidly replenish muscle glycogen stores, reversed the insulin-sensitizing effects of prior exercise, suggesting an importance of muscle glycogen stores on insulin action post-exercise (32, 55).

One of the most explored signaling pathways that connect the energetic changes of the cell with exercise to improved substrate metabolism is the AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric enzyme composed of α-catalytic subunit along with two regulatory subunits (β and γ). The enzyme was first discovered by two separate groups in 1973 (9, 30), but it took until 1994 for AMPK to be purified and sequenced (92, 125). The enzyme is considered the energy sensor of the cell, responding to lower ATP

production and increased energy expenditure associated with cellular stress (i.e., muscle contraction) (114). Activation of AMPK by muscle contraction was first discovered by Winder and Hardie (140). Since this seminal study, the activation of AMPK via muscle contraction or treatment with AICAR, an AMPK agonist, has been extensively shown to improve several aspects of skeletal muscle metabolism, including improved insulin action (4, 17, 31, 55, 80).

AMPK activity is controlled by covalent and allosteric mechanisms. AMPK is covalently activated by upstream kinases (LKB1 and CaMKK) phosphorylating the Thr172 site on the α-subunit. However, research has suggested an integral role of allosteric activation to not only improve AMPK activity, but also inducing a conformational change in the enzyme to allow easier access to the Thr172 site (114, 141). With exercise, there is a greater turnover rate of ATP hydrolysis, with the ADP derived from ATP hydrolysis rapidly converted to AMP via the adenylate cyclase reaction. AMP binding to the γ-subunit of AMPK stimulates AMPK activity, as well as inducing a conformational change in the enzyme to improve phosphorylation of the Thr172 site (29, 64, 120). Along with AMP levels, muscle glycogen levels can also regulate AMPK activity. The β-subunit of AMPK contains a glycogen binding domain that acts as a glycogen sensor. When glycogen levels were increased in a dose-response manner, McBride et al. showed that rat liver AMPK activity was consequently decreased, suggesting an integral role of glycogen content in mediating AMPK activity (89). In skeletal muscle, lowered muscle glycogen levels resulted in enhanced AMPK activity (44, 126, 144), showing that the in vitro studies by McBride et al. translate to human skeletal muscle physiology.

Collectively, these studies suggest a critical role of allosteric mediators on activation of AMPK.

AMPK acts as an upstream kinase associated with various metabolic processes in skeletal muscle (141). The first downstream target of AMPK discovered was acetyl CoA carboxylase (ACC) (30), which catalyzes the carboxylation of acetyl CoA to malonyl CoA. Winder and Hardie showed that phosphorylation of ACC by AMPK in skeletal muscle resulted in reduced ACC activity, as indicated by a reduction on malonyl CoA (140). Malonyl CoA is an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme of fatty acid entry into the mitochondria. A greater ability of fatty acids to enter the mitochondria to be further oxidized has great implications for insulin sensitivity, as greater fatty acid oxidation eliminates lipotoxic intermediates which can impede insulin signal transduction (10, 17). Along with its role on fatty acid oxidation, phosphorylation of ACC has recently been shown to affect insulin-stimulated glucose metabolism in skeletal muscle. In transgenic mice with mutation in the phosphorylation site of ACC, O'Neill et al. showed whole-body insulin sensitivity was greatly reduced in these mice, which was attributed to reduced skeletal muscle glucose uptake (100). These data suggest an important role of ACC phosphorylation on skeletal muscle insulin action.

Along with ACC, AMPK activation via AICAR treatment or muscle contraction has also been shown to phosphorylate AS160 in skeletal muscle (81, 133–135). Using in vitro studies along with mass spectrometry, Treebak et al. have shown that AMPK directly phosphorylates AS160 at the Ser704 residue (Ser711 in mouse tissue) (135). Interestingly, insulin also phosphorylated this site; however, in vitro work confirmed that insulin-stimulated AS160 phosphorylation of Ser704 was independent of Akt (135).

Human-based studies have confirmed the rodent and in vitro work by Treebak et al., showing that the Ser704 of AS160 site is phosphorylated following exercise, and is further enhanced following a rise in insulin, suggesting an important role of the Ser704 site on the insulin-sensitizing effects of exercise (102, 134). However, Treebak et al. showed that transfection of mutation of the Ser704 site of AS160 to an alanine to prevent phosphorylation did not inhibit glucose uptake in skeletal muscle (135). It appears that phosphorylation of Ser704 requires phosphorylation of another site on AS160, as transfection of the 4P mutant of AS160 also reduced Ser704 phosphorylation. An attractive candidate would be the Thr642 site of AS160, which is phosphorylated following muscle contraction and insulin stimulation (102, 132, 134).

Exercise Training

In comparison to acute exercise, chronic exercise training remodels the molecular machinery in skeletal muscle. Exercise training results in changes in total protein content and activity of key enzymes associated with substrate metabolism (48). Furthermore, exercise increases the abundance and function of mitochondria, subcellular organelles that are critical for oxidative metabolism (67). Collectively, these adaptations lead to a more oxidative profile in skeletal muscle, allowing for sustained physical activity over longer periods of time. The oxidative profile of skeletal muscle is also critical for disease progression, as obesity is associated with a lower oxidative profile, as indicated by a lower percentage of type I oxidative fibers (130). As mitochondria are the critical mediators of oxidative metabolism, understanding how exercise training invokes changes in mitochondrial function/abundance is critical to understand the mechanisms to improve the oxidative profile of skeletal muscle.

Mechanistic View of Exercise Training on Insulin Action: Role of PGC1α

The induction of genes that regulate substrate metabolism following exercise training appear to be mediated by the peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α). Originally discovered in brown adipose tissue upon cold stimulation (110), PGC1α has been shown to control various aspects of substrate metabolism in skeletal muscle, including mitochondrial biogenesis, substrate oxidation, and fiber type switching (85). Exercise training invokes ~1.5-2.5-fold increase in PGC1α (21). Interestingly, while the effects of mitochondrial content/function have received great attention, PGC1α also plays a critical role on changes in insulin sensitivity following exercise training. The enhancements in insulin sensitivity appear to originate from an increase in GLUT4 abundance. In cultures muscle cells, PGC1α overexpression resulted in increased GLUT4 mRNA expression, leading to increased glucose uptake (90). PGC1α binds to the muscle-specific transcription factor MEF2C, leading to increased transcription of GLUT4 (90). Interestingly, the authors observed that the majority of GLUT4 resided at the membrane even without insulin stimulation (90), suggesting PGC1α may also regulate GLUT4 trafficking.

Despite its apparent role on insulin sensitivity, various reports contradict the importance of PGC1α to improve insulin action in skeletal muscle. In mice with ~10-13-fold overexpression of PGC1α, whole body insulin sensitivity has been shown to not change or even be suppressed (36, 93). A possible explanation for this is that overexpression of PGC1α beyond physiological levels may induce compensatory mechanisms (i.e., increased lipid disposition) that lead to insulin resistance (36). In order to examine whether physiological overexpression of PGC1α can improve insulin

sensitivity, Benton and colleagues transiently overexpressed PGC1α in rat skeletal muscle using in vivo electroporation, which resulted in ~20-25% increase in PGC1α protein content (11, 12). These studies showed that a physiological increase in PGC1α resulted in improved insulin sensitivity, as indicated by increased glucose uptake (11, 12). Furthermore, beyond the increase in GLUT4 content, modest overexpression of PGC1α also improved insulin-stimulated phosphorylation of AS160 (11), which corresponds to the data from Michael et al. that showed enhanced membrane-bound GLUT4 protein abundance (90). These data suggest a physiological increase in PGC1α, as observed with exercise training, improves insulin sensitivity in skeletal muscle via increased GLUT4 protein abundance and insulin-stimulated AS160 phosphorylation.

Exercise Resistance

Interestingly, not everyone responds favorably to exercise. In a review by Stephens and Sparks, after examining several published (5, 37, 124) and unpublished exercise training studies in individuals with comorbidities (i.e., severe obesity and type 2 diabetes), observed that 15-20% of these individuals do not improve glucose homeostasis following exercise training (127). Furthermore, Bouchard et al. have shown that 7% of individuals in a large cohort (1700 participants) actually have reductions in glucose homeostasis following exercise training (23). These shocking findings have shifted the focus on determining the inherent characteristics of these individuals that are exercise resistant.

In order to examine this, Stephens et al., using microarray analysis of skeletal muscle biopsies prior to exercise training, showed that of the 186 genes examined, 70%

of these genes were differentially regulated between responders and non-responders, with a downregulation of these genes shown in the non-responder group (128). Furthermore, nearly 25% of these genes were associated with substrate utilization and mitochondrial biogenesis (128). These data suggest that exercise resistance is associated in inherent characteristics of skeletal muscle; however, the mechanism for altered gene expression with exercise resistance is unclear. Kirchner et al. have suggested that epigenetic modifications may play a role in the etiology of metabolic diseases (79). Stephens and Sparks also suggest that these epigenetic modifications may lead to exercise resistance (127). Therefore, in order to remove the resistance to exercise, clinical interventions are required that alter the myocellular environment (i.e., changes in the epigenome) to respond to exercise.

Roux-en-Y Gastric Bypass Surgery and Metabolic Improvements

Unfortunately, exercise training alone only results in ~3% weight loss (103). As many metabolic derangements are associated with excess weight, further therapeutic strategies, along with exercise, are required. Since 1980, the Roux-en-Y gastric bypass (RYGB) procedure has been consistently used to induce weight loss in severely obese individuals that cannot lose weight with normal lifestyle interventions (104). The surgery consists of a restriction of the stomach by creating a 20-30 ml proximal gastric pouch, along with bypassing the remainder of the stomach and the proximal small intestine (104, 113). This eliminates nutrients from reaching the majority of the stomach, as well as the duodenum and part of the jejunum (45). The surgery results in weight loss of over 100 lbs. in these individuals, which is sustained nearly 15 years after the surgery was

performed (109, 121). Furthermore, the surgery has been shown to help control hypertension, along with allowing the patients to function physically better (108).

An interesting observation that came from studies with RYGB patients was the improvement in glycemic control post-surgery. In patients that presented type 2 diabetes prior to surgery, nearly 83% of these individuals had full remission of the disease (109, 121). It was originally thought that the excessive weight loss induced by the surgery led to improved glycemic control; however, Pories and Dohm suggest that remission of type 2 diabetes occurs almost immediately post-surgery, as they described a patient that, only 6 days post-surgery, maintained euglycemia without the need of antidiabetic medications (106). These data suggest that RYGB itself can improve glycemic control.

These exciting findings have led to the investigation of how RYGB improves glycemic control. The gold standard for examining insulin sensitivity is utilizing the hyperinsulinemic-euglycemic clamp, which is considered a surrogate for peripheral tissue (i.e., adipose and skeletal muscle) insulin action. Interestingly, despite the improvements in glycemic control in the matter of days to weeks post-RYGB, previous studies have shown that insulin sensitivity is unaltered acutely (2, 28, 47, 138). This has also been confirmed by our group using an intravenous glucose tolerance test (113). Based on these results, the authors suggest that earlier adaptations in glycemic control following RYGB surgery were due to energy restriction, which improved hepatic insulin action, gut hormone secretion and β-cell function (28, 45, 47, 113), while significant and sustained weight loss (> 6 months post-RYGB) leads to improved insulin sensitivity (2, 28). Interestingly, de Weijer and colleagues observed that two weeks following RYGB surgery, there was a significant increase in lipolysis in these patients, resulting in an increase in

plasma free fatty acids (138). A rise in plasma free fatty acids has been shown to induce insulin resistance in skeletal muscle due to impaired insulin signaling (18); thus, acute changes in skeletal muscle insulin action following RYGB surgery may be masked by negative adaptations in the systemic environment.

Despite the importance of skeletal muscle on glycemic control, there are few studies that have examined glucose metabolism in skeletal muscle following RYGB. Studies from Friedman (54) and Bikman (16) have observed improved glucose transport in muscles derived from RYGB patients ~1-year post-surgery. These improvements appear to be due to altered intracellular signaling, as GLUT4 protein content was unchanged post-RYGB (54), while proximal insulin signaling is improved (2, 16). However, these studies were performed in patients ~1-year post-RYGB, when substantial weight loss was achieved. It is unclear how RYGB alters the myocellular environment during the acute period (weeks-months) post-RYGB. Recently, the intracellular signaling mechanisms have been examined acutely (1-week to 3-months) following RYGB surgery. Severino et al. observed a reduction in basal phosphorylation of Akt (Ser473) 1-month following RYGB (122), which would be in line with data from Reed et al. that showed plasma insulin levels were reduced acutely post-RYGB (113). Unfortunately, the authors did not examine phosphorylation of key signaling proteins under insulin-stimulated conditions; thus it is impossible to understand whether these results impact insulin action in skeletal muscle. To examine this, Albers et al. examined key signaling proteins associated with skeletal muscle glucose metabolism under insulin-stimulated conditions (hyperinsulinemic-euglycemic clamp) (2). In line with whole-body measures of insulin sensitivity, the authors observed no change in the insulin signaling cascade either 1-week

or 3-months following RYGB surgery. However, as stated above, the changes in skeletal muscle insulin action may be masked by the systemic environment which is altered immediately post-surgery (138); therefore, the muscle-specific effects acutely following RYGB on insulin action remain unclear.

Interestingly, following RYGB surgery, skeletal muscle appears to adapt in some similar ways to exercise. Holmes et al. observed an increase in the phosphorylation status of AMPK (Thr172) 6-months following RYGB surgery (66), which was also observed 3-months post-surgery in the study by Albers et al. in normal glucose tolerant individuals (2). The authors suggest a role of adiponectin in inducing these changes in AMPK activity; however, at these stages following RYGB, the body is an energy-restricted state due to lower nutrient absorption. Therefore, it is possible that the energy-restricted environment may invoke a lowered energetic state in skeletal muscle which would increase AMPK activity similar to acute exercise (114). Furthermore, Barres et al. observed a physiological (~50%) increase in PGC1α mRNA expression in skeletal muscle 6-months following RYGB surgery (7). Similar to exercise training, a physiological increase in PGC1α may invoke improvements in insulin action in skeletal muscle following RYGB surgery. Furthermore, the changes in PGC1α expression were due to alterations in the methylation status of PGCα1, suggesting RYGB may alter the inherent defects associated with metabolic diseases (7). Collectively, these data suggest RYGB surgery invokes similar improvements in skeletal muscle to acute (AMPK activity) and chronic (PGC1α expression) exercise that would elicit improvements in insulin action.

Human Primary Skeletal Muscle Cell Culture Model

Based on the findings by de Weijer et al. (138), changes in skeletal muscle insulin action following RYGB may be masked by systemic factors that impede insulin signaling. Therefore, different methodologies are required to understand the time course changes in insulin action of skeletal muscle following RYGB surgery. Previous work has utilized skeletal muscle strips from the rectus abdominus from patients before and ~1-year post-surgery to examine changes in insulin-stimulated glucose metabolism (16, 54); however, this method is invasive as each strip would have to be collected during a surgical procedure, making time course evaluations almost impossible. The use of human primary skeletal muscle cells has provided an excellent tool to examine skeletal muscle metabolism. The procedure, which requires a small (~50-100 mg) amount of muscle, involves isolation of satellite cells and proliferation of myoblasts in culture. Upon confluence (70-90% of the cell plate covered with myoblasts), myoblasts are treated with a low serum media void of growth factors, allowing fusion of the myoblasts to form myotubes, which have similar characteristics of muscle fibers, including myosin expression and the appearance of sarcomere striations (14, 94).

The use of skeletal muscle cells have many advantages that allow the investigator to examine whether different treatments remodel the cell to improve metabolism. To understand the mechanism in which PPAR-α improve lipid homeostasis in humans, Muoio et al. treated human skeletal muscle cells with a PPAR-α agonist (GW7647) and observed an improvement in fatty acid oxidation (94). Furthermore, Bikman et al. showed that treatment of muscle cells with the AMPK agonist AICAR prevented lipid-induced insulin resistance (17). As myotubes contain contractile filaments, recent studies have examined whether muscle contraction can elicit muscle-specific changes in metabolism.

Electrical stimulation of muscle cells can mimic motor neuron activation of muscle fibers, and has been utilized as an in vitro model of exercise, as shown by increased fuel oxidation and insulin action (83, 99, 112). Lambernd et al. have shown that electrical stimulation of muscle cells for 24-hours can prevent insulin resistance induced by treatment with condition media (83). These interesting results show that human skeletal muscle cells are an excellent tool to examine whether pharmacological or physiological (i.e., muscle contraction) treatments can improve insulin action in skeletal muscle.

Not only can this model be used to test different treatments, human skeletal muscle cells are also a valuable tool to understand inherent characteristics of skeletal muscle metabolism (14). Studies have shown that the metabolic characteristics of the subject are retained in cell culture. As an example, whole-body fatty acid oxidation is suppressed ~40% with severe obesity in comparison to lean controls (131). Interestingly, in vitro fatty oxidation was suppressed at a similar ~40% in skeletal muscle cells derived severely obese subjects, which were grown in culture for ~1-month void of systemic factors (70). Further studies have shown that skeletal muscle cells derived from obese patients are metabolically inflexible and insulin resistant, which is consistent with their whole-body phenotype (17, 136). Human skeletal muscle cell culture studies have also shown that whole-body treatments translate to changes in the inherent characteristics of skeletal muscle, as Bourlier et al. observed improved glucose metabolism in muscle cells derived from obese patients that exercise trained (24). These data suggest that human skeletal muscle cell cultures can provide an excellent tool to examine whether whole-body interventions can improve the inherent characteristics of skeletal muscle.

Statement of Problem

RYGB surgery induces various metabolic benefits that improve whole-body metabolism, including improved glycemic control only a few days post-surgery. Despite these improvements, the mechanism in which RYGB improves metabolism is unclear. It appears that there are acute and chronic adaptive responses to RYGB surgery; however, how different tissues respond acutely and chronically to RYGB are unknown. Skeletal muscle is a major site of insulin-stimulated glucose disposal, and appears to be associated with the chronic adaptations to RYGB once substantial weight loss has been achieved. However, the lack of acute changes in skeletal muscle glucose metabolism may be masked by an altered systemic environment that is conducive for impaired muscle insulin action. In order to remove the systemic influence, previous research has utilized muscle strips from the rectus abdominus to examine metabolic and molecular adaptations following RYGB (16); however, the ability to understand the muscle-specific adaptations acutely and chronically following RYGB make this method not feasible. While a rodent model may be used to understand the molecular adaptations that occur following RYGB surgery, this method removes the translational aspect of the human benefits that occur following RYGB. Collectively, this indicates that new methodologies are required to examine muscle-specific alterations in insulin action. Despite being an excellent model to examine inherent defects in skeletal muscle metabolism, there has only been one study that examined the metabolic changes in skeletal muscle cells derived from RYGB patients (96), and the acute changes (1-month post-RYGB) have not been examined with this model. Furthermore, skeletal muscle from severely obese patients may have inherent characteristics that blunt the positive adaptations of muscle contraction to improve insulin

action. As RYGB can alter inherit characteristics, it is possible that the surgery may alter the skeletal muscle phenotype to allow for positive responses to muscle contraction. With a drastic increase of potential patients due to a greater rise in severe obesity, we feel it is crucial to determine whether RGB surgery alters the myocellular milieu to positively respond to various metabolic perturbations.

CHAPTER TWO

Alterations in Insulin Action in Primary Myotubes Following Roux-en-Y Gastric Bypass Surgery

ABSTRACT

Insulin resistance is a metabolic derangement evident with severe obesity (BMI $\geq$ 40 kg/m^2). In the severely obese, Roux-en-Y gastric bypass (RYGB) surgery has been shown to induce positive metabolic adaptations, including improved peripheral insulin action. However, the underlying cellular mechanisms involved in tissues such as skeletal muscle are uncertain. To examine this, primary human skeletal muscle cells were isolated from muscle biopsies obtained from individuals prior to, 1-month, and 7-months following RYGB. Insulin-stimulated glycogen synthesis, an index of insulin action, improved in myotubes derived from subjects at 1-month following RYGB (27% vs. 36% insulin stimulation pre vs. 1-month, $P<0.05$), which was sustained at 7-months post-surgery (41%). At 1-month post-RYGB, muscle glycogen levels were lower (-23%) and phosphorylation of acetyl CoA carboxylase (ACC) was elevated (+16%), suggesting that an alteration in energy state was linked with enhanced insulin action. At 7-months post-RYGB, glycogen content returned to pre-surgery levels; however, there was a significant increase in peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) protein content (+54%). These data indicate that insulin action intrinsically improves in skeletal muscle with RYGB; however, the cellular mechanisms involved appear to consist of distinct acute and chronic components.

INTRODUCTION

Severe obesity (BMI $\geq$ 40 kg/m^2) is associated with numerous metabolic defects, including insulin resistance and type 2 diabetes. In terms of intervention, Roux-en-Y gastric bypass surgery (RYGB) leads to improved metabolic health, as indicated by enhanced insulin action and reversion of type 2 diabetes (104, 107). While the clinical efficacy of RYGB is clearly evident, the time course of resolution of various deficiencies in carbohydrate metabolism is variable. For example, improved glycemic control and overt reversal of type 2 diabetes are evident almost immediately (~1-wk) after RYGB (28, 113). Conversely, peripheral insulin action appears to improve ~3 months or longer after RYGB, and seems to be connected with substantial weight loss (2, 16). These data suggest that distinct cellular mechanisms are involved with the improvements in metabolic health seen with RYGB.

Skeletal muscle encompasses ~40% of total body mass, and is a major site for insulin-stimulated glucose disposal. Skeletal muscle from severely obese individuals display numerous defects in carbohydrate metabolism, including impaired insulin-stimulated glucose transport, glycogen synthesis, and insulin signal transduction (16, 53, 59); however, these defects are reversed following RYGB (2, 16). Primary human skeletal muscle cells have been extensively utilized to study muscle-specific adaptations in substrate metabolism (14), and our group has reported that defects in substrate metabolism evident *in vivo* are retained in cells from severely obese individuals (10, 17, 68). Recently, Nascimento and colleagues reported an increase in basal glycogen synthesis in primary muscle cells derived from patients at 6-months after gastric bypass

surgery, suggesting that RYGB alleviates inherent defects in carbohydrate metabolism in skeletal muscle evident with severe obesity (97).

While adaptations in skeletal muscle appear to be a major factor responsible for improved whole-body insulin sensitivity, it is not evident if RYGB alters the intrinsic phenotype of skeletal muscle in a manner which is retained in primary skeletal muscle cells which proliferate and differentiate in an environment void of *in vivo* factors which can influence insulin action (i.e., hormones, blood lipids, etc.). It is also not evident when muscle-specific changes in insulin action occur following RYGB (i.e., acutely or chronically), and the cellular mechanisms involved. The purpose of the current study was to use a primary human skeletal muscle cell culture model to examine if RYGB alters insulin action in skeletal muscle and, if so, the time course of any changes. Our data reveal that RYGB improves the inherent characteristics of insulin action in skeletal muscle as early as 1-month post-surgery, which is retained at 7-months post-surgery. Furthermore, it appears that different cellular mechanisms are involved in inducing changes in insulin action at 1-month versus 7-months post-surgery.

MATERIALS AND METHODS

Roux-en-Y gastric bypass and primary human muscle cell cultures.

Skeletal muscle biopsies were obtained from the vastus lateralis of severely obese (BMI $\geq$ 40 kg/m^2) female patients before, 1-month, and 7-months following RYGB using the percutaneous needle biopsy technique. We were not able to obtain samples in two of the subjects at 1-month after the surgery. Data were excluded from one subject that displayed an abnormal insulin response (e.g., two standard deviations from the mean). A fasting venous blood sample was obtained prior to the muscle biopsy for analysis of plasma glucose and insulin. RYGB surgery, previously described in detail (107), consists of a reduction in the size of the stomach and bypassing a portion of the proximal small intestine (16). Primary skeletal muscle cells were isolated from the muscle biopsies and cultured into myoblasts as described previously (14, 15, 94). Myoblasts were subcultured onto 12-well (insulin-stimulated glycogen synthesis) and 6-well (immunoblot analysis and muscle glycogen content) type-I collagen-coated plates at densities of 40 x 10^3 or 60 x 10^3 cells per well, respectively. Upon reaching 80-90% confluency, differentiation to myotubes was induced by switching from growth media to differentiation media (Dulbecco's Modified Eagle's Medium supplemented with 2% horse serum, 0.3% bovine serum albumin, 0.05% fetuin, and 100 mg/ml penicillin/streptomycin). Experiments were performed on day 7 of differentiation, and all procedures were approved by the East Carolina University Institutional Review Board.

Insulin-stimulated glycogen synthesis.

The rate of glycogen synthesis was determined using previously described methods (3). Briefly, following 3-hour serum starvation, cells were treated with media containing D-[U-^{14}C] glucose (Perkin-Elmer, MA) (1 µCi/ml, 5.0 mM glucose) in the presence or absence of 100 nM insulin for 2-hours at 37°C. Following incubation, cells were washed with ice-cold PBS and solubilized in 0.05% SDS. An aliquot was transferred to a 2 ml tube containing carrier glycogen (2 mg) and heated for 1-hour at 100°C. The remaining lysate was used to assess protein concentration (bicinchoninic acid assay, Pierce Biotechnology, Rockford, IL). Glycogen was precipitated by the addition of 100% ethanol and overnight incubation at 4°C. Glycogen pellets were centrifuged (11,100 x g for 15-minutes at 4°C), washed once with 70% ethanol, and resuspended in dH_2O. Incorporation of radioactive glucose into glycogen was determined with liquid scintillation.

***Immunoblot Analysis*.**

Myotubes were serum-starved for 3-hours, followed by treatment with 100 nM of insulin for 10-minutes. Cells were harvested in ice-cold lysis buffer containing 50 mM HEPES, 12 mM sodium pyrophosphate, 100 mM sodium fluoride, 100 mM EDTA, 10 mM sodium orthovanate, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Lysates were sonicated for 5-seconds, rotated for ~1-hour at 4°C, and centrifuged at 12,000 rpm for 15-minutes at 4°C. The supernatants were used for immunoblot analysis as described previously (51). The following primary antibodies were used: phospho-acetyl CoA carboxylase (ACC) (Ser79) (Cell Signaling, Beverly, MA), phospho-Akt (Ser473 and Thr308) (Cell Signaling), total Akt (Cell Signaling), phospho-AMP-activated protein kinase (AMPK) (Thr172) (Cell Signaling), phospho-Akt-substrate at 160 kDa (AS160) (Thr642) (Abcam, Cambridge,

MA), total AS160 (Millipore, Billerica, MA), beta actin (housekeeping protein; LI-COR Biosciences, Lincoln, NE), GLUT4 (Millipore), phospho-glycogen synthase kinase 3α (GSK-3α) (Ser21) (Cell Signaling), total GSK-3α (Cell Signaling), hexokinase II (Santa Cruz Biotechnology, Dallas, TX) mitofusin 2 (MFN2) (Abnova, Walnut, CA), and peroxisome proliferator-activated receptor γ coactivator α (PGC1α) (Abcam). Membranes were probed with IRDye secondary antibodies (LI-COR Biosciences) and band intensities quantified using Odyssey software (LI-COR Biosciences).

Muscle glycogen content.

Myotubes were serum-starved for 24-hours and lysates were harvested as described above (*Immunoblot analysis*). Muscle glycogen content in cell lysates was examined by adapted methods previously described (88). Briefly, 2N HCl was added to lysates and were hydrolyzed for 2-hours at 95°C. The samples were neutralized with 2N NaOH and 1M Tris-HCl (pH 7.4). Glycogen content was measured using a hexokinase reagent (Thermo Fisher Scientific, Waltham, MA), and data are expressed as glucose content per µg protein.

Statistical Analysis*.*

Comparisons of insulin action (% or fold change), as well as body mass, blood chemistry measures, and basal changes in protein expression between pre and 1-month, and 7-months post-RYGB were performed by repeated measures ANOVA. *Post hoc* testing was performed using the Student's *t* test when appropriate. Data are expressed as means ± SEM. Significance was set as $P < 0.05$.

RESULTS

Subject characteristics are presented in Table 1. Subjects lost weight at 1-month (~13%), with further reductions (~21%) at 7-months following surgery. Fasting insulin and glucose levels were significantly lower at 7-months compared to 1-month.

Insulin-stimulated glycogen synthesis.

The relative increase over basal in insulin stimulated glycogen synthesis improved ($P < 0.05$) at both the 1-month (~36% insulin stimulation) and 7-month (~41%) time points compared to before surgery (~27%) (Fig 2.1). No differences in basal glycogen synthesis were observed over time (data not shown).

Insulin signaling cascade.

Despite the improvement in metabolic function, insulin-stimulated phosphorylation of Akt (Fig 2.2.a and 2.2.b) and GSK3α (Fig 2.2.c) were not enhanced following surgery. Phosphorylation of AS160 showed a trend for improvement 7-months following surgery ($P=0.14$, Fig 2.2.d). Total GLUT4 protein content was also unchanged following surgery (Fig 2.2.e). There were no changes in the total amount of Akt, GSK3α, or AS160 with RYGB (Fig 2.2.f).

Muscle glycogen content and energetic signaling.

To determine if RYGB alters the energetic state of the cell, we examined muscle glycogen content, and the phosphorylation status of AMPK and its downstream target ACC. At 1-month, we observed a ~23% decrease in muscle glycogen content ($P < 0.05$); however there were no difference in muscle glycogen content between pre and 7-months

post-surgery (Fig 2.3). While phospho-AMPK was not significantly altered following surgery (Fig 2.4.a), the phosphorylation status of ACC increased at 1-month following RYGB (Fig 2.4.b) ($P < 0.05$).

PGC1α and downstream targets.

While the energetic state appeared to be linked with changes in insulin action 1-month post-surgery (low glycogen content and increased AMPK activity), this did not explain the improvements in insulin action at 7-months after the intervention. We thus examined whether PGC1α, a major transcriptional coactivator involved in substrate utilization, was altered following surgery. As shown in Fig 2.5.a, while there was no change at the 1-month time point, PGC1α protein content increased by ~54% at 7-months following RYGB ($P < 0.05$). MFN2, a downstream target of PGC1α involved with fusion of mitochondria (149), was unaltered following surgery (Fig 2.5.b). However, hexokinase protein content tended ($P<0.10$) to increase following RYGB surgery (Fig 2.5.c).

DISCUSSION

RYGB surgery induces metabolic benefits in severely obese individuals, including improved whole-body insulin action at 3-12 months post-intervention (2, 16, 104, 107). Methods assessing whole-body insulin action (i.e., glucose clamp) not only take into account skeletal muscle, but other insulin-responsive tissues such as adipose tissue. Furthermore, blood-borne factors such as hormones, lipids, cytokines, and others can affect whole-body insulin action (117). To eliminate the influence of these confounding factors, we utilized primary muscle cells derived from patients before, 1-month, and 7-months after RYGB surgery to examine muscle-specific adaptations. We observed that insulin-stimulated glycogen synthesis, which has been used as an index of insulin action in human cell culture systems (3), improved as early as 1-month post-surgery, and remained elevated at 7-months following surgery (Fig 2.1). These novel data suggest that skeletal muscle-specific improvements may occur relatively early after RYGB. The reported lack of a similar acute improvement in whole-body insulin sensitivity (2, 28, 113) could possibly be due to tissue systems other than skeletal muscle not responding in such a relatively rapid manner or acute changes in the systemic environment that may impede skeletal muscle insulin action.

While the chronic effects of various disorders (i.e., severe obesity and type 2 diabetes) on metabolic function of myotubes has been examined well (1, 69), few studies have examined whether acute interventions that improve whole-body metabolism in patients may also be reflected in the physiology of these cells. Bourlier et al. observed that in obese individuals, 8-weeks of exercise training improved in vitro glucose metabolism in muscle cells derived from these subjects (24). Furthermore, Nascimento

et al. have shown improvements in basal glycogen synthesis in skeletal muscle cells derived from RYGB patients 6-months following surgery (96). The current study, which showed improved insulin-stimulated glycogen synthesis, expands on the growing knowledge that acute interventions (i.e., exercise training and RYGB surgery) can improve the inherent characteristics of skeletal muscle metabolism. Furthermore, along with the results of the exercise training study by Bourlier et al. (24), our results suggest that the inherent metabolic parameters of skeletal muscle can vastly change in a relatively acute manner (1-2 months) following the intervention.

Insulin enhances carbohydrate metabolism by binding to its respective receptor which initiates a signaling cascade involving an increase in Akt activity. Akt activation provides an upstream signal resulting in increased glucose entry into the cell through translocation of insulin-responsive GLUT4 vesicles to the membrane, as well as enhanced activity of glycogen synthase, the major regulator of glycogen synthesis (8). In the present study, we did not observe changes in insulin-induced phosphorylation of Akt or its major downstream target associated with glycogen synthesis, GSK3α (Fig 2.2). This lack of change is supportive of the *in vivo* findings of Albers et al. who reported that insulin-stimulated Akt activity was not potentiated in skeletal muscle at either 1-week or 3-months following gastric bypass surgery (2). The role of insulin-stimulated phosphorylation of GSK3α on skeletal muscle glycogen synthesis has been questioned, as Bouskila et al. have shown that mice with mutated GSK3, which could not be phosphorylated following insulin stimulation, have normal insulin-stimulated glycogen synthesis (25).

As Akt activity and GSK3α phosphorylation were unchanged following surgery, the mechanism for enhanced insulin action in our study was unclear. Activation of AMPK,

the major energy sensor in skeletal muscle, has been shown to improve insulin action in skeletal muscle (4, 17, 31, 55, 80). Furthermore, Hunter et al. have shown that enhanced AMPK-mediated glucose uptake allosterically activates glycogen synthesis in mice (71). To examine whether the improvement in insulin action at 1-month after RYGB was linked to enhanced AMPK activity, we examined the phosphorylation status of AMPK and its downstream target ACC. Phosphorylation of ACC increased following surgery (Fig 2.4.b), suggesting improved activity of AMPK which in turn may have contributed to enhanced insulin action. A relatively acute increase in kinase activity appears to occur in various tissues following RYGB, as Xu et al. have recently observed an increase in AMPK activity in adipose tissue only 3-months following surgery (147). These data (147), along with the current finding, suggest that improvements in peripheral insulin action in the early stages following RYGB may be due to enhanced AMPK activity.

Despite improved AMPK activity following RYGB, the mechanism in which AMPK becomes activated was not evident. Previous data has suggested a role of systemic adiponectin (2, 66, 147), via increasing the viability of upstream kinases (LKB1 and CaMKK) which phosphorylate and activate AMPK (148). As our cell culture model minimizes the effect of systemic factors on muscle metabolism, the increase in AMPK activity we observed appears to involve different mechanisms. Previous data has shown a role of muscle glycogen levels in controlling AMPK, with lower muscle glycogen content increasing the activity of the kinase (6, 126), potentially due to removal of allosteric inhibition of glycogen (89) or inhibiting the activity of phosphatases, resulting in maintained phosphorylation and increased kinase activity (120). In agreement with the reports (6, 89, 120, 126), muscle glycogen levels decreased in cells derived from patients

1-month post-surgery (Fig 2.3), in conjunction with improved ACC phosphorylation and sustained phosphorylation of AMPK (Fig 2.4).

At 7-months post-surgery, glycogen content returned to pre-surgery levels (Fig 2.3), which suggests that another mechanism was involved with enhanced insulin-stimulated glycogen synthesis. PGC1α, a transcriptional coactivator, is a key regulator of substrate utilization in skeletal muscle. A physiological increase in PGC1α protein content (20-150%), similar to what we observed at 7-months after RYGB (Fig 2.5.a), can enhance glucose utilization (85). PGC1α has been shown to regulate genes associated with carbohydrate metabolism, most notably inducing an increase in GLUT4 expression (46). However, we did not observe an increase in GLUT4 abundance post-surgery (Fig 2.2.e), implicating that the increase in PGC1α may affect insulin action through other mechanisms. Benton et al. reported that modest overexpression of PGC1α (~25%) in rat skeletal muscle improved insulin-stimulated phosphorylation of AS160 (11). However, we did not observe a robust increase in insulin-stimulated AS160 phosphorylation at 7-months post-surgery (Fig 2.2.d).

We did observe a trend ($P<0.10$) for an increase in hexokinase protein content following RYGB surgery. The increase in hexokinase corresponding with increased PGC1α content is similar to the results by Wende et al. that observed in mice with transgenic overexpression of PGC1α in skeletal muscle an increase in hexokinase protein abundance, which led to an increase in glycogen synthesis (139). While PGC1α activates transcription of various genes (85), it is unclear whether the increase in hexokinase was directly or indirectly due to PGC1α activity. Future research should examine the mechanism(s) in which PGC1α can enhance hexokinase protein content.

Based on our findings, allosteric activation of glycogen synthesis appears to be a major regulator of improved insulin action following RYGB surgery. At 1-month post-RYGB, muscle glycogen levels were significantly reduced in myotubes. Similarly, during intense exercise, another intervention that lowers the energetic state of the cell, glycogen levels greatly diminish (73, 98). Several studies have shown a relationship between reduced muscle glycogen levels and insulin action post-exercise in humans (19, 20, 116). Thus, our results suggest an important role of muscle glycogen in mediating improved insulin action acutely following RYGB surgery. Furthermore, hexokinase protein content tended to increase at both 1-month and 7-months following RYGB surgery. Along with glycogen stores, glycogen synthesis is also allosterically controlled by intracellular G6P (84). An increase in glucose entry following insulin stimulation, along with increased phosphorylation of glucose by hexokinase, increases G6P levels in skeletal muscle, which is then partitioned towards glycogen synthesis. Collectively, the results from this study suggest a potential integral role of allosteric activation of glycogen synthesis in mediating insulin action following RYGB surgery.

In primary human skeletal muscle cell cultures, the evident phenotype is likely due to genetic and/or epigenetic alterations. Epigenetic modifications are responsive to environmental cues, including conditions of nutrient excess or deprivation (79), and can occur rapidly following a change in the nutrient state. For example, Jacobsen et al. indicated that high fat feeding for only 5 days alters the epigenetic profile in human skeletal muscle (72). At 1-month after RYGB, patients are essentially in a catabolic state due to the reduction in energy intake. It is possible that this change in the nutrient environment elicits metabolic changes such as a reduction in glycogen content, possibly

via epigenetic mechanisms. Similarly, changes in PGC1α protein content 7-months following RYGB surgery may have originated from epigenetic modifications, as Barres et al. observed that promoter methylation of PGC1α in skeletal muscle biopsies was reduced 6-months following gastric bypass surgery, which led to an increase in PGC1α mRNA expression (7). Future research should aim to identify potential epigenetic modifications that may occur post-surgery that alter carbohydrate metabolism.

In conclusion, by using human primary skeletal muscle cells, we were able to examine muscle-specific changes in insulin action following Roux-en-Y gastric bypass surgery. Our findings suggest that improvements in insulin-stimulated glycogen synthesis, a proxy for insulin action, occur as early as 1-month following surgery due to a lower muscle glycogen levels and enhanced ACC phosphorylation. Furthermore, though the energetic state returns to normal, further improvements in insulin-stimulated glycogen synthesis appear to be linked to an increase in PGC1α protein content 7-months following surgery. These data indicate that insulin action intrinsically improves in skeletal muscle with RYGB; however, the cellular mechanisms involved appear to consist of distinct acute and chronic components.

Tables and Figures

	Pre (n = 8)	1-month (n = 6)	7-month (n = 8)
Weight (kg)	**139.3 ± 6.7**	**120.3 ± 9.1***	**100.4 ± 5.3*,†**
BMI (kg / m^2)	**50.2 ± 2.0**	**43.2 ± 2.8***	**35.7 ± 2.2*,†**
Glucose (mmol/l)	**5.1 ± 0.1**	**4.8 ± 0.2**	**4.4 ± 0.2*,†**
Insulin (pmol/l)	**95 ± 8**	**76 ± 21**	**51 ± 10***

Table 2.1: Subject characteristics. Data ± SEM. *, $p < 0.05$ vs. Pre; †, $p < 0.05$ vs. 1-month.

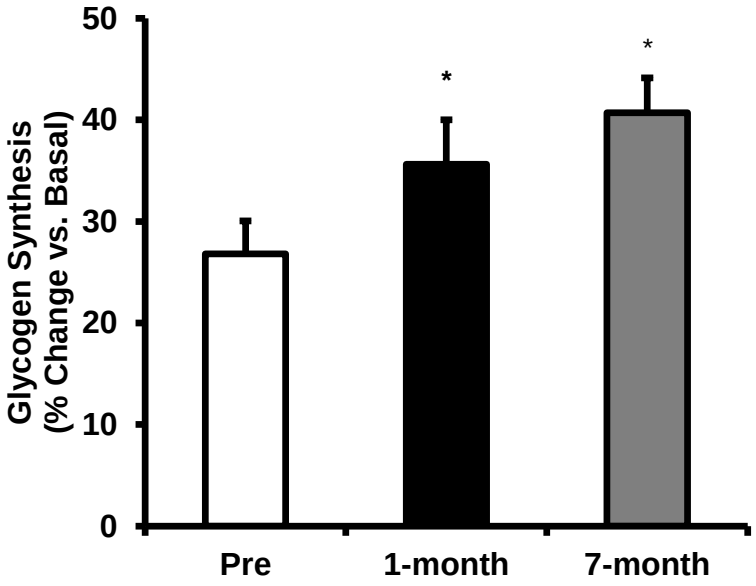

Fig 2.1: Relative increase in insulin-stimulated glycogen synthesis rates in myotubes derived from patients before (Pre), 1-month and 7-months after RYGB surgery. Data ± SEM. N = 5-7 per group; *p < 0.05 vs. Pre.

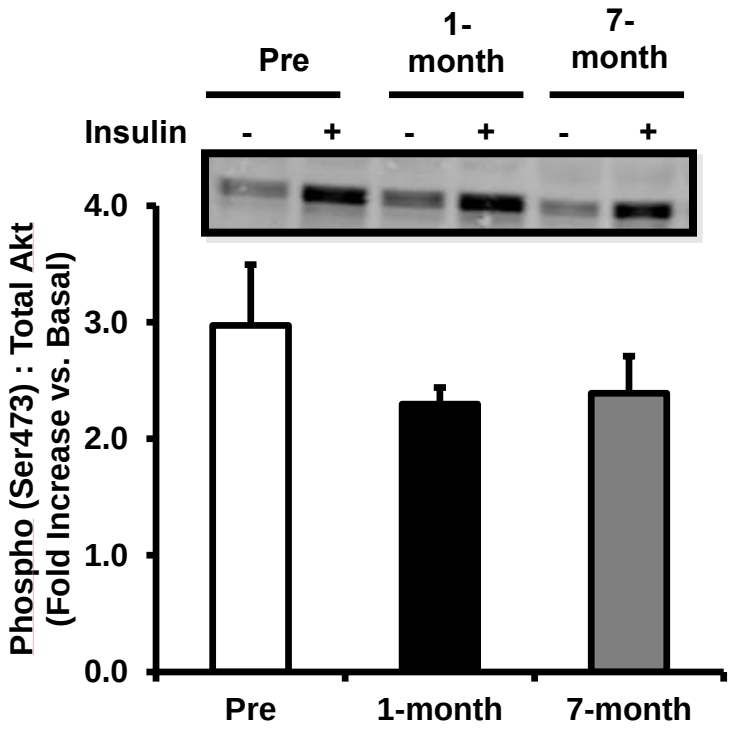

Fig 2.2.a: Insulin-stimulated phosphorylation of Akt (Ser473) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-7 per group.

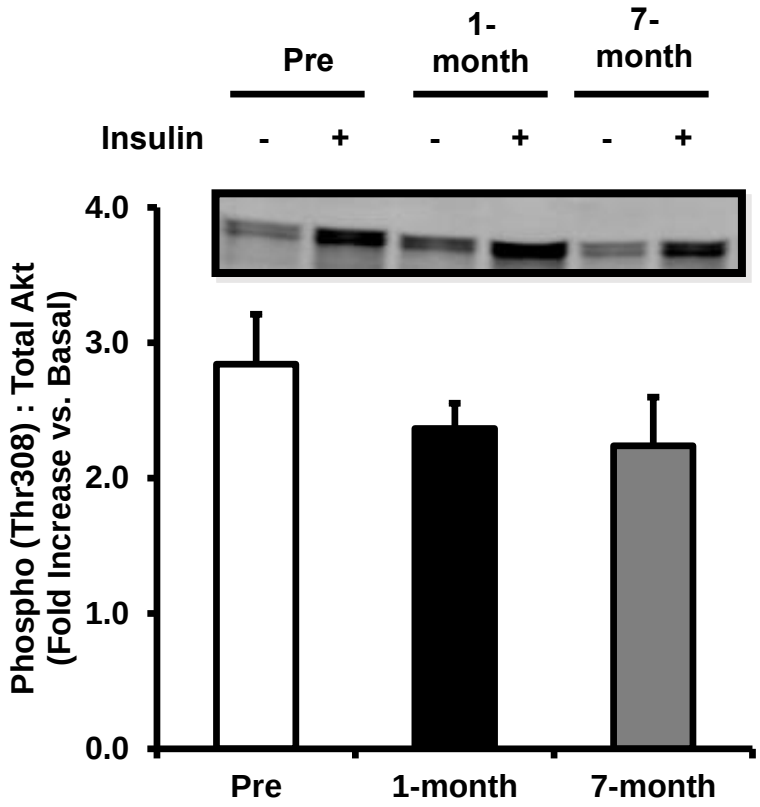

Fig 2.2.b: Insulin-stimulated phosphorylation of Akt (Thr308) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-7 per group.

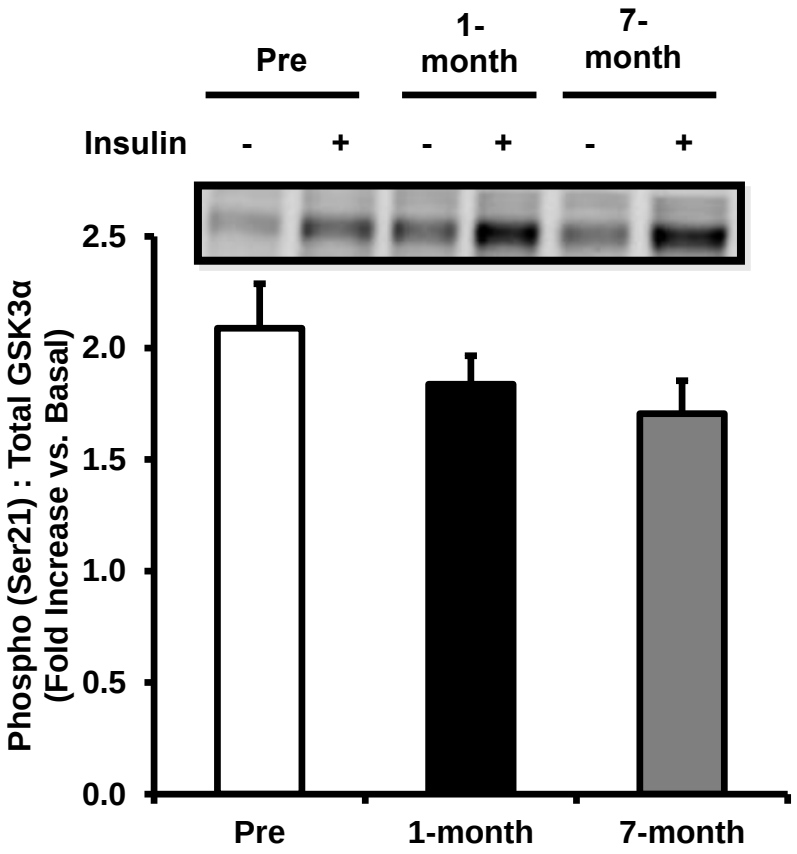

Fig 2.2.c: Insulin-stimulated phosphorylation of GSK3α (Ser21) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-7 per group.

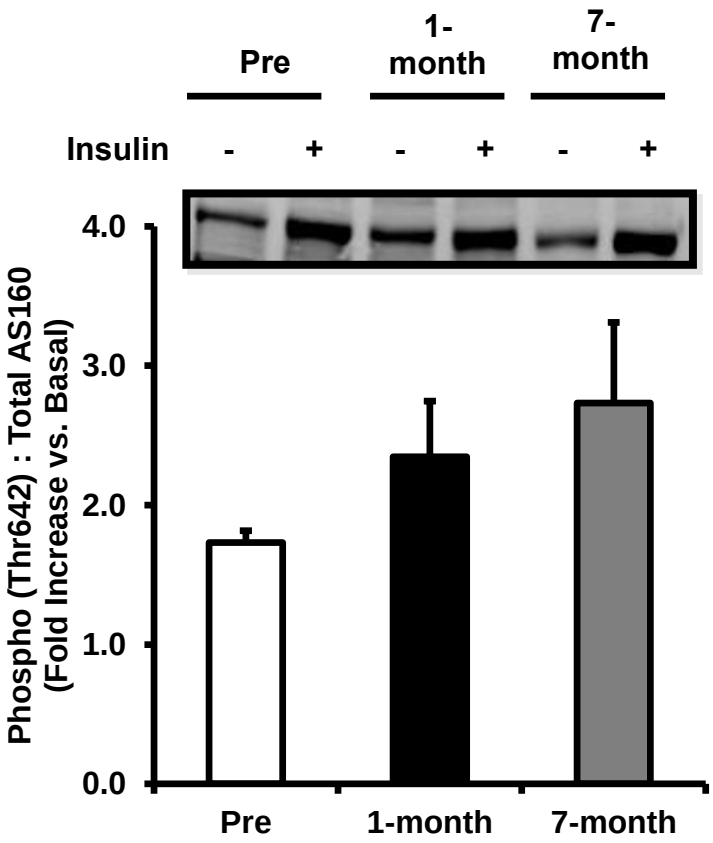

Fig 2.2.d: Insulin-stimulated phosphorylation of AS160 (Thr642) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-7 per group.

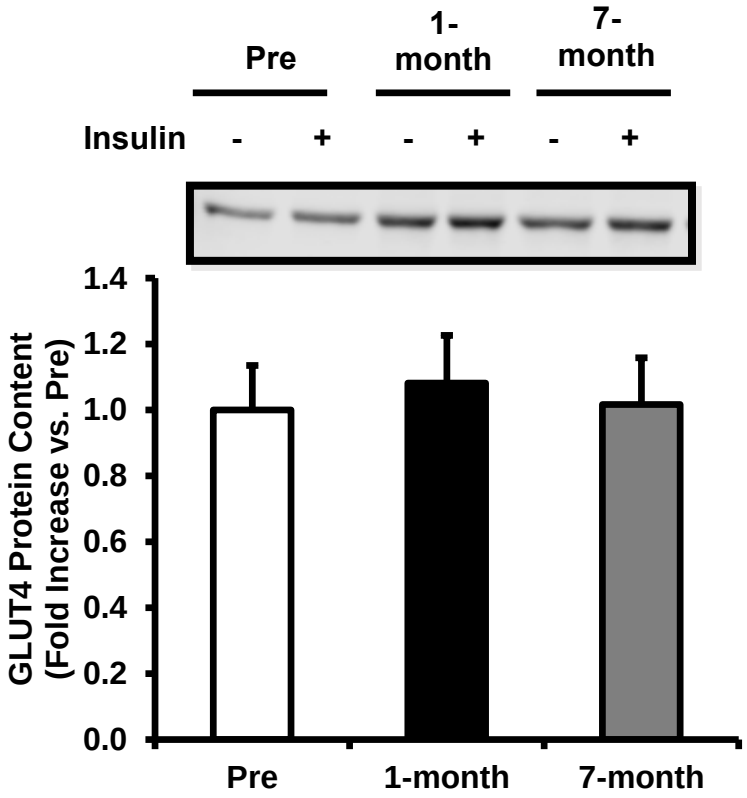

Fig 2.2.e: GLUT4 protein content in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Basal bands were quantified, N.S. basal vs. insulin. Data ± SEM. N = 5-7 per group.

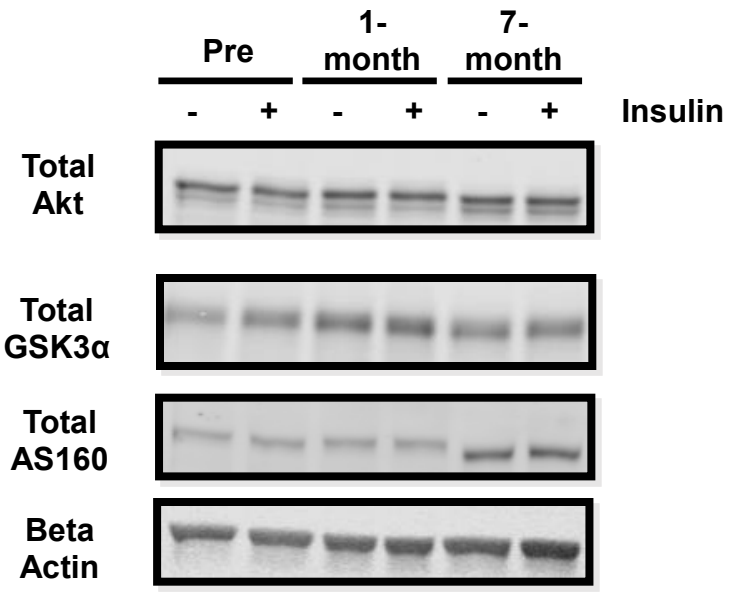

Fig 2.2.f: Total protein content of Akt, GSK3α, AS160, and Beta Actin in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-7 per group

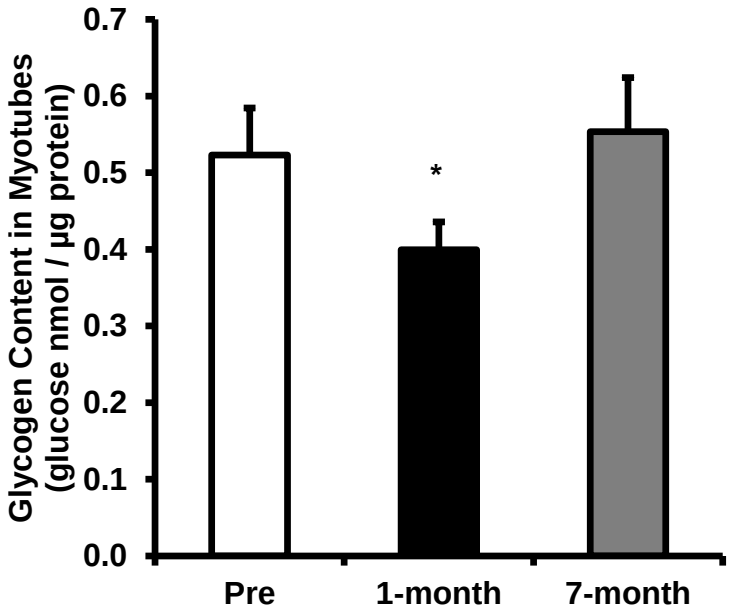

Fig 2.3: Basal muscle glycogen content in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-6 per group; *, $p < 0.05$ vs. Pre.

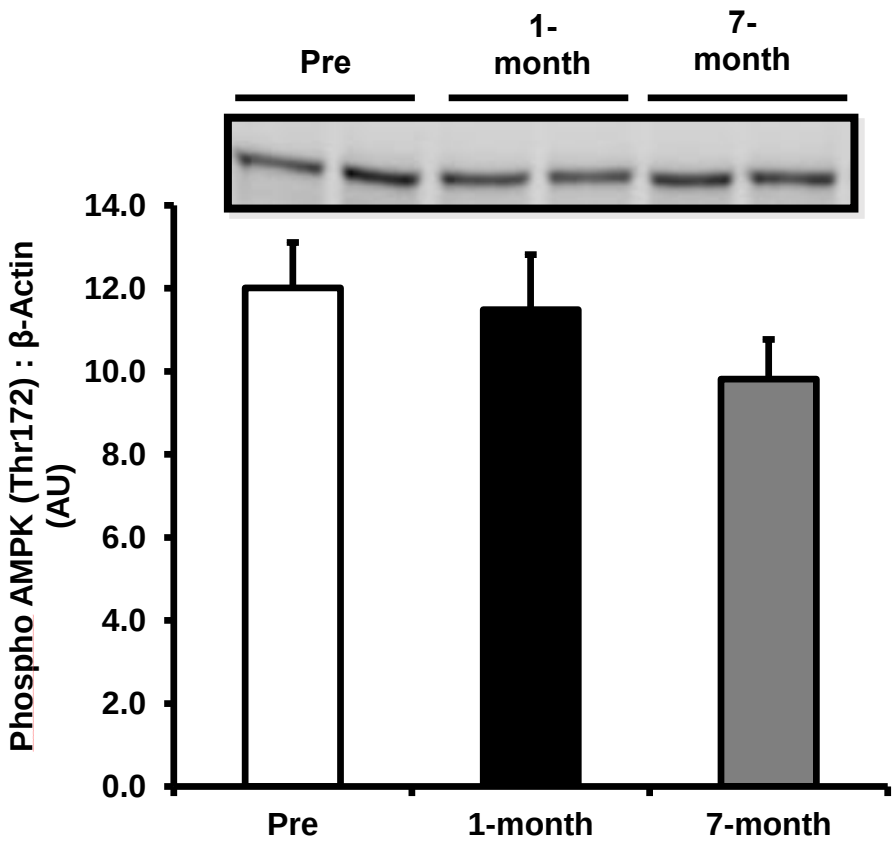

Fig 2.4.a: AMPK phosphorylation (Thr172) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-6 per group; *, $p < 0.05$ vs. Pre.

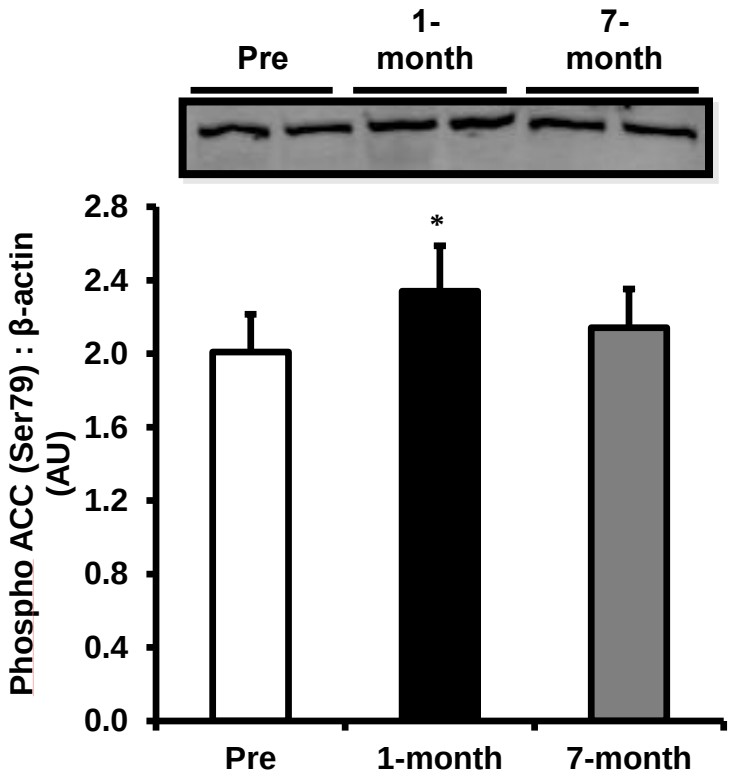

Fig 2.4.b: ACC phosphorylation (Ser79) in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 5-6 per group; *, $p < 0.05$ vs. Pre.

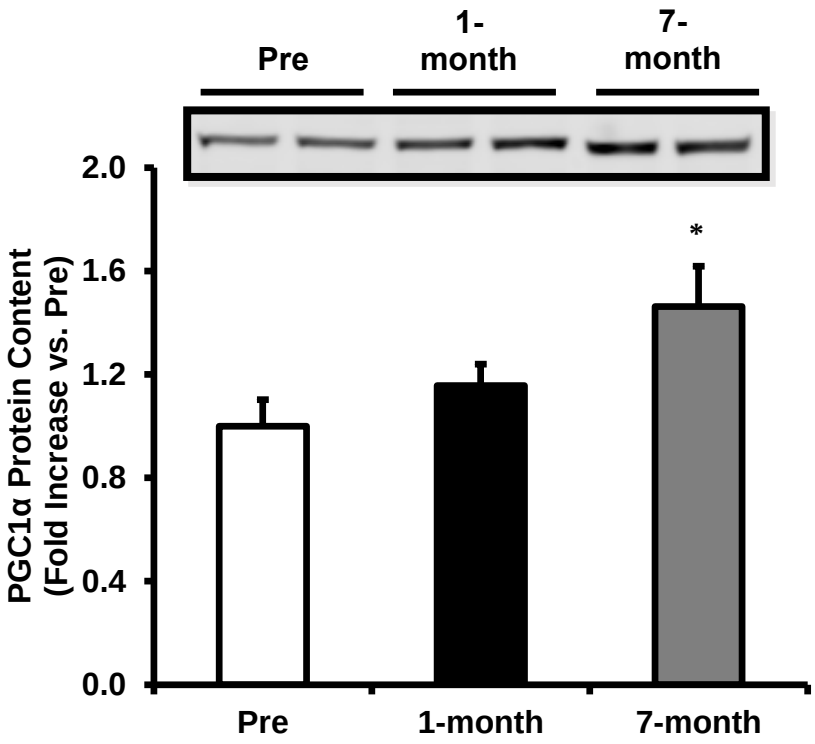

Fig 2.5.a: PGC1α protein content in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 per group; *, $p < 0.05$ vs. Pre.

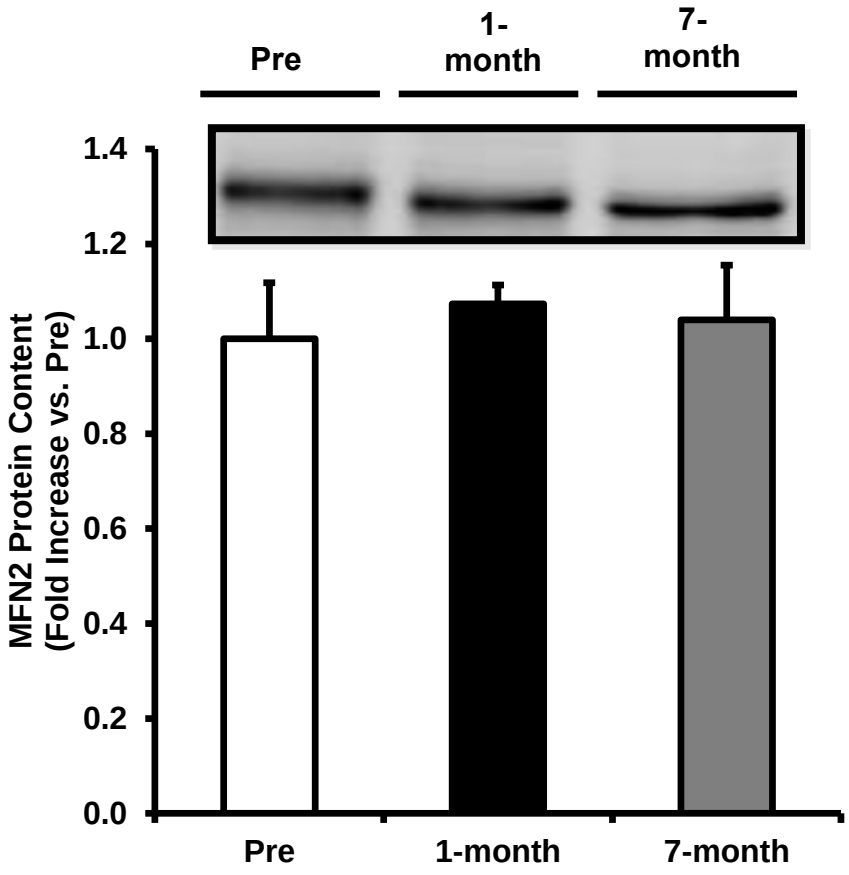

Fig 2.5.b: MFN2 protein content in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 per group.

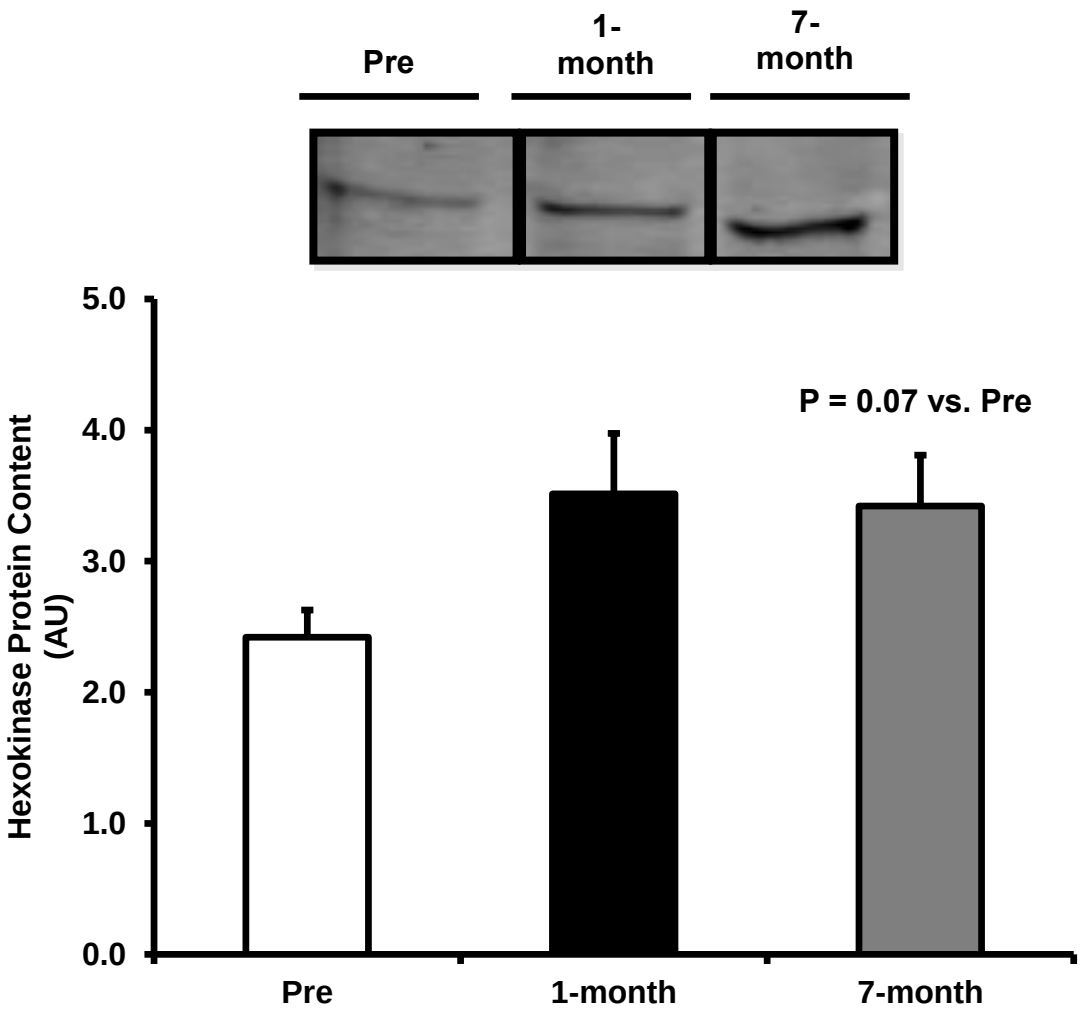

Fig 2.5.c: Hexokinase protein content in myotubes derived from gastric bypass patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 per group.

	1-month	7-month
Insulin-Stimulated Glycogen Synthesis	↑	↑
Insulin Signaling Cascade	↔	↔
Glycogen Levels	↓	↔
AMPK Phosphorylation	↔	↔
ACC Phosphorylation	↑	↔
PGC1α Protein Content	↔	↑
GLUT 4 Protein Content	↔	↔
MFN2 Protein Content	↔	↔
Hexokinase Protein Content	↔	↑ (P = 0.07)

Table 2.2: Summary of results. Data are presented as change in comparison to before surgery (PRE).

CHAPTER THREE

Roux-en-Y Gastric Bypass Surgery Enhances Contraction-Mediated Glucose Metabolism in Primary Human Myotubes

ABSTRACT

Contractile activity (e.g., exercise) invokes various metabolic adaptations in skeletal muscle, including improved insulin action and substrate oxidation. However, individuals with metabolic perturbations (i.e., severely obese [BMI $\geq$ 40 kg/m^2]) and type 2 diabetics) appear to have inherent defects in the ability to respond to contractile activity. Along with significant weight loss, Roux-en-Y gastric bypass (RYGB) surgery elicits positive metabolic adaptations; however, it is uncertain whether this clinical intervention alters the myocellular milieu to respond to contractile activity. To examine this, skeletal muscle cells were isolated from muscle biopsies obtained from patients before and after RYGB surgery, and differentiated into myotubes. Once fully differentiated, myotubes were electrically stimulated to contract for 24 hours (2 ms bipolar pulse, 1 Hz, and 11.5 V), after which changes in metabolic function and intracellular signaling were examined. Myotubes from severely obese patients prior to RYGB surgery were unresponsive to the metabolic benefits of muscle contractile activity, as indicated by a lack of change in insulin-stimulated glycogen synthesis (38.6% vs. 38.5% increase over basal for control vs. electrical stimulation) and basal glucose oxidation (4.5 nmol glucose/mg/hr vs. 4.6 nmol glucose/mg/hr control vs. electrical stimulation). Interestingly, myotubes derived from the same patients 1-month post-RYGB were responsive to muscle contraction, as indicated by an ~1.4-fold increase in insulin-stimulated glycogen synthesis (36.9% vs.

52.9% increase over basal for control vs. electrical stimulation) and an ~1.5-fold increase in basal glucose oxidation (3.9 nmol glucose/mg/hr vs. 5.5 nmol glucose/mg/hr control vs. electrical stimulation). At the molecular level, while unresponsive before surgery, muscle contraction improved insulin-stimulated phosphorylation of the AS160 (Thr642 and Ser704) 1-month following RYGB surgery. These data indicate that RYGB surgery inherently improves the ability of skeletal muscle to respond to contractile activity, suggesting an exercise program should be immediately implemented in these patients to further improve substrate metabolism.

INTRODUCTION

Obesity and type 2 diabetes are considered “lifestyle-related diseases” (48), as these diseases can be prevented by altering the lifestyle habits of the individual (22). Decreases in physical activity, along with increased caloric intake, are the main attributes that have led to an increased rate of obesity, and consequently insulin resistance and type 2 diabetes, in the United States. In order to reduce the risk of these diseases, clinical interventions are required to reduce the obesity epidemic. Exercise, which is the accumulation of intense skeletal muscle contractions over time, induces a wide range of benefits that can improve whole-body metabolism, and has been considered a cornerstone target to treat various metabolic disorders (41, 48, 65). At the level of skeletal muscle, chronic exercise training improves insulin sensitivity, fuel oxidation, and mitochondrial function (38, 48). Beyond the benefits of long-term exercise training, each acute training bout induces a consequential post-exercise situation in skeletal muscle which improves various metabolic benefits (146), most notably an increased sensitivity to insulin action. Thus, even a single bout of exercise can improve the metabolic profile of skeletal muscle.

However, despite performing regular exercise, severely obese (BMI $\geq$ 40 kg/m^2) patients may still retain a phenotype that is conducive to metabolic disorders. Being overweight or obese is associated with a higher all-cause mortality (52). Interestingly, despite the metabolic improvements, exercise training alone only results in ~3% weight loss (103); thus, these individuals are still at risk. Furthermore, individuals with metabolic disorders may not be as responsive to the benefits of exercise. In a review by Stephens and Sparks, after examining several published (5, 37, 124) and unpublished exercise

training studies in individuals with comorbidities (i.e., severe obesity and type 2 diabetes), the authors observed that 15-20% of these individuals do not improve glucose homeostasis following exercise training (127). Collectively, these data suggest that exercise training combined with interventions that induce significant weight loss are required to improve metabolic health with these individuals.

Roux-en-Y gastric bypass surgery (RYGB) is a surgical procedure employed to treat severe obesity via gastric restriction and bypass of the proximal small intestine (104, 113). Beyond substantial weight loss, RYGB surgery leads to an improvement in metabolic health, including the reversion of type 2 diabetes (104, 107). However, in comparison to healthy lean individuals, metabolic derangements remain with these patients, including insulin resistance and impaired fuel oxidation (13, 113). As exercise can improve these metabolic parameters, it has recently been suggested that exercise may improve the remaining metabolic defects following RYGB surgery. Recently, Coen et al. have shown that 6-months of exercise training in patients that recently underwent RYGB surgery (1-3 months post) improved insulin sensitivity to a greater extent than surgery alone (40). The changes in insulin sensitivity may be due to improvements in skeletal muscle metabolism, as the combined effects of exercise training and RYGB surgery results in improved fuel oxidation, mitochondrial function, and lipid diversion (13, 39).

Primary human skeletal muscle cells derived from muscle biopsies, which are grown in culture void of systemic factors such as hormones and insulin, are an excellent tool to examine muscle-specific alterations in various metabolic parameters. Furthermore, it has been shown that metabolic parameters of the donor are retained at

the level of the cell (10, 14, 17, 27, 42, 136); thus providing an excellent model to examine whether therapeutic interventions can improve muscle-specific derangements in metabolism. Electrical stimulation of muscle cells can mimic motor neuron activation of muscle fibers, and has been utilized as an in vitro model of exercise, as shown by increased fuel oxidation and insulin action (83, 99, 112). In order to examine whether RYGB alters the myocellular environment to respond to muscle contraction, we employed the electrical stimulation model with muscle cells derived from patients before, 1-month, and 7-months post-RYGB surgery.

MATERIALS AND METHODS

Primary human muscle cell cultures and electrical stimulation.

All procedures were approved by the East Carolina University Institutional Review Board. Skeletal muscle biopsies were obtained from the vastus lateralis of severely obese (BMI $\geq$ 40 kg/m^2, N=6) female patients before, 1-month, and 7-months following RYGB using the percutaneous needle biopsy technique. Primary skeletal muscle cells were isolated from the muscle biopsies and cultured into myoblasts as described previously (94). Myoblasts were subcultured onto 6-well type-I collagen-coated plates at densities of 60 x 10^3 or 40 x 10^3 cells per well for metabolic function and immunoblot analysis, respectively. Upon reaching 80-90% confluency, differentiation to myotubes was induced by switching from growth media to differentiation media (Dulbecco's Modified Eagle's Medium supplemented with 2% horse serum, 0.3% bovine serum albumin, 0.05% fetuin, and 100 mg/ml penicillin/streptomycin). On *day 7* of differentiation, myotubes were electrically stimulated to contract for 24-hours (11.5 V and 1 Hz) using a cell culture stimulator (C-PACE EP, IonOptix, Westwood, MA)

In vitro glucose metabolism.

The rate of glucose oxidation and glycogen synthesis was determined using adapted methods previously described (3). Briefly, following 3-hour serum starvation, cells were incubated in a sealed plate with reaction media containing D-[U-^{14}C] glucose (Perkin-Elmer, MA) (1 µCi/ml, 5.0 mM glucose) in the presence or absence of 100 nM insulin for 2-hours at 37°C. Following incubation, reaction media was transferred to a modified 48-well microtiter plate with fabricated grooves between two adjoining wells to

allow for acid-driven $^{14}CO_2$ from media to be trapped by 1M NaOH (78). Cells were washed with ice-cold PBS and solubilized in 0.05% SDS; after which an aliquot was transferred to a 2 ml tube containing carrier glycogen (2 mg) and heated for 1-hour at 100°C. The remaining lysate was used to assess protein concentration (bicinchoninic acid assay, Pierce Biotechnology, Rockford, IL). Glycogen was precipitated by the addition of 100% ethanol and overnight incubation at 4°C. Glycogen pellets were centrifuged (11,100 x g for 15-minutes at 4°C), washed once with 70% ethanol, and resuspended in dH_2O. Incorporation of radioactive glucose into CO_2 or glycogen was determined with liquid scintillation.

In vitro TCA cycle flux.

To determine whether electrical stimulation alters flux through the tricarboxylic acid (TCA) cycle, we examined alterations oxidation of 2-^{14}C-pyruvate. Briefly, following contraction, cells were serum-starved for 3-hours, after which cells were treated with reaction media containing 2-^{14}C-pyruvate (0.5 µCi/ml, 1 mM sodium pyruvate) for 2-hours. In comparison to 1-carbon labeled pyruvate, which provides an indication of pyruvate dehydrogenase complex activity, 2-^{14}C-pyruvate provides an indication of TCA cycle flux as the CO_2 is derived exclusively from the TCA cycle. Following the 2-hour incubation, acid-driven CO_2 production was examined as described above.

Immunoblot analysis.

Myotubes were serum-starved for 3-hours, followed by treatment with 100 nM of insulin for 10-minutes. Cells were harvested in ice-cold lysis buffer containing 50 mM HEPES, 12 mM sodium pyrophosphate, 100 mM sodium fluoride, 100 mM EDTA, 10 mM

sodium orthovanate, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Lysates were sonicated for 5-seconds, rotated for ~1-hour at 4°C, and centrifuged at 12,000 rpm for 15-minutes at 4°C. The supernatants were used for immunoblot analysis as described previously (51). The following primary antibodies were used: phospho-Akt (Ser473) (Cell Signaling), total Akt (Cell Signaling), phospho-Akt-substrate at 160 kDa (AS160) (Thr642) (Abcam, Cambridge, MA), total AS160 (Millipore, Billerica, MA), hexokinase II (Santa Cruz Biotechnology), GLUT4 (Millipore), mitofusin 2 (MFN2) (Abnova), myosin heavy chain slow isoform (Developmental Studies Hybridoma Bank), and peroxisome proliferator-activated receptor γ coactivator α (PGC1α) (Abcam). Generation and validation of the phospho-specific antibody for Ser704 of AS160 utilized for this study has been previously described (135). Membranes were probed with IRDye secondary antibodies (LI-COR Biosciences) and band intensities quantified using Odyssey software (LI-COR Biosciences).

Statistical Analysis.

Two-way ANOVA with repeated measures was used to compare differences between control and electrically stimulated cells before and after (1-month and 7-month) RYGB surgery. Comparison of insulin action (% or fold change) was performed by repeated measures ANOVA. *Post hoc* testing was performed using the Student's *t* test when appropriate. Statistical significance was defined as $P < 0.05$, and data are presented as mean ± SEM.

RESULTS

Insulin Action.

In order to examine alterations in insulin action following electrical stimulation, we examined insulin-stimulated glycogen synthesis. There were no changes in basal glycogen synthesis following electrical stimulation amongst the groups. However, following insulin stimulation, there appeared to be a greater effect of electrical stimulation in cells derived from patients 1-month post-RYGB. When plotted as a relative to the control plate, cells derived from patients 1-month post-RYGB were more responsive to insulin stimulation following electrical stimulation (Fig 3.1).

To examine the mechanism for improved contraction-induced insulin action 1-month post-RYGB, we examined the phosphorylation status of Akt, GSK3α, and AS160, two key regulators of insulin-stimulated glucose metabolism in skeletal muscle. There were no differences in contraction-mediated insulin-stimulated phosphorylation of Akt (Ser473) and GSK3α (Ser21) amongst the groups (Figure 3.2.a and 3.2.b). However, insulin-stimulated phosphorylation of AS160 at two different residues (Thr642 and Ser704) was enhanced following electrical stimulation 1-month (Thr642 and Ser704) and 7-months (Thr642 only) following RYGB (Fig 3.2.c and 3.2.d. GLUT4 protein content was unaltered following electrical stimulation at any time point (Fig 3.2.e). Interestingly, while hexokinase increased following electrical stimulation before and 7-months post-RYGB, the protein was not increased following electrical stimulation 1-month following RYGB surgery; however, in comparison to before surgery, hexokinase protein content tended to

be greater at 1-month post-RYGB under control and electrically stimulated conditions (Fig 3.2.f).

Substrate Oxidation.

Figure 3.3 shows alterations in basal glucose oxidation following 24-hours of electrical stimulation in cells derived from patients before and after RYGB surgery. Basal glucose oxidation was unaltered following electrical stimulation in cells derived from patients before surgery. At the 1-month time point, glucose oxidation tended to increase (3.9 nmol / mg / hr vs. 5.5 nmol / mg / hr for Pre and 1-month, respectively), while there was no change at the 7-month time point. In order to examine whether the changes in basal glucose oxidation were due to increased TCA cycle flux, we examined the rate of second carbon labeled pyruvate following electrical stimulation. We were unable to detect a change in 2-pyruvate oxidation following electrical stimulation amongst the groups (Fig 3.4).

As changes in oxidation may be due to alterations in mitochondrial content and/or dynamics, we next examined protein abundance of key regulators of mitochondria size/number. There were no changes in the protein abundance of PGC1α (Fig 3.5.a), MFN2 (Fig 3.5.b), or citrate synthase (Fig 3.5.c). Furthermore, the oxidative profile of cells, as indicated by the abundance of myosin heavy chain slow isoform was unaltered following electrical stimulation amongst the groups (Figure 3.6).

DISCUSSION

Despite improvements following RYGB surgery, patients are still deficient in various metabolic parameters in comparison to lean controls, including insulin resistance and impaired fuel oxidation (13, 113). A lack of physical activity post-RYGB has been suggested as a possible explanation for further improvements in metabolism in these patients. Recent data has suggested an integral role of exercise following RYGB surgery to aid in improvements in insulin action and mitochondrial function (13, 39, 40). To examine the muscle-specific effects of the combined role of muscle contraction and RYGB surgery, we utilized a cell culture model along with electrical stimulation to examine alterations in insulin action and fuel oxidation. Our results reveal that, at 1-month post-RYGB, muscle cells were more responsive to muscle contraction, as indicated by an increase in insulin-stimulated glycogen synthesis (Fig 3.1) and basal glucose oxidation (Fig 3.3). Furthermore, 7-months following RYGB surgery, cells from these patients were not as response to muscle contraction. Our data indicate that, in order to receive the positive muscle-specific alterations in insulin action and fuel oxidation, an exercise program should commence as early as 1-month following RYGB surgery.

The results of the current study are in line with recent data from Coen and colleagues, who showed improvements in insulin sensitivity following an exercise training intervention (40). The authors initiated a 6-month exercise program 1-3 months following RYGB surgery. While the authors initially designed the experiments to conclude at a time point when weight loss improves peripheral insulin sensitivity (6-9 months following RYGB), their results, along with those in the current study, would suggest that in order to maximize the effects of muscle contraction on skeletal muscle metabolism, an exercise

program should begin as earlier as possible post-RYGB surgery (1-3 months post). While we did not observe any improvements in the metabolic profile of cells derived from patients 7-months following RYGB surgery with electrical stimulation, exercise programs commencing greater than 6-months post-RYGB still provide additional metabolic benefits. Berggren et al. have shown that 1-year following RYGB surgery, patients who participated in a 10-day exercise training program improved skeletal muscle lipid oxidation to an extent similar to lean controls (13). While we did not examine lipid oxidation in these patients, it is possible that initiating an exercise program at early and late stages following RYGB surgery elicit differential metabolic responses, with changes in carbohydrate metabolism occurring when exercise begins immediately following RYGB, while changes in fat metabolism occur if exercise is started later (~6-12 months post).

Similar to Coen et al. (40), we also observed enhanced contraction-mediated insulin action in muscle cells following RYGB surgery, as indicated by improved insulin-stimulated glycogen synthesis. The mechanism in which electrical stimulation improved insulin action appears to be due to enhanced insulin-stimulated phosphorylation of AS160. AS160, also known as TBC1D4, is a Rab-GTPase that is associated with translocation of insulin-responsive GLUT4 transporters to the cell membrane and t-tubules (119). AS160 has been considered a major target site to improve insulin sensitivity, as prior muscle contraction has been shown to enhance insulin-stimulated AS160 phosphorylation at the Thr642 site up to 27-hours post-exercise (4, 55). The mechanism for enhanced insulin-stimulated phosphorylation of Thr642 site of AS160 post-contraction may be due to enhanced activity of AMPK following muscle contraction. AMPK, the energy sensor of the cell, is activated following the high energetic demands

brought upon by muscle contraction. While AMPK activation itself is insufficient to phosphorylate the Thr642 site (135), data from Kjobsted et al. suggest that AMPK phosphorylates the Ser704 site of AS160 (Ser711 in mouse tissue), which allows the Thr642 site to be more accessible (80). Furthermore, Treebak et al. have also shown that the Ser704 site is responsive to insulin, with a greater effect post-exercise (134, 135). In line with this, we observed enhanced insulin-stimulated phosphorylation of the Ser704 site following muscle contraction in cells derived from patients 1-month post-RYGB. These data suggest a potential synergistic role of phosphorylated AS160 at the Thr642 and Ser704 sites in improved insulin-stimulated glucose metabolism following muscle contraction.

The mechanism in which electrical stimulation enhances fuel oxidation of muscle cells 1-month post-RYGB is unclear. Similar to Coen et al. (39), we did not observe a change in mitochondrial content following electrical stimulation. However, the authors observed changes in electron transport chain efficiency following an exercise training intervention post-RYGB, which may have led to improvements in insulin sensitivity (39). In order to examine whether mitochondria were more efficient, we examined the oxidation of 2-carbon labeled pyruvate, which provides an indication of TCA cycle flux. Unfortunately, 2-pyruvate oxidation was unchanged with electrical stimulation, which suggests TCA flux was unaltered. Another possible explanation for improved basal glucose oxidation following electrical stimulation could be enhanced basal disposal of glucose into the cell. Along with changes in insulin action, Coen and colleagues also observed an increase in insulin-independent glucose disposal, as indicated by increase S_G (40). S_G is the effectiveness of glucose alone to enhance its own disposal into the

muscle, and has been shown to be reduced in various metabolic disorders (86). While the mechanism for this phenomena is unclear, the authors suggest a greater mass action effect of glucose into the cell. GLUT4 is the major glucose transporter in skeletal muscle, and is involved in both basal and insulin/contraction mediated glucose disposal (117). Similar to Coen et al. (39), we did not observe a change in GLUT4 protein content following electrical stimulation amongst the groups; however, acute exercise is associated with enhanced GLUT4 translocation to the cell membrane. Therefore, it is possible that electrical stimulation invoked GLUT4 translocation to the membrane, leading to increased glucose entry into the cell under basal conditions and enhanced glucose oxidation.

It has been shown that 15-20% of individuals with metabolic disorders (severely obese and type 2 diabetics) do not improve glucose homeostasis following exercise training (127). The results of the current study expand on the knowledge of exercise resistance, as we observed no change in insulin-stimulated glycogen synthesis, as well as basal glucose oxidation following 24-hour electrical stimulation of myotubes derived from RYGB patients prior to surgery. The inherent defect to respond to exercise signals has been previously shown utilizing pharmacological and genetic manipulation of myotubes derived from severely obese patients. Bikman et al. observed that treatment of cells derived from severely obese subjects with AICAR, an AMPK agonist, did not rescue the insulin resistant phenotype of the muscle (17). Furthermore, Consitt et al. showed that, using adenoviral expression in myotubes, a physiological increase in PGC1α similar to exercise training did not prevent the inherent defect in skeletal muscle lipid oxidation of severely obese individuals (42). Collectively, these results suggest that

myotubes of severely obese individuals are potentially resistant to the signals associated with chronic muscle contraction (i.e., exercise training).

As myotubes present an exercise resistant phenotype, it is suggested that the inherent characteristics stem from genetic and/or epigenetic mechanisms. To examine the possible mechanisms for exercise resistance, Stephens et al., using microarray analysis of skeletal muscle biopsies prior to exercise training, showed that various genes were differentially regulated between responders and non-responders, with a downregulation of several genes associated with substrate utilization and mitochondrial biogenesis (128). Stephens and Sparks suggest that epigenetic modifications may lead to exercise resistance (127); thus interventions that alter the epigenetic profile of skeletal muscle may improve the ability to respond to muscle contraction. Barres et al. have shown RYGB surgery induces epigenetic modifications in skeletal muscle (7); however, the authors suggest that the modifications were due to the substantial weight loss 6-months following the surgery. Interestingly, we observed that the changes in the ability of myotubes to respond to electrical stimulation occurred as earlier as 1-month following RYGB surgery. Along with the results from Chapter 2, these results suggest that RYGB surgery acutely alters the metabolic profile of skeletal muscle. Whether these changes were due to epigenetic modifications is unknown and requires future examination.

In conclusion, the results of the current study suggest cells derived from patients 1-month following RYGB surgery are more metabolically responsive when electrically stimulated to contract in comparison to before surgery. While the mechanism for enhanced basal glucose oxidation are unclear, improved insulin action 1-month post-RYGB appear to be due to enhanced insulin-stimulated AS160 phosphorylation following

electrical stimulation. Collectively, our results, along with those from Coen and colleagues (39, 40), suggest that an exercise program should be implemented immediately following RYGB (1-3 months) to induce muscle-specific improvements in metabolism.

Tables and Figures

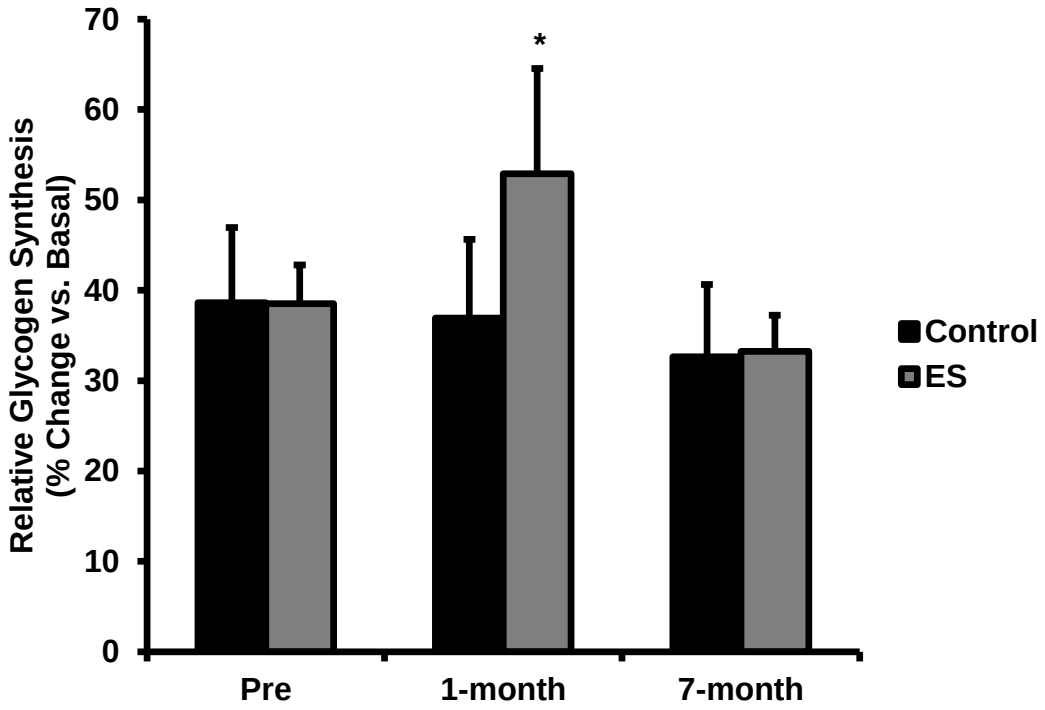

Fig 3.1: Relative insulin stimulated glycogen synthesis following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months following surgery. Data ± SEM. N = 6 / group. *, $P < 0.05$ vs. Control.

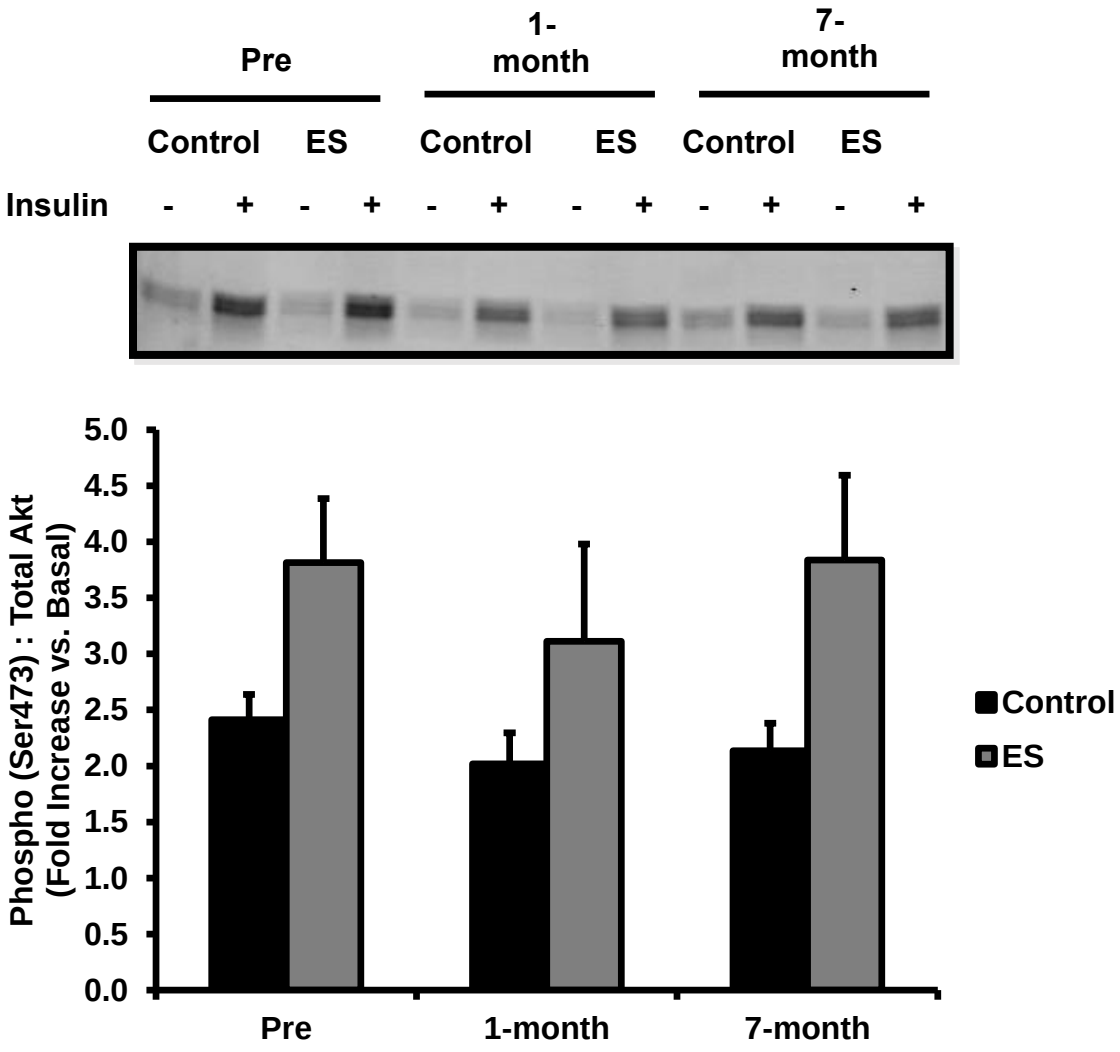

Fig 3.2.a: Insulin-stimulated phosphorylation of Akt (Ser473) following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

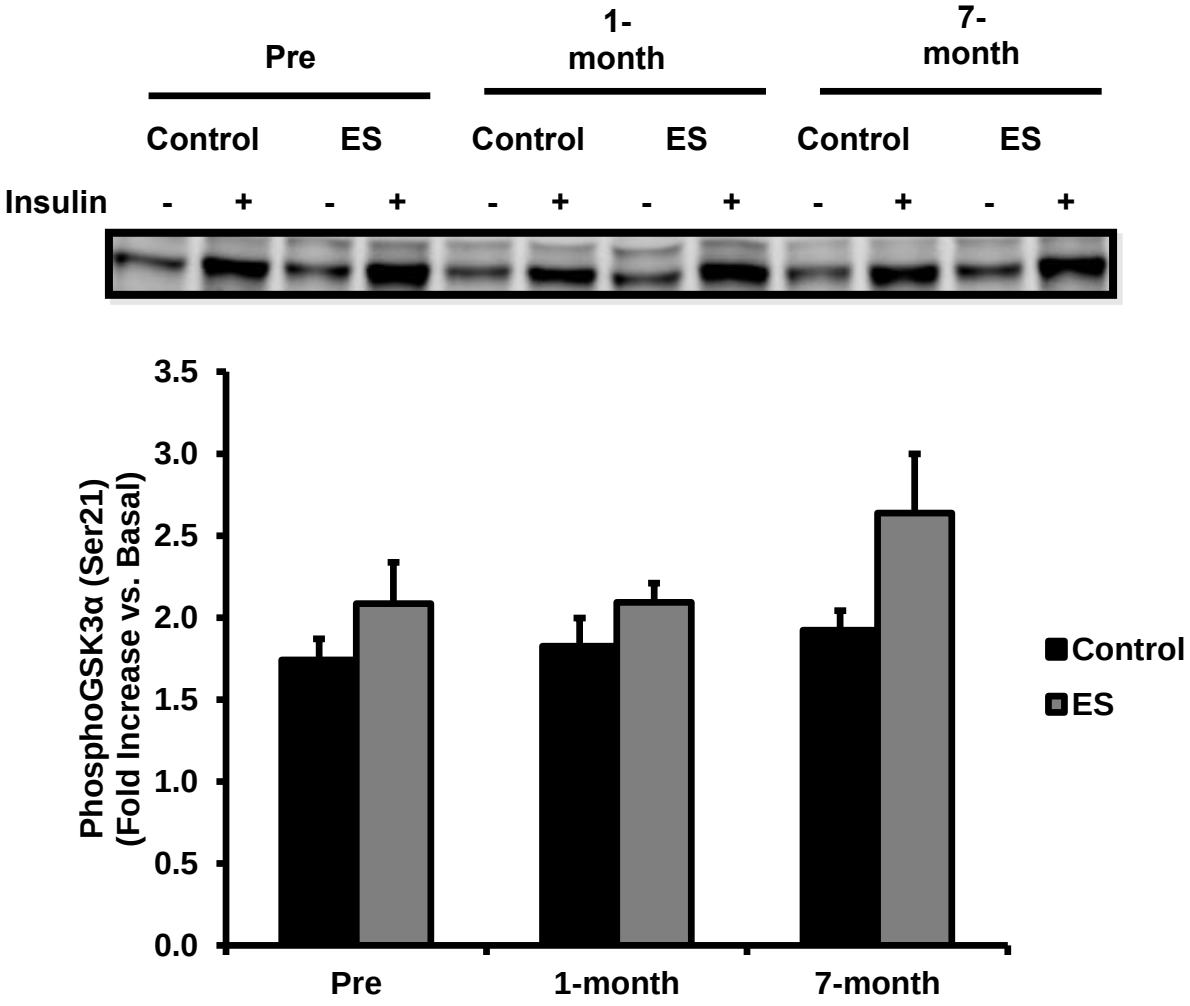

Fig 3.2.b: Insulin-stimulated phosphorylation of GSK3α (Ser21) following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

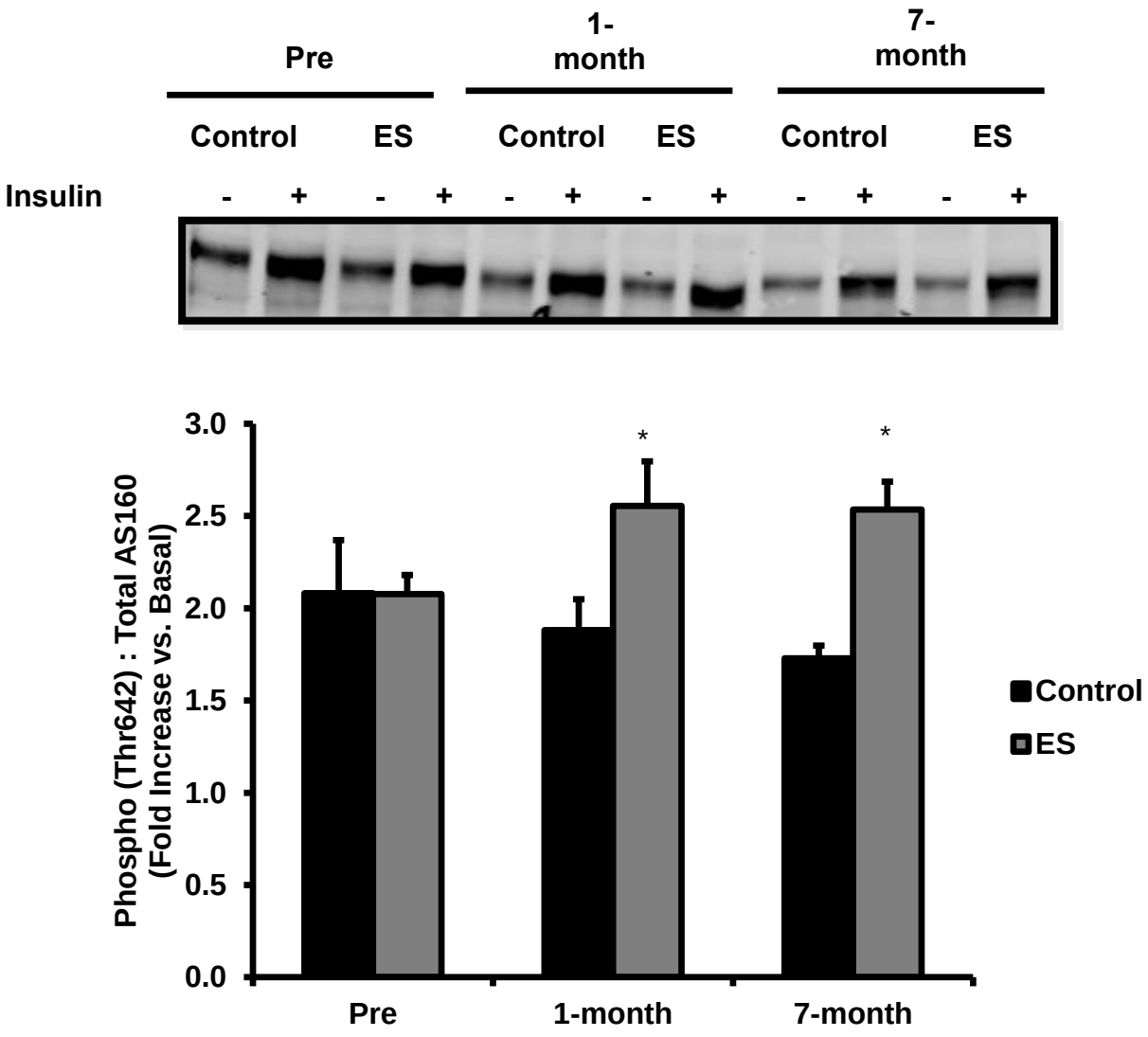

Fig 3.2.c: Insulin-stimulated phosphorylation of AS160 (Thr642) following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group. *, $P < 0.05$ vs. Control.

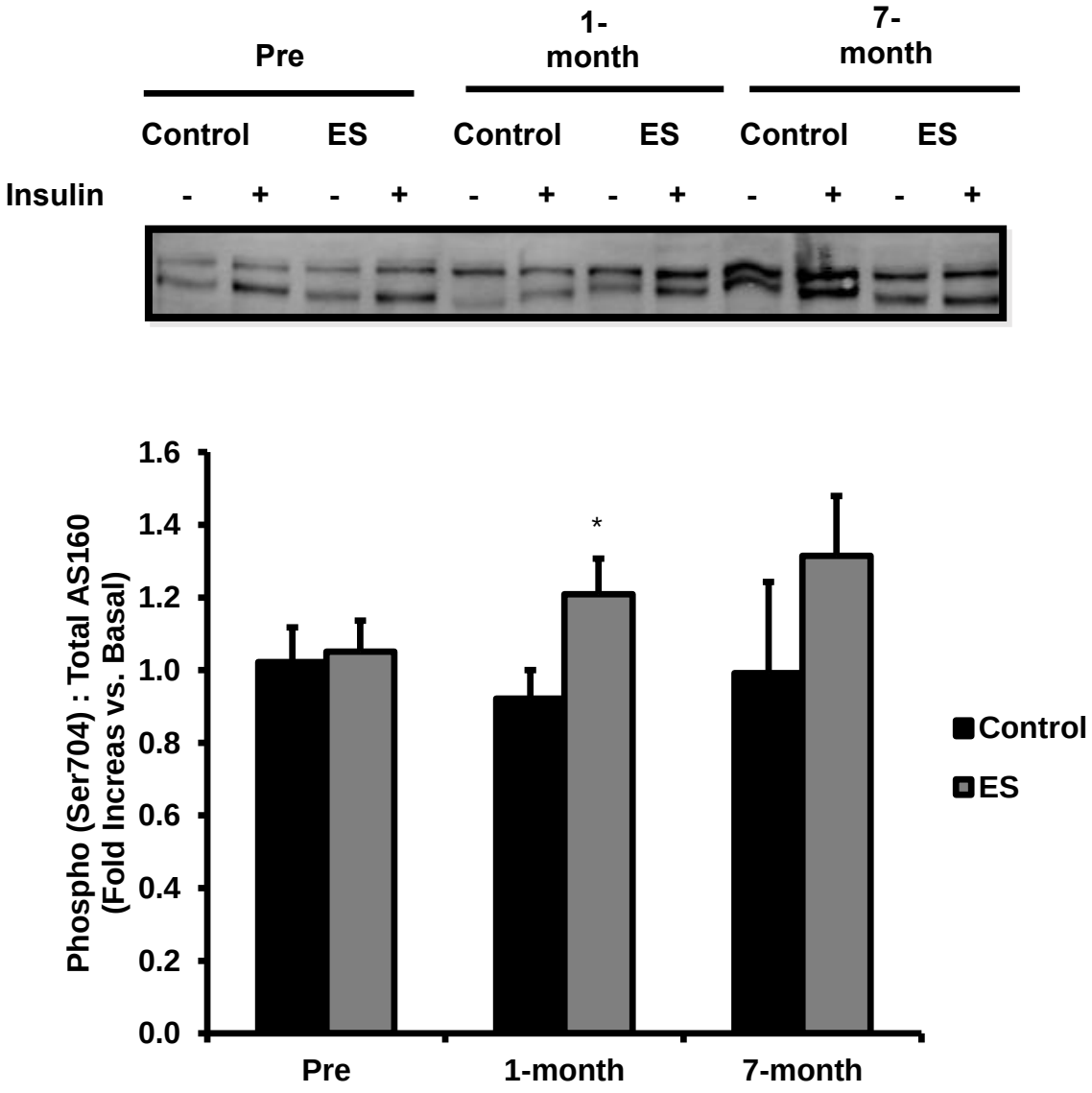

Fig 3.2.d: Insulin-stimulated phosphorylation of AS160 (Ser704) following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

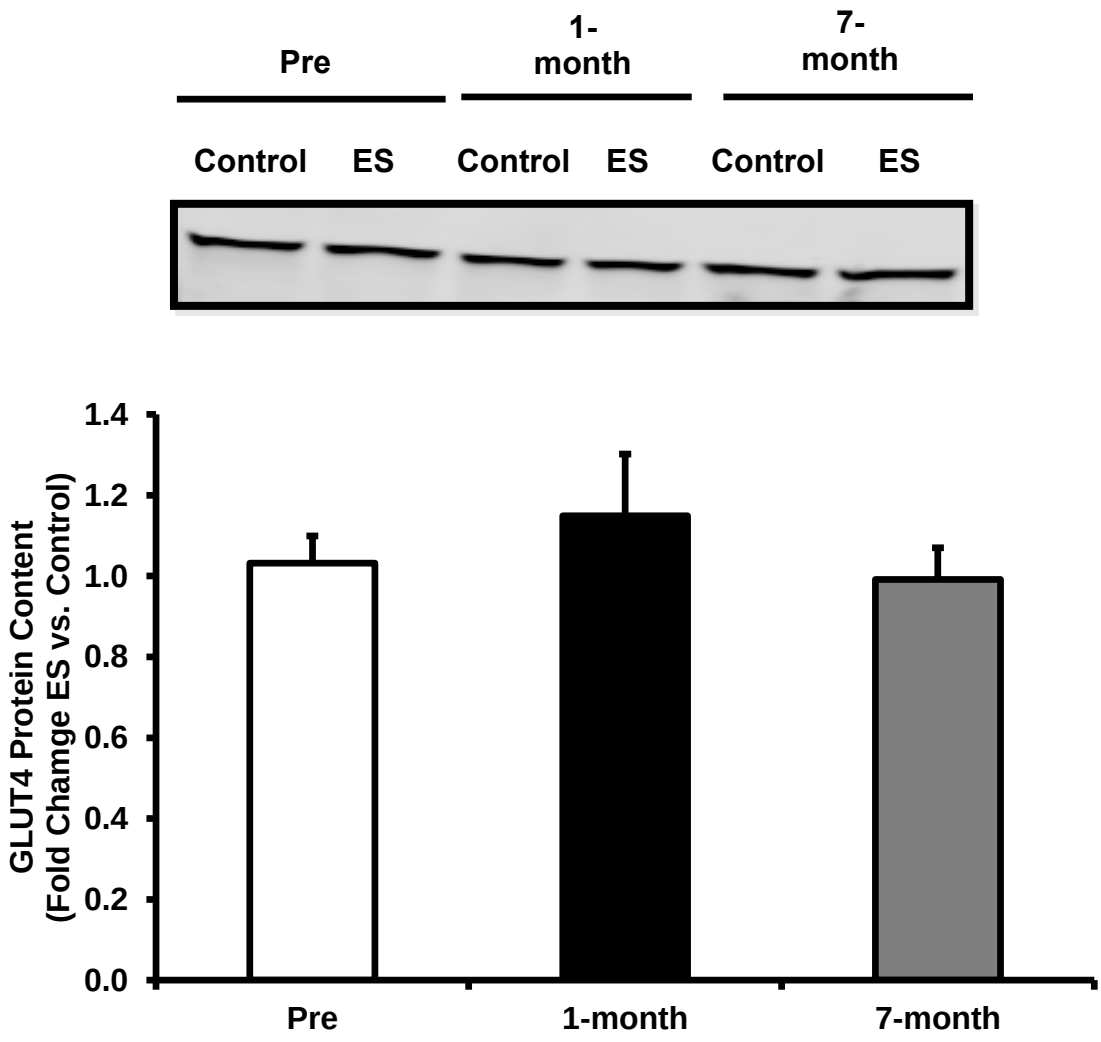

Fig 3.2.e: Protein content of GLUT4 following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

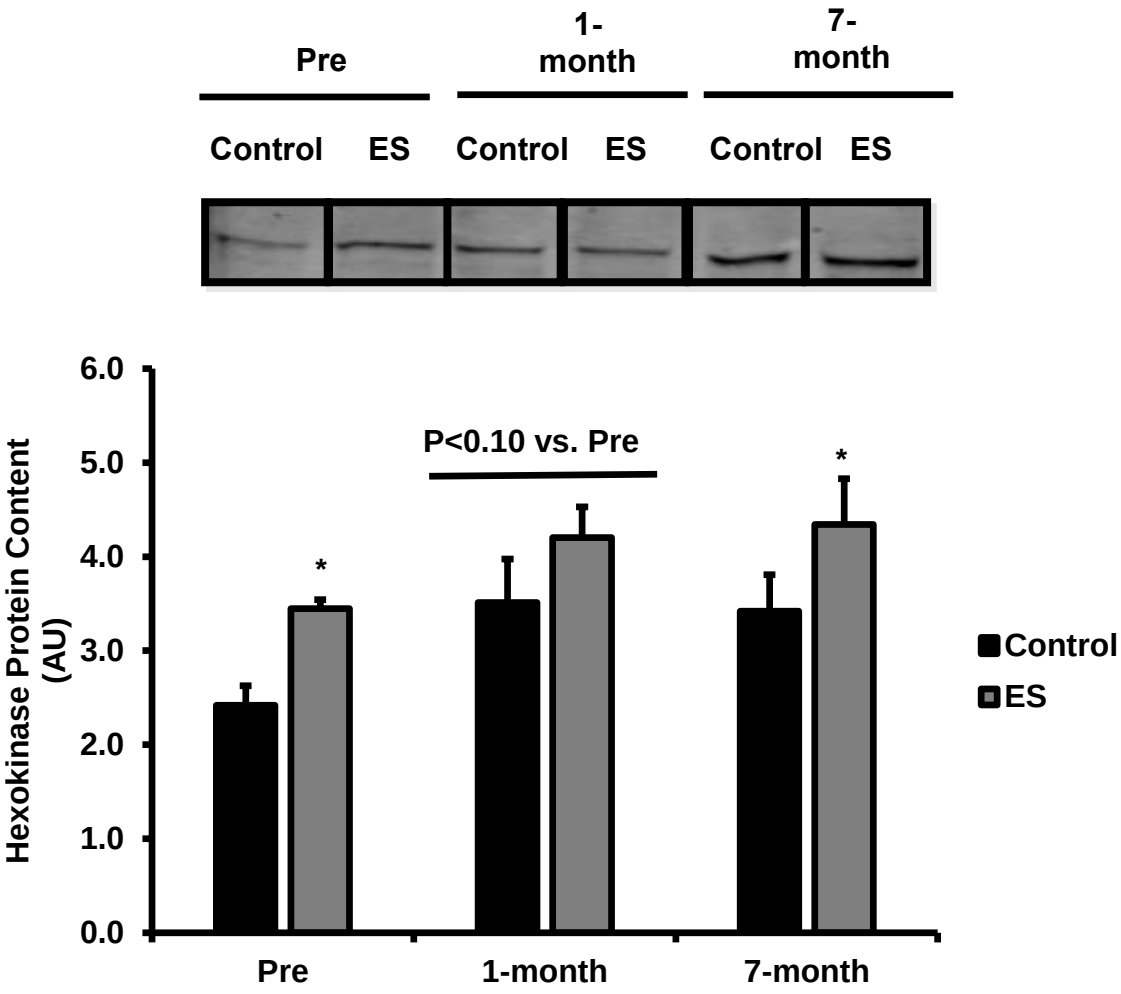

Fig 3.2.f: Protein content of hexokinase following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group. *, P < 0.0 vs. Control.

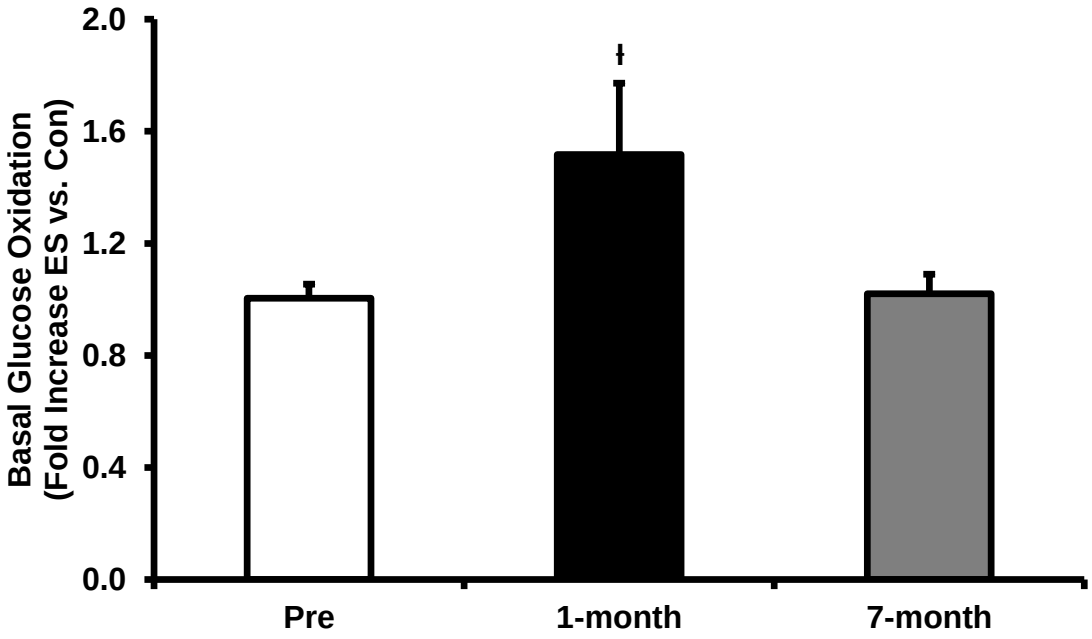

Fig 3.3: Relative changes in basal glucose oxidation following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group. ł, P < 0.10 vs. Control.

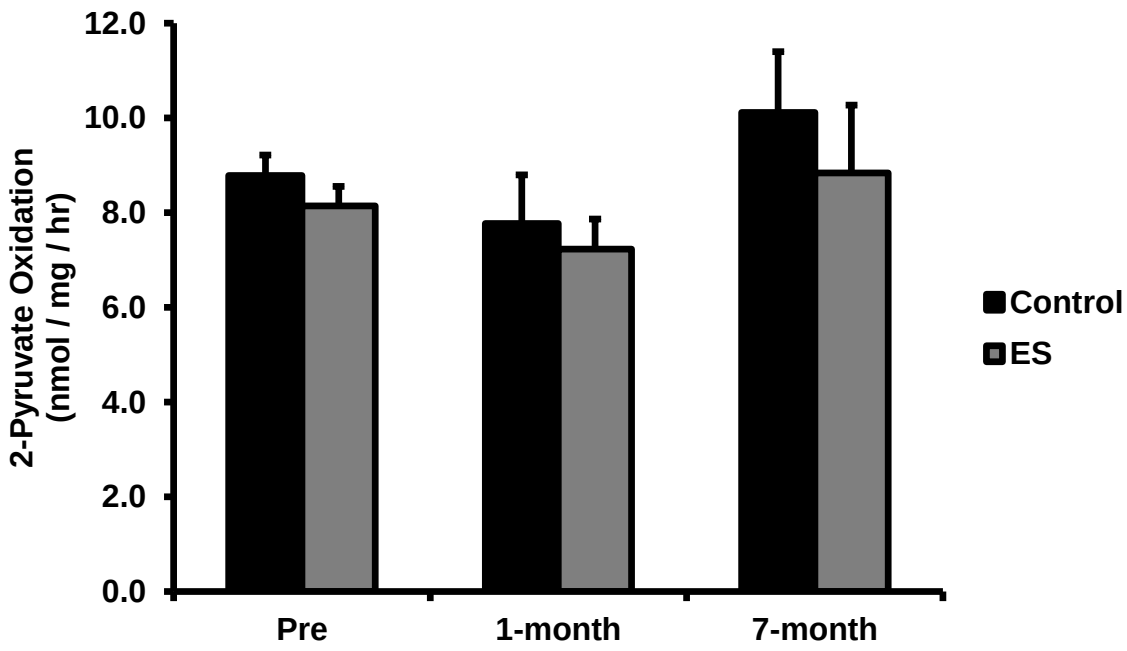

Fig 3.4: 2-pyruvate oxidation following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

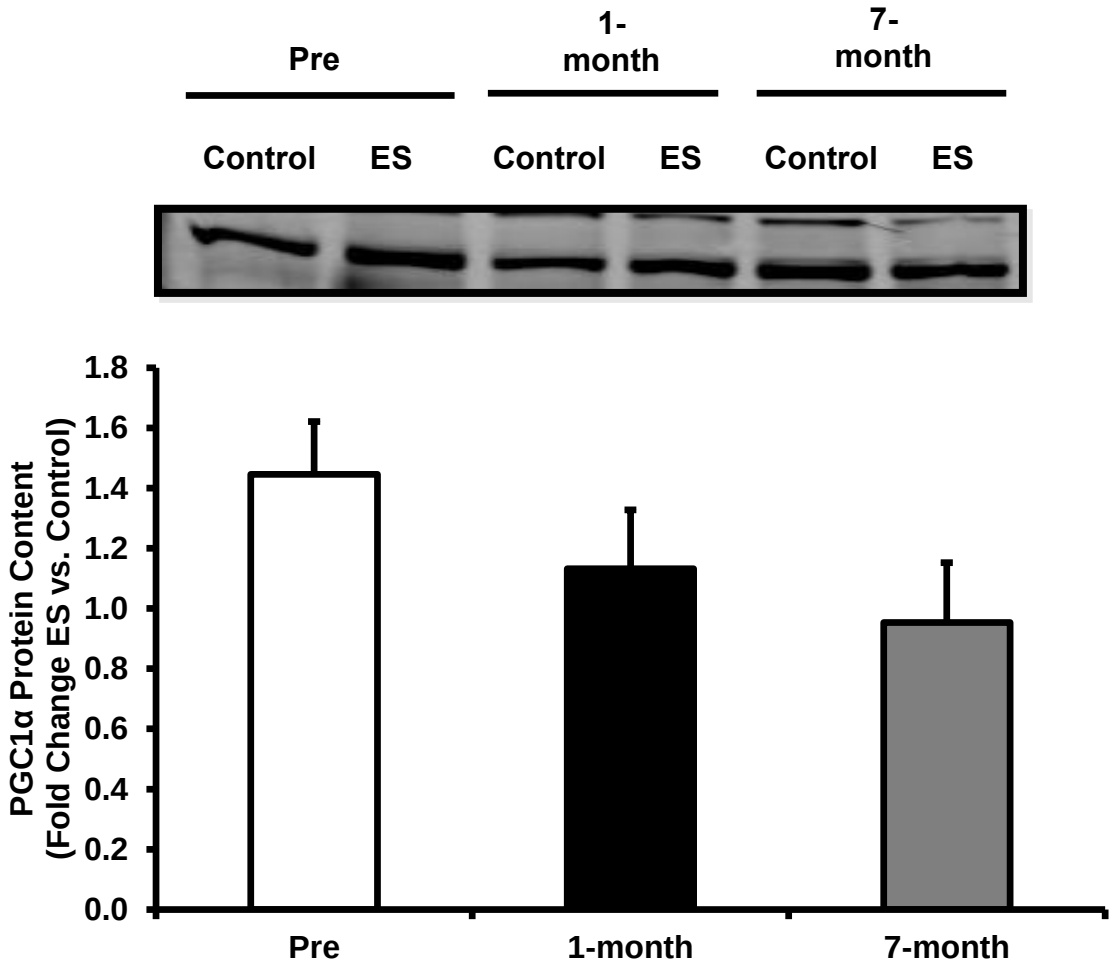

Fig 3.5.a: Protein content of PGC1α following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

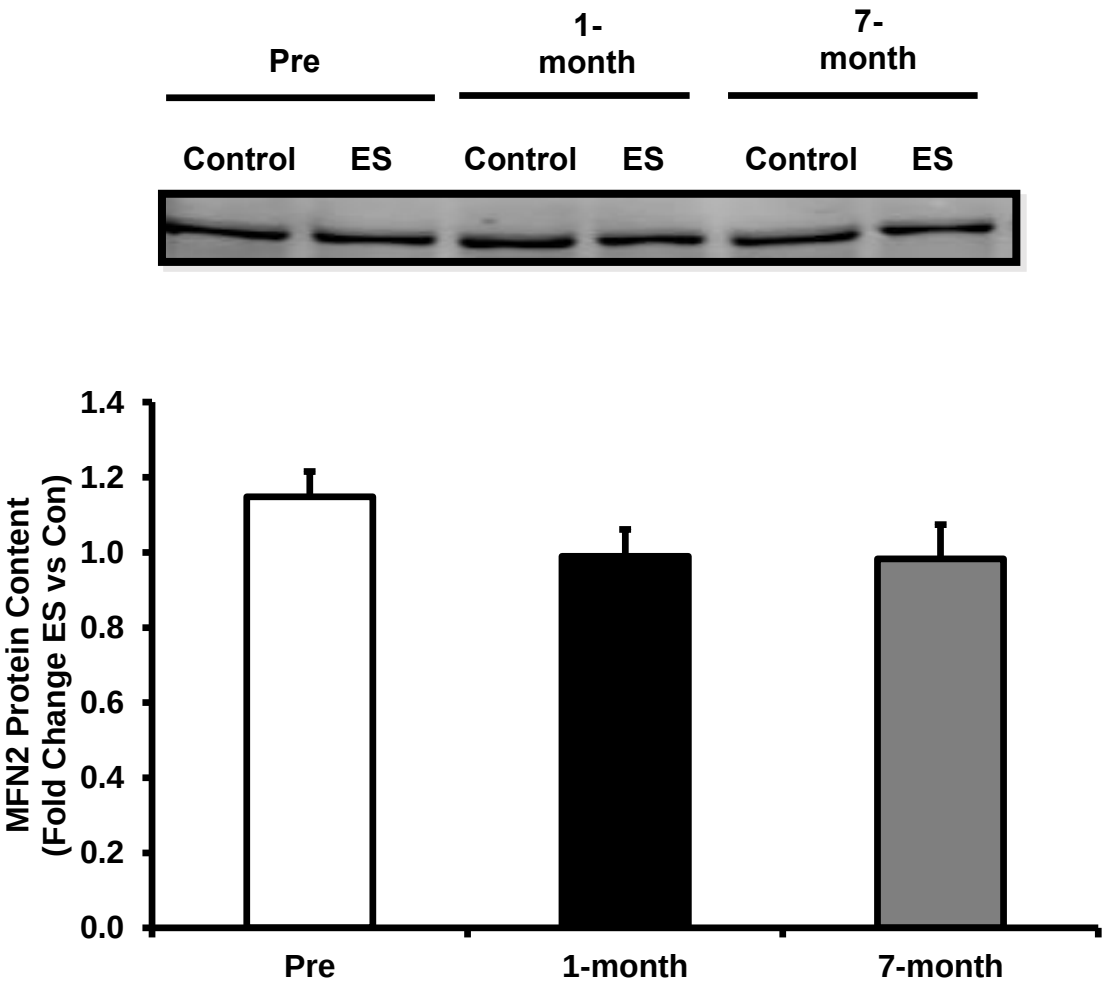

Fig 3.5.b: Protein content of MFN2 following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

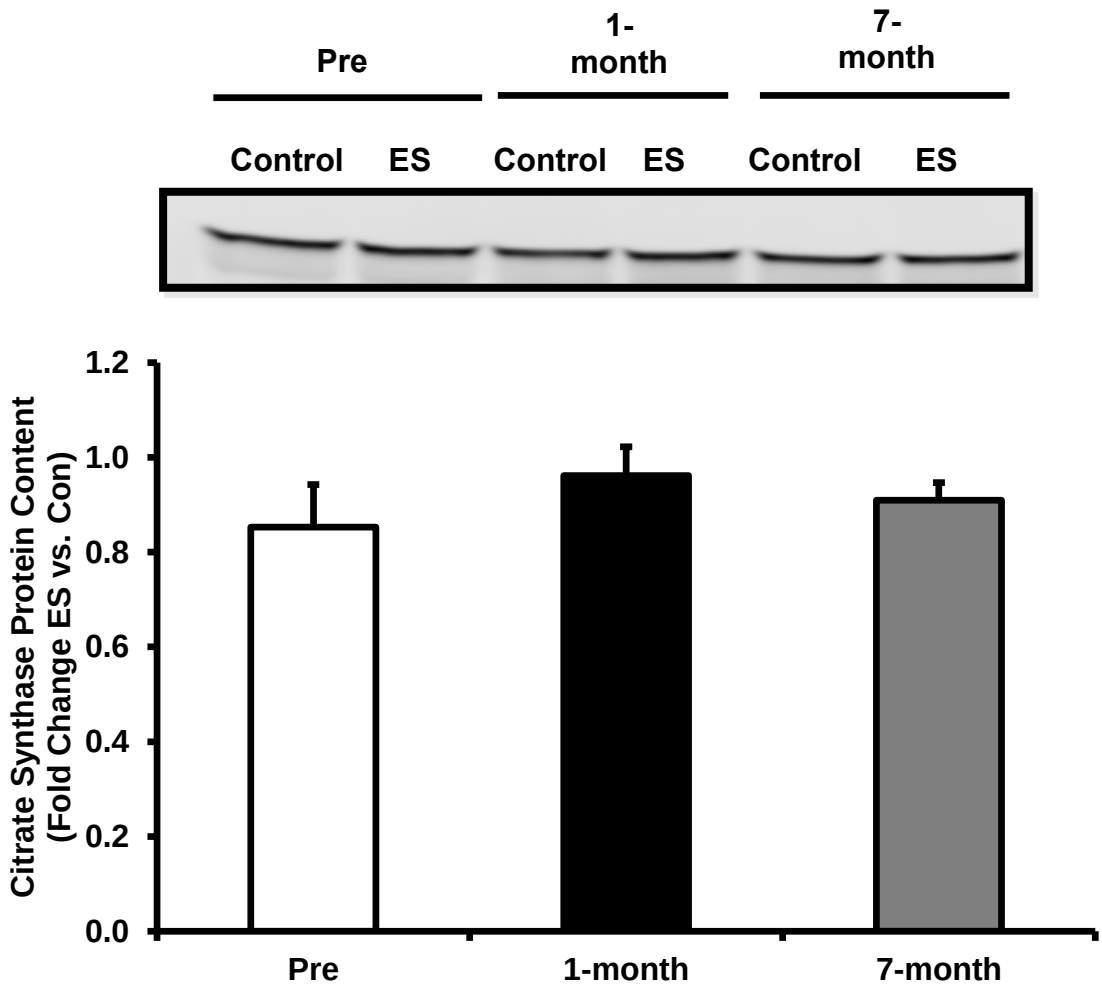

Fig 3.5.c: Protein content of citrate synthase following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

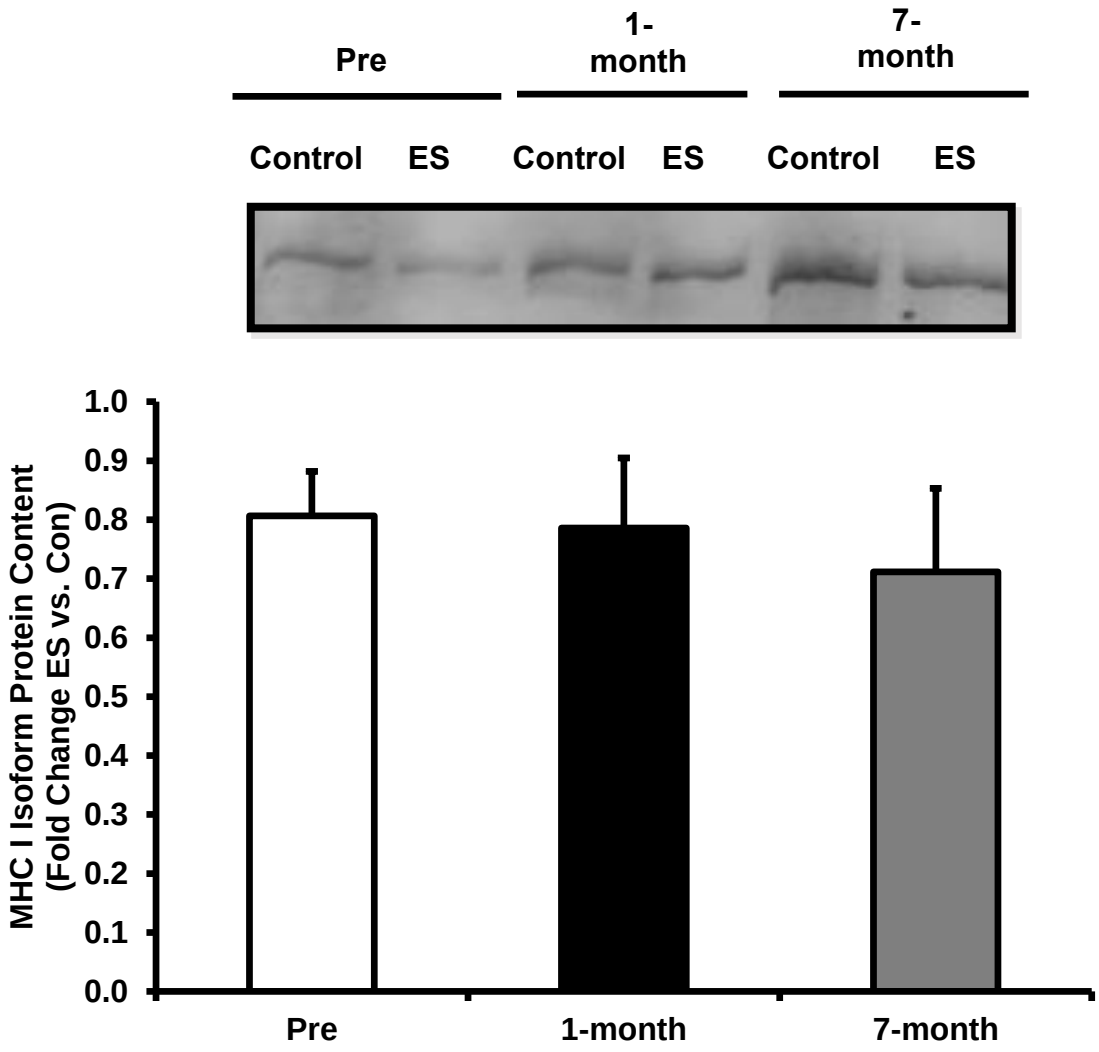

Fig 3.6: Protein content of MHC slow isoform following electrical stimulation in cells derived from RYGB patients before (Pre), 1-month, and 7-months post-surgery. Data ± SEM. N = 6 / group.

	Pre	1-month	7-month
Insulin-Stimulated Glycogen Synthesis	↔	↑	↔
Insulin-Stimulated Akt Phosphorylation (Ser473)	↔	↔	↔
Insulin-Stimulated GSK3α Phosphorylation (Ser21)	↔	↔	↔
Insulin-Stimulated AS160 Phosphorylation (Thr642)	↔	↑	↑
Insulin-Stimulated AS160 Phosphorylation (Ser704)	↔	↑	↔
GLUT4 Protein Content	↔	↔	↔
Hexokinase Protein Content	↑	↑ ($P < 0.10$)	↑
Basal Glucose Oxidation	↔	↑	↔
TCA Cycle Flux	↔	↔	↔
PGC1α Protein Content	↔	↔	↑
MFN2 Protein Content	↔	↔	↔
Citrate Synthase Protein Content	↔	↔	↔
MHC I (Slow) Isoform Protein Content	↔	↔	↔

Table 3.1: Summary of results. Data are presented as change following electrical stimulation (ES).

CHAPTER FOUR

In Vitro Metabolic Flexibility in Myotubes Derived from Patients following Roux-en-Y Gastric Bypass Surgery

ABSTRACT

The inability of the mitochondria to switch substrate preference in the face of increased supply, also known as metabolic inflexibility, is a hallmark for many metabolic disorders, including severe obesity (BMI $\geq$ 40 kg/m2) and type 2 diabetes. Interestingly, various groups have shown that the ability of skeletal muscle to switch substrate preference is retained in primary muscle cells, suggesting metabolic flexibility is an inherent characteristic of skeletal muscle. Roux-en-Y gastric bypass surgery has been shown to improve whole-body metabolic flexibility; however, it is uncertain whether this characteristic is retained in vitro. To examine this, primary human skeletal muscle cells were isolated from muscle biopsies obtained from individuals prior to, 1-month, and 7-months following RYGB and differentiated into myotubes. The ability of 24-hour treatment of myotubes with fatty acids (200 µM oleate: palmitate mixture) to increase lipid oxidation (FAO adaptability) was similar at each time point. However, a relationship between FAO adaptability and basal insulin 1-month following RYGB surgery was evident, as a greater reduction in basal insulin levels tended (P=0.14) to be associated with a greater ability to increase lipid oxidation following fatty acid treatment. The acute ability of fatty acids (600 µM oleate: palmitate mixture) or glucose (5 mM) to suppress pyruvate and lipid oxidation, respectively, was minimally altered following RYGB surgery. The results of the current

study suggest that alterations in metabolic flexibility at the whole-body level following RYGB surgery are not retained in primary myotubes.

INTRODUCTION

Mitochondria are critical organelles associated with fuel selection, as carbon intermediates from key nutrients such as lipids and glucose are directed towards mitochondria in order to be oxidized to produce energy in the form of ATP. As nutrient availability shifts throughout the course of the day, the ability of mitochondria to handle different substrate preferences at specific times is critical to maintain normal energy homeostasis. This periodical shift in substrate utilization is known as metabolic flexibility. It has been suggested that an inability for mitochondria to properly shift nutrient preference, also known as metabolic inflexibility, is a trademark for many metabolic disorders, such as obesity and type 2 diabetes (57, 95); thus examination of key tissues involved in metabolic flexibility is critical to understand the pathogenesis of these diseases.

Skeletal muscle, a metabolically active organ, encompasses ~40% of total body mass, and is considered a metabolic sink, as it has the capability to not only oxidize but also store excess substrates (i.e., lipid and glucose); therefore, targeting muscle is critical in treating whole body metabolic derangements in which systemic substrate levels are increased. Under fasting conditions, skeletal muscle relies on lipids as a fuel source to spare glucose for tissues with limited lipid oxidation capacity (i.e., brain) (95). However, after a meal in which systemic glucose and insulin levels drastically increase, muscle switches to glucose utilization to avoid hyperglycemic conditions. Using indirect calorimetry across the leg, Kelley and Mandarino have shown that during a hyperinsulinemic-euglycemic clamp, lean individuals dramatically switch from lipid oxidation to glucose oxidation; however, obese individuals show an inability to switch

substrate utilization (77). Furthermore, this metabolic derangement appears to be retained at the level of the cell, as previous reports have suggested myotubes derived from obese individuals have an inability to switch fuel utilization (27, 58, 136). These data collectively suggest that metabolic inflexibility is an inherent characteristic of skeletal muscle.

To aid in weight loss associated with severe obesity, clinical interventions, such as Roux-en-Y gastric bypass (RYGB) surgery, are commonly employed in severely obese patients (BMI $\geq$ 40 kg/m^2). Along with drastic weight loss, RYGB has been shown to improve various metabolic parameters, including a decreased incidence of diabetes (104, 107). Furthermore, it has recently been suggested that the early adaptations following RYGB consist of improved metabolic flexibility (62). While skeletal muscle is a key determinant of whole-body metabolic flexibility, it is currently uncertain whether RYGB surgery induces muscle-specific alterations in substrate switching. Thus, the purpose of this study was to use a primary human skeletal muscle cell culture model to examine if RYGB alters metabolic flexibility in vitro. To do this, muscle cells were derived from RYGB patients before, 1-month, and 7-months post-surgery, and utilized to examine chronic and acute alterations in substrate switching.

MATERIALS AND METHODS

Roux-en-Y gastric bypass and primary human muscle cell cultures.

Skeletal muscle biopsies were obtained from the vastus lateralis of severely obese (BMI $\geq$ 40 kg/m^2, N=6) female patients before, 1-month, and 7-months following RYGB using the percutaneous needle biopsy technique. A fasting venous blood sample was obtained prior to the muscle biopsy for analysis of plasma glucose and insulin. RYGB surgery, previously described in detail (107), consists of a reduction in the size of the stomach and bypassing a portion of the proximal small intestine (16). Primary skeletal muscle cells were isolated from the muscle biopsies and cultured into myoblasts as described previously (94). Myoblasts were subcultured onto 12-well type-I collagen-coated plates at densities of 40 x 10^3 cells per well, respectively. Upon reaching 80-90% confluency, differentiation to myotubes was induced by switching from growth media to differentiation media (Dulbecco's Modified Eagle's Medium supplemented with 2% horse serum, 0.3% bovine serum albumin, 0.05% fetuin, and 100 mg/ml penicillin/streptomycin). Experiments were performed on days 7-8 of differentiation, and all procedures were approved by the East Carolina University Institutional Review Board.

In Vitro Metabolic Flexibility Assays.

To examine the chronic effects of lipid treatment to increase fatty acid oxidation, cells were treated for 24-hours with 200 µM oleate:palmitate mixture, after which fatty acid oxidation was examined as previously described (42, 70, 94). Briefly, cells were incubated in a sealed plate with reaction media containing 12.5 mM HEPES, 0.5% BSA, 1 mM carnitine, 200 µM oleate, and 1 µCi/ml [1-^{14}C] oleate (Perkin-Elmer, MA) for 3-hours. After the 3-hour incubation at 37°C, reaction incubation media was transferred to

a modified 48-well microtiter plate with fabricated grooves between two adjoining wells to allow for acid-driven $^{14}CO_2$ from media to be trapped by 1M NaOH (78). Following ~60-minute incubation, an aliquot of NaOH was counted for evolved $^{14}CO_2$ by liquid scintillation. Cells were washed with PBS and solubilized in 0.05% SDS for determination of protein concentration (bicinchoninic acid assay, Pierce Biotechnology, Rockford, IL).

In a second set of cells, the ability of glucose to acutely suppress fatty acid oxidation was assessed as previously described (136). Briefly, after 90-minute treatment with glucose- and serum-starvation media, cells were incubated with media containing 12.5 mM HEPES, 0.5% BSA, 200 µM oleate, radiolabeled oleate ([1-^{14}C]oleate, 1 µCi/ml), and 1 mM carnitine, in the presence or absence of 5 mM glucose for 3-hours at 37°C. Acid-driven $^{14}CO_2$ was examined as described above.

In a third set of cells, the ability of fatty acids to suppress pyruvate oxidation was assessed essentially as described above, except the incubation media contained DMEM with no glucose supplemented with 1 mM pyruvate, radiolabeled pyruvate ([1-^{14}C]pyruvate, 0.5 µCi/ml), and either 0 or 600 µM of oleate:palmitate mixture with 1 mM carnitine.

Statistical Analysis.

Two-way ANOVA with repeated measures was used to compare differences between control and treated cells before and after (1-month and 7-month) RYGB surgery. Comparison between metabolic flexibility (% increase or decrease in fatty acid oxidation) amongst groups was performed by repeated measures ANOVA. *Post hoc* testing was performed using the Student's *t* test when appropriate. Relationship between whole-body measures and in vitro metabolic flexibility was examined by Pearson product moment

correlation. Statistical significance was defined as $P < 0.05$, and data are presented as mean ± SEM.

RESULTS

Subject characteristics.

Weight was significantly reduced 1-month following RYGB surgery (139.3 ± 6.7 vs. 120.3 ± 9.1 kg Pre vs. 1-month), with further reductions 7-months post-RYGB (100.4 ± 5.3 kg). Though there was significant weight loss, subjects were still classified as obese, as BMI was >30 kg/m^2 (43.2 ± 2.8 and 35.7 ±2.2 kg/m2 1-month and 7-months post-RYGB, respectively).

In vitro metabolic flexibility.

We first examined if the treatment of cells for 24-hours with a physiological mixture of fatty acids (200 µM palmitate: oleate mixture) would induce an increase in lipid oxidation. As shown in Fig 4.1.a, lipid oxidation was suppressed 1-month and 7-months following RYGB under lipid-treated conditions. There was an effect of fatty acid-treatment to increase lipid oxidation in each group (113% vs. 135% vs. 107% increase for Pre, 1-month, and 7-months post-RYGB, respectively); however, there were no differences between groups. When plotted as a function of metabolic flexibility (i.e., relative increase over control in lipid oxidation, Fig 4.1.b), an apparent increase in metabolic flexibility was observed 1-month following RYGB; however this was not statistically significant as there was an even distribution between subjects that improved (N=3) and those that did not (N=3). To determine whether the groups that either increased or decreased in in vitro metabolic flexibility were associated with differing whole-body metabolic adaptations post-RYGB, we examined how these two groups differed in various metabolic parameters following RYGB. There were no differences between groups in percent loss of weight or BMI 1-month post-RYGB; however, there was a trend (P=0.13) for a greater decrease in

blood insulin levels in the group that improved metabolic flexibility in comparison to those that did not. Along with this, when changes in fasting blood insulin were plotted against changes in metabolic flexibility post-RYGB, there was a trend for a negative correlation (Fig 4.2, r=-0.68, P=0.14).

As metabolic flexibility is considered an acute shift in substrate utilization, we next examined whether acute treatment of glucose or fatty acids (3-hour) could suppress lipid and glucose oxidation, respectively. Glucose significantly reduced lipid oxidation in each group (Fig 4.3.a and 4.3.b). While there were no differences 1-month post-RYGB (64% suppression for Pre and 1-month), there was a small (~3%), yet statistically significant, improvement for glucose to suppress lipid oxidation 7-months following RYGB surgery (67%). There were no changes between groups in the ability of fatty acids to suppress pyruvate oxidation (57% vs. 50% vs. 53% suppression for Pre, 1-month, and 7-months post-RYGB, respectively; Fig 4.4).

DISCUSSION

Metabolic flexibility was first discovered by Sir Philip Randle, who observed that fatty acids suppress glucose oxidation in rat skeletal muscle (111). Since this seminal work, various groups have examined the importance of substrate switching on whole-body metabolism. Metabolic inflexibility, a derangement in the ability to properly switch fuel utilization, is associated with many metabolic diseases, including obesity and type 2 diabetes (76). In order to improve derangements in whole-body metabolism associated with these diseases, new therapeutic strategies are required. Along with substantial weight loss, RYGB surgery improves various metabolic parameters, including remission of type 2 diabetes and improved insulin action. Interestingly, the remission in type 2 diabetes occurs almost immediately post-RYGB, and does not appear to be due to improved insulin action in peripheral tissues (107, 113). Recent data from Hansen et al. suggest that metabolic flexibility is improved following RYGB surgery (62). As skeletal muscle is a critical factor associated with metabolic flexibility, we utilized muscle cells derived from RYGB patients 1-month and 7-months following surgery to examine muscle-specific changes in metabolic flexibility. Despite the improvements at the whole-body level, we were unable to observe robust changes in in vitro metabolic flexibility following RYGB surgery.

The lack of change in in vitro metabolic flexibility was surprising, as it has been shown that changes in whole-body metabolism are retained at the level of the cell (24, 61, 68, 136). Ukropcova and colleagues have shown that in vitro metabolic flexibility is related to many metabolic parameters of the individual, including body mass (136). However, this study utilized subjects that were lean to slightly obese (range: 20.1-32.9

kg/m^2). The subjects in the current study were classified as severely obese prior to surgery (BMI > 40 kg/m^2), and, though they lost significant weight post-RYGB, subjects were still larger than in the study by Ukropcova (~36 kg/m^2 at 7-months post-RYGB). Severely obese individuals appear to have a metabolic program that not only differs from their lean counterparts, but also from those classified as overweight or obese (BMI between 30-39.9 kg/m^2) (68). It is possible that there is a plateau effect of metabolic flexibility once individuals attain a certain body mass. Future research should continue with the findings by Ukropcova et al. (136), and extend to individuals with severe obesity.

Upon closer examination, two groups emerged within the 1-month post-RYGB patients in their response to 24-hour lipid treatment, one (N=3) that improved, while the other (N=3) did not. While there were no differences in the amount of weight loss between each group, there was a trend (P=0.13) in the reduction in fasting insulin between groups, with the group that had improved metabolic flexibility having the greater reduction in insulin in comparison to the group that did not improve. In order to determine the role of fasting insulin on in vitro metabolic flexibility, we plotted the change in fasting insulin between before and 1-month post-RYGB to the change in in vitro metabolic flexibility. A trend for a negative correlation was evident (r=-0.68) between the change in blood insulin and in vitro metabolic flexibility 1-month following RYGB. Despite not necessarily having a role in the ability of fatty acids to increase lipid oxidation, our data are consistent to the results of Ukropcova et al., who showed a negative correlation between the ability of fatty acids to increase lipid oxidation and fasting blood insulin (136). It has been suggested that hyperinsulinemia, rather than hyperglycemia, is responsible for the development of type 2 diabetes (105). Furthermore, Reed et al. have shown that the immediate remission

of type 2 diabetes corresponded to reduced fasting insulin levels (113). Our results further support the potential role of reduced fasting insulin levels on improved metabolic function following RYGB surgery.

Glucose suppresses lipid oxidation by the reverse-Randle cycle, in which an influx of acetyl CoA derived from glycolysis invokes and increase in malonyl CoA levels, which in turn reduces lipid oxidation by suppressing CPT1, the major regulator of lipid entry into the mitochondria (118). While glucose acutely suppressed lipid oxidation in each group, there was either no change (1-month) or a small change (~3% greater ability 7-months post-RYGB). These results may be due to the model utilized in this study. At the whole-body level, glucose ingestion will invoke an increased insulin response. To avoid hyperglycemia, insulin will bind to receptors found on insulin-responsive tissues, such as skeletal muscle. Consequently, this will increase the permeability of the muscle to glucose through increased translocation of the insulin-responsive glucose transporter, GLUT4, to the cell membrane. Glucose uptake appears to be critical for acute metabolic flexibility, as Galgani et al. have shown that, when corrected for glucose disposal rates, metabolic flexibility is similar between lean and diabetics (56). In cell culture models, muscle cells differ from their in vivo counterparts. GLUT1, an exclusively membrane-bound transporter which is found in low abundance from muscle biopsies (129), is greatly enhanced in muscle cells. Al-Khalili et al. have shown that the ratio of GLUT1 to GLUT4 is 12:1 in myotubes (3), which would invoke a greater glucose entry into the cell. It is possible that the lack of change in acute metabolic flexibility following RYGB surgery is due to similar rates of glucose entry into the cell, suppressing lipid oxidation to a similar extent.

While it was a small change (~3%), there was a statistically significant improvement in the ability of glucose to suppress lipid oxidation at 7-months post-RYGB. The mechanism behind this improvement may be due to alterations in PGC1α protein content 7-months following RYGB. We have previously observed that cells derived from patients 7-months post-RYGB have a ~54% increase in PGC1α protein content (see chapter 2, Figure 2.5.a). PGC1α, a transcriptional coactivator, is critical for fuel utilization in skeletal muscle. Wende et al. have shown the important role PGC1α in fuel diverting, as PGC1α-deficient mice have a reduction in fuel diverting following an acute bout of exercise (139). This would suggest a potential role of PGC1α in metabolic flexibility, and future research should aim to focus on how PGC1α alters skeletal muscle metabolic flexibility in vitro.

While the assays used in this study have been utilized by others to examine in vitro metabolic flexibility (27, 58, 136), it is possible that our assays were not sensitive enough to determine differences in metabolic flexibility following RYGB surgery. In regards to acute changes in metabolic flexibility, we utilized concentrations of glucose (5 mM) and fatty acids (600 μM) previously described (58, 136), but is possible that these concentrations may have maximized the metabolic response of cells, masking any potential inherent differences groups. Titrations with lower concentrations of these substrates may have elicited differences between groups in regards to metabolic flexibility. Furthermore, while Ukropcova and colleagues suggest insulin did not have an effect on in vitro metabolic flexibility, our subjects were considered insulin resistant prior to surgery. As the characteristics of the donor are retained in vitro, it is possible that inherent differences in metabolic flexibility may have been distinguished if insulin was

added to the incubation media. While we believed that the assays chosen for this study were the most ideal to examine in vitro metabolic flexibility, future work should distinguish whether small manipulations in reaction buffer concentrations may elicit inherent differences between groups.

In conclusion, the results of the current study suggest that in vitro metabolic flexibility is minimally altered following RYGB surgery. Though negative as far as understanding whether muscle specifically is involved in alterations in metabolic flexibility immediately post-RYGB, there were observations that warrant further investigation. Most notably, there appears to be a relationship between the ability of cells to respond to lipid treatment and fasting blood insulin levels 1-month following surgery. Also, while small, adaptations to acutely switch between glucose and lipid oxidation may be due to changes in PGC1α protein content. Collectively, these data suggest important mechanistic roles on alterations in metabolic flexibility following RYGB surgery.

Figures

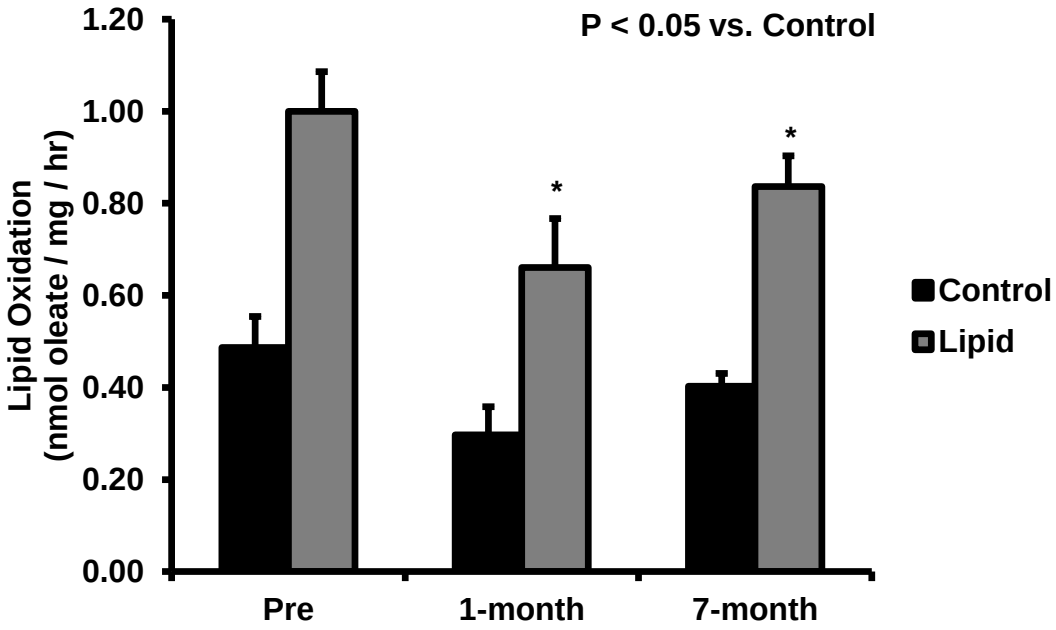

Fig 4.1.a: Ability in fatty acids to increase in vitro lipid oxidation following RYGB surgery. Data ± SEM. N = 6 / group. *, P < 0.05 vs. Pre Lipid.

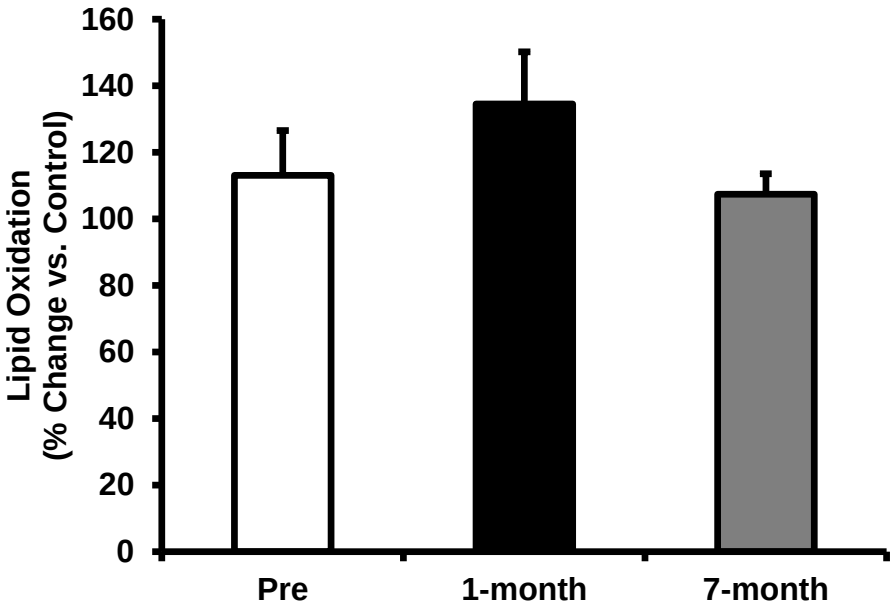

Fig 4.1.b: Relative increase in lipid oxidation following fatty acid treatment in myotubes derived from RYGB patients. Data ± SEM. N = 6 / group.

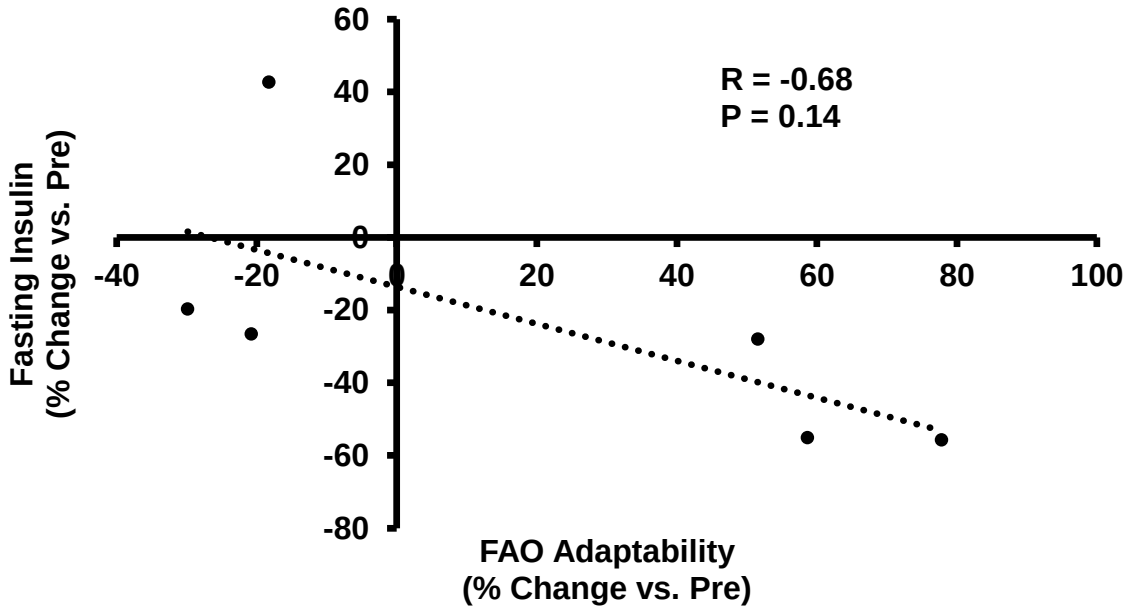

Fig 4.2: Correlation between changes in in vitro metabolic flexibility and fasting blood insulin 1-month following RYGB surgery.

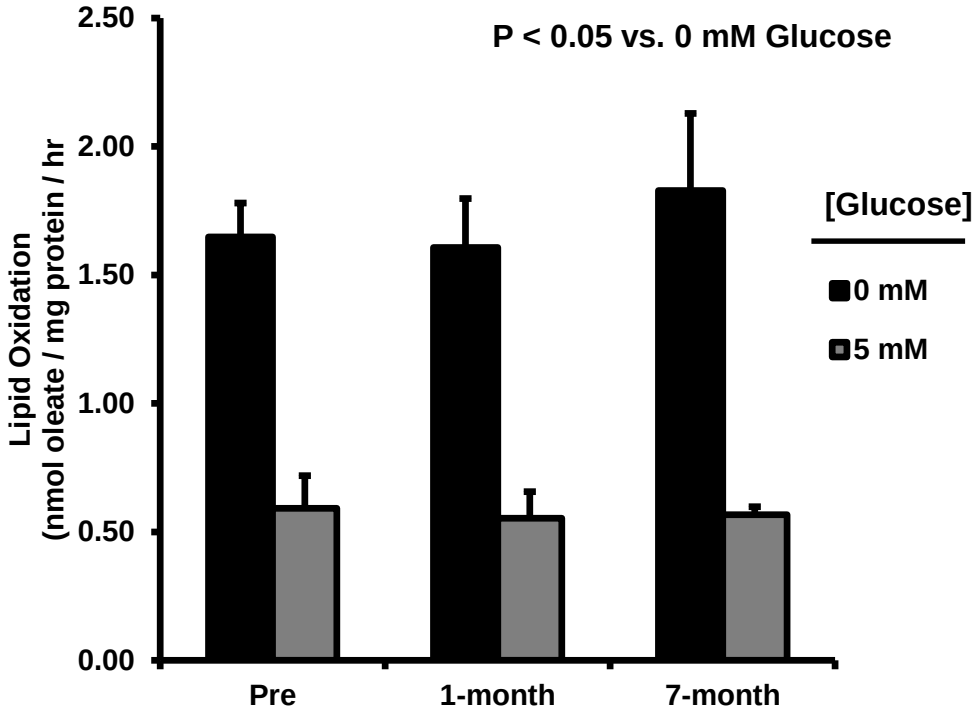

Fig 4.3.a: Acute ability of glucose to suppress in vitro lipid oxidation following RYGB surgery. Data ± SEM. N = 6 / group.

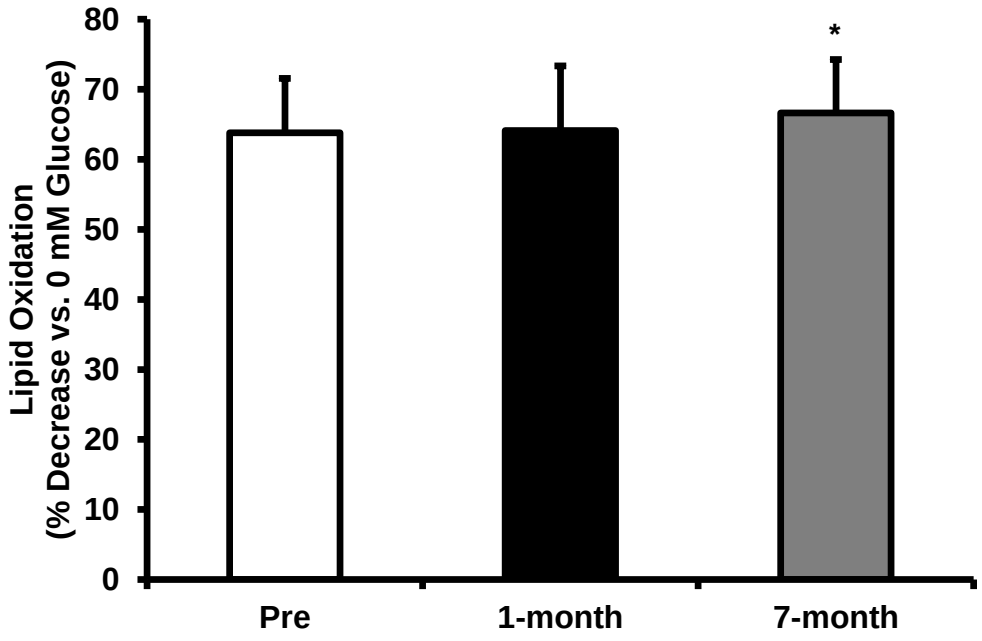

Fig 4.3.b: Acute ability of glucose (5 mM) to suppress in vitro lipid oxidation following RYGB surgery. Data ± SEM. N = 6 / group. *. P < 0.05 vs. Pre.

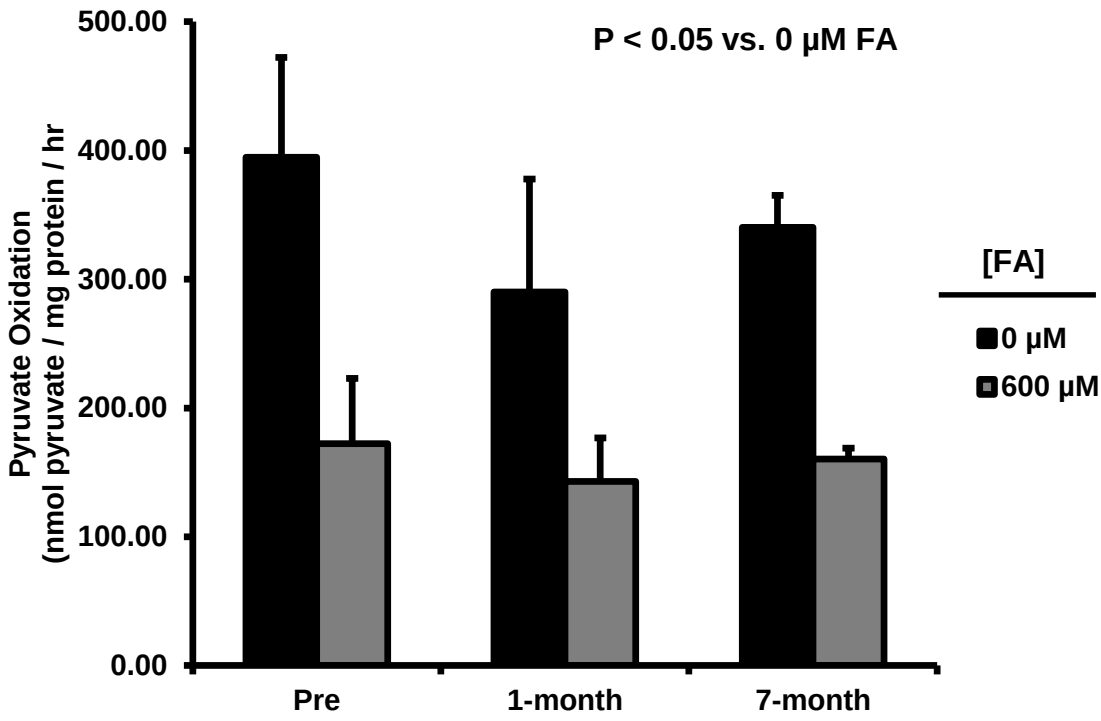

Fig 4.4: Ability of fatty acids to suppress in vitro pyruvate oxidation following RYGB surgery. Data ± SEM. N = 6 / group.

CHAPTER FIVE

Summary and Conclusions

Since 1980, the RYGB surgical procedure has been consistently used to induce weight loss in severely obese individuals that cannot lose weight with normal lifestyle interventions (i.e., exercise training) (104). Beyond substantial weight loss (> 100 lbs.), RYGB surgery induces various metabolic benefits in severely obese individuals, including improved whole-body insulin action and remission of type 2 diabetes (2, 16, 104, 107). Furthermore, recent evidence suggest that exercise training immediately following RYGB surgery (1-3 months) can elicit additional benefits in whole-body and skeletal muscle metabolism (39, 40). These data collectively suggest the importance of RYGB surgery and exercise training in improving metabolism in severely obese patients. Despite the importance in controlling whole-body metabolism, it is unclear how skeletal muscle adapts following RYGB surgery.

The purpose of this project was to i) determine whether RYGB surgery elicits muscle-specific and time-dependent changes in insulin action, ii) determine whether the combined effects of RYGB surgery and muscle contraction can elicit improvements in skeletal muscle metabolism, and iii) determine whether RYGB surgery improves skeletal muscle metabolic flexibility. To eliminate the confounding systemic factors that may impede substrate metabolism in skeletal muscle, we utilized a primary human skeletal muscle cell culture model to examine these questions.

Collectively, these studies serve to i) reveal muscle-specific changes in insulin action occur earlier than previous data has suggested, ii) understand the mechanism(s) in which RYGB induces acute and chronic metabolic adaptations in skeletal muscle, iii) further understand how exercise following RYGB can further enhance substrate metabolism in a muscle-specific manner, iv) confirm an important role of AS160 phosphorylation on contraction-mediated insulin action, and v) indicate metabolic flexibility is potentially not an inherent characteristic of skeletal muscle.

An interesting finding from this study was the rapid response in insulin action of myotubes derived from patients immediately following RYGB (1-month post). Utilizing either hyperinsulinemic-euglycemic clamps or an oral glucose tolerance test, previous studies have shown that insulin sensitivity is unaltered acutely (2, 28, 47, 113, 138). In fact, most studies suggest that improved insulin sensitivity in skeletal muscle occurs ~3-12 months following RYGB surgery once substantial weight loss has been achieved (2, 16, 104, 107). However, the acute adaptations in skeletal muscle insulin action may be masked by the systemic environment. de Weijer and colleagues observed that two weeks following RYGB surgery, there was a significant increase in lipolysis in these patients, resulting in an increase in plasma free fatty acids (138). A rise in plasma free fatty acids has been shown to induce insulin resistance in skeletal muscle due to impaired insulin signaling (18); thus any positive adaptations in skeletal muscle insulin action acutely following RYGB surgery may be impeded by these systemic factors.

RYGB surgery consists of a restriction of the stomach along with bypassing the remainder of the stomach and the proximal small intestine (104, 113), which eliminates nutrients from reaching the majority of the stomach, as well as the duodenum and part of

the jejunum (45). The lowered energetic state invoked by the surgery may lead to the acute adaptations in whole-body glycemic control, as indicated by improved hepatic insulin action and β-cell function (28, 45, 47, 113). The results from the current study confirm the importance of a lowered energetic state in improved peripheral insulin action, as we observed lower muscle glycogen levels 1-month following RYGB surgery. Similarly, during intense exercise, another intervention that lowers the energetic state of the cell, glycogen levels greatly diminish (73, 98). Several studies have shown a relationship between reduced muscle glycogen levels and insulin action post-exercise in humans (19, 20, 116). Thus, our results suggest an important role of muscle glycogen in mediating improved insulin action acutely following RYGB surgery.

It has recently been suggested that initiating an exercise program immediately following RYGB surgery may provide additional benefits in substrate metabolism in comparison to surgery alone. In a recent study from Coen and colleagues, the authors, who initiated a 6-month exercise program acutely following RYGB surgery (1-3 months post-surgery), observed improvements in insulin sensitivity and mitochondrial function following the combined intervention (39, 40). Similarly, following electrical stimulation of myotubes to contract, we observed an improvement in insulin-stimulated glycogen synthesis and basal glucose oxidation in myotubes derived from RYGB patients 1-month post-surgery. While the authors initially designed the experiments to conclude at a time point when weight loss improves peripheral insulin sensitivity (6-9 months following RYGB), their results, along with those in the current study, would suggest that in order to maximize the effects of muscle contraction on skeletal muscle metabolism, an exercise program should begin as earlier as possible post-RYGB surgery (1-3 months post).

The mechanism for improved contraction-mediated insulin action appears to be due to improved phosphorylation of AS160. Also known as TBC1D4, AS160 is a Rab-GTPase that is associated with translocation of insulin-responsive GLUT4 transporters to the cell membrane and t-tubules (119). Previous data has shown muscle contraction enhances insulin-stimulated AS160 phosphorylation at the Thr642 site up to 27-hours post-exercise (4, 55). The mechanism for enhanced insulin-stimulated phosphorylation of Thr642 site of AS160 post-contraction may be due to enhanced activity of AMPK following muscle contraction. AMPK, the energy sensor of the cell, is activated following the high energetic demands brought upon by muscle contraction. While AMPK activation itself is insufficient to phosphorylate the Thr642 site (135), data from Kjobsted et al. suggest that AMPK phosphorylates the Ser704 site of AS160 (Ser711 in mouse tissue), which allows the Thr642 site to be more accessible (80). Furthermore, Treebak et al. have also shown that the Ser704 site is also responsive to insulin, with a greater effect post-exercise (134, 135). In line with this, we saw enhanced insulin-stimulated phosphorylation of the Ser704 site following muscle contraction in cells derived from patients 1-month post-RYGB. These data suggest a potential synergistic role of phosphorylated AS160 at the Thr642 and Ser704 sites in improved insulin-stimulated glucose metabolism following muscle contraction.

Interestingly, before RYGB surgery, severely obese patients were unresponsive to contraction-mediated changes in metabolism, as indicated by a lack of change in insulin-stimulated glycogen synthesis and basal glucose oxidation. These data expand to a growing notion of exercise resistance with metabolic disorders. It has been shown that 15-20% of individuals with metabolic disorders (severely obese and type 2 diabetics) do

not improve glucose homeostasis following exercise training (127). To examine the possible mechanisms for exercise resistance, Stephens et al., using microarray analysis of skeletal muscle biopsies prior to exercise training, showed that various genes were differentially regulated between responders and non-responders, with a downregulation of several genes associated with substrate utilization and mitochondrial biogenesis (128). These data suggest that exercise resistance is associated in inherent characteristics of skeletal muscle. RYGB surgery has been shown to alter the inherent characteristics of skeletal muscle, as indicated by changes in substrate metabolism of myotubes, which indicate the genetic influence on metabolism (current study and from Nascimento et al. (97)) as well as epigenetic modifications in skeletal muscle (7). Therefore, it is possible that RYGB surgery alters the myocellular milieu which is conducive to adapt to muscle contraction.

The mechanism for improved insulin action during the later stages following RYGB (7-months post, once substantial weight loss was achieved) appear to be due to enhanced PGC1α protein content. PGC1α, a transcriptional coactivator, is a key regulator of substrate utilization in skeletal muscle. A physiological increase in PGC1α protein content (20-150%), similar to what we observed at 7-months after RYGB, can enhance glucose utilization (85). A similar physiological increase in PGC1α was also observed by Barres et al., who observed ~50% increase 6-months following RYGB (7). PGC1α has been shown to regulate genes associated with carbohydrate metabolism, most notably inducing an increase in GLUT4 expression (46). However, we did not observe an increase in GLUT4 abundance post-surgery (Fig 2e), implicating that the increase in PGC1α may affect insulin action through other mechanisms. In mice with

transgenic overexpression of PGC1α in skeletal muscle, Wende et al. observed an increase in hexokinase protein abundance, which led to an increase in glycogen synthesis (139). Similarly, we observed a trend ($P<0.10$) for an increase in hexokinase protein content following RYGB surgery. Following insulin-stimulated glucose entry into the cell, a greater amount of hexokinase would convert glucose to G6P, which is an allosteric activator for glycogen synthase. Collectively, these data would suggest a potential role of hexokinase protein content in inducing glycogen synthesis following RYGB. While PGC1α activates transcription of various genes (85), it is unclear whether the increase in hexokinase was due directly or indirectly to PGC1α activity. Future research should examine the mechanism(s) in which PGC1α can enhance hexokinase protein content.

Recently, metabolic flexibility, the ability to switch substrate utilization, has been considered a key determinant of metabolic health and insulin sensitivity (95). At the whole-body level, RYGB surgery has been shown to invoke an improvement in metabolic flexibility (62). However, in the current study, in vitro metabolic flexibility was minimally altered following RYGB surgery. Though negative as far as understanding whether muscle specifically is involved in alterations in metabolic flexibility immediately post-RYGB, there were observations that warrant further investigation. Most notably, there appears to be a relationship between the ability of cells to increase lipid oxidation after fatty acid treatment and fasting blood insulin levels 1-month following surgery. These observations are consistent to the results of Ukropcova et al., who showed a negative correlation between the ability of fatty acids to increase lipid oxidation and fasting blood insulin (136). It has been suggested that hyperinsulinemia, rather than hyperglycemia, is responsible for the development of type 2 diabetes (105). Furthermore, Reed et al. have

shown that the immediate remission of type 2 diabetes corresponded to reduced fasting insulin levels (113). Our results further support the potential role of reduced fasting insulin levels on improved metabolic function following RYGB surgery.

In conclusion, the results from this study suggest RYGB surgery alters the inherent characteristics of skeletal muscle that invoke improved insulin action and exercise-responsiveness. While previous research suggest insulin action in skeletal muscle is improved once substantial weight loss is achieved, we show that, when utilizing a muscle-specific model (e.g., human skeletal muscle cells), improvements in insulin action occur as early as 1-month following RYGB surgery. Furthermore, it appears that acute adaptations in skeletal muscle following RYGB surgery invoke a cellular environment that is more responsive to the additional benefits of muscle contraction. Collectively, the results of this study provide valuable mechanisms in which RYGB surgery and muscle contraction can improve insulin action in skeletal muscle.

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Appendix A: Institutional Review Board Approval Document

EAST CAROLINA UNIVERSITY

University & Medical Center Institutional Review Board Office 4N70
Brody Medical Sciences Building· Mail Stop 682

600 Moye Boulevard · Greenville, NC 27834

Office **2527442914**· Fax **2527442284**· **www.ecu.edu/irb**

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC:

Gabriel Dubis
Date: 10/29/2015
Re: CR00003321
UMCIRB 060080
Lipid Metabolism in Obesity, Weight Loss and Exercise (2): Muscle Cell Studies

I am pleased to inform you that at the convened meeting on 10/28/2015 of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 10/28/2015 to 10/27/2016.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document	Description
060080 HIPAA Authorization.pdf(0.01)	HIPAA Authorization
Acute Exercise Training(0.05)	Consent Forms
Advertisments(0.04)	Recruitment Documents/Scripts
AuthorizationforFutureEffect of Weight Loss.HighFat Diet(0.02)	HIPAA Authorization
Brief Protocol(0.02)	Study Protocol or Grant Application
Diabetic Ad 060080.docx(0.01)	Recruitment Documents/Scripts
ECUDesignatedHealthCareComponentsApril2014.pdf(0.01)	HIPAA Authorization
Effect of Exercise Training(0.02)	HIPAA Authorization
Effect of Exercise Training.High Fat Diet(0.02)	HIPAA Authorization

Effect of Weight Loss.High Fat Diet(0.03)	HIPAA Authorization
Full Protocol(0.02)	Study Protocol or Grant Application
Full Protocol Version 4 Track Changes.docx(0.01)	Study Protocol or Grant Application
Full Protocol Version 4.docx(0.01)	Study Protocol or Grant Application
Genetics Consent(0.03)	Consent Forms
HIPAAAuthorizationEffect of Acute Exercise Training(0.02)	HIPAA Authorization
HIPAAAuthorizationforFutureHigh Fat Diet Study(0.03)	HIPAA Authorization
HIPAAAuthorizationforFutureResearch Genetics Database(0.02)	HIPAA Authorization
HIPAAAuthorizationforFutureResearchEffect of Acute Exercise Training(0.02)	HIPAA Authorization
HIPAAAuthorizationforFutureResearchEffect of Exercise Training(0.02)	HIPAA Authorization
HIPAAAuthorizationforFutureResearchEffects of Exercise Training.High Fat Diet(0.02)	HIPAA Authorization

HIPAAAuthorizationforFutureResearchMuscle Cell Studies(0.01)	HIPAA Authorization
HIPAAAuthorizationGenetics Database(0.02)	HIPAA Authorization
HIPAAAuthorizationHigh Fat Diet Study(0.03)	HIPAA Authorization
HIPAAAuthorizationMuscle Cell Studies(0.01)	HIPAA Authorization
Lipid metabolism Ad Women 1845.docx(0.01)	Recruitment Documents/Scripts
Lipid metabolism Ad Women 2844.docx(0.02)	Recruitment Documents/Scripts
Medical History(0.01)	Data Collection Sheet
Version 5 exercise training.doc(0.03)	Consent Forms
Version 5 cell culture studies.doc(0.02)	Consent Forms
Version 5 cell culture studies.doc tracked changes(0.01)	Consent Forms
Version 5 exercise training.doc tracked changes(0.01)	Consent Forms
Version 5 HFD and exercise.doc(0.02)	Consent Forms
Version 5 HFD and exercise.doc tracked changes(0.01)	Consent Forms
Version 5 HFD and weight loss.doc(0.02)	Consent Forms
Version 5 HFD and weight loss.doc tracked changes(0.01)	Consent Forms
Version 5 HFD.doc(0.02)	Consent Forms
Version 5 HFD.doc tracked changes(0.01)	Consent Forms

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:

R. Hickner & M. Pories

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None