Abstract

Potential Mechanisms For Amelioration of Type 2 Diabetes Following Bariatric Surgery

By: Melissa Anne Reed

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Directors: G. Lynis Dohm and Timothy P. Gavin

Major Department: Department of Kinesiology

Severe obesity and Type 2 Diabetes (T2DM) are worldwide health concerns reaching epidemic proportions. Roux-en-Y gastric bypass (RYGB) has been shown to elicit a rapid and durable improvement and/or remission of T2DM in as little as one week following surgery in approximately 80% of patients, however the exact mechanism(s) responsible for this remission remain unknown. It was the central hypothesis of this project that T2DM is rapidly ameliorated following RYGB in part by increasing skeletal muscle insulin sensitivity

Study One: To test this hypothesis we examined insulin sensitivity and insulin secretion in severely obese non-diabetic and T2DM women prior to RYGB and 1 week and 3 months post-RYGB using an insulin-modified intravenous glucose tolerance test (IVGTT) with Minimal Model analysis. Insulin sensitivity and insulin secretion were measured in lean women as a control group. One week after RYGB, insulin sensitivity in T2DM doubled compared to pre-RYGB but was still impaired (S_I approximately 50% of lean controls) and insulin secretion was improved but not restored (AIRg approximately 50% of lean controls). Remarkably, fasting insulin decreased one week post-RYGB and was no different from lean controls in both the T2DM and non-diabetic groups in spite of continued elevated glucose in the T2DM patients compared to lean,

suggesting that there is no longer a relationship between fasting insulin and glucose following RYGB.

The main finding in this study was that RYGB reverses hyperinsulinemia, which is beneficial for a multitude of reasons. Hyperinsulinemia is a central factor in the cluster of disorders of the metabolic syndrome and is associated with increases in cardiovascular disease and overall all-cause mortality. Consistent with previous reports, approximately 25% of patients in Study One did not experience an improvement in their T2DM post-RYGB. The rapid reversal of hyperinsulinemia following RYGB and 25% non-resolution of T2DM led to two further questions: 1) Is it possible, pre-RYGB, to predict T2DM resolution following RYGB? and 2) Can hyperinsulinemia induce skeletal muscle insulin resistance?

Study Two: To follow-up question number one we examined the T2DM cohort from Study One to determine the remission of T2DM following RYGB. Three patients exhibited complete T2DM remission 3 months post-RYGB (defined as fasting plasma glucose (FPG) < 100mg/dL), three experienced improvement but FPG remained impaired (FPG of 100-125 mg/dL) and two patients did not experience improvement in their T2DM (FPG > 125 mg/dL). We found that pre-RYGB insulin sensitivity and duration of diabetes were significantly correlated with FPG at 3 months post-RYGB suggesting that preserving insulin sensitivity may be essential in the remission of T2DM following RYGB.

Study Three: To investigate the effects of hyperinsulinemia on skeletal muscle, primary skeletal myotubes from lean (LN) (23.0 $\pm$ 1.7 kg/m^2) insulin sensitive (HOMA-IR: 1.3 $\pm$ 0.3) and severely obese (OB) (41.4 $\pm$ 3.8 kg/m^2) insulin resistant (HOMA-IR: 5.8 $\pm$

1.1) individuals were exposed to physiological levels of hyperinsulinemia for 4 days. It was hypothesized that exposure to chronic hyperinsulinemia (5000 pM) would down-regulate insulin signaling. Following the 4-day incubation, cells were exposed to a 15 minute acute supraphysiological (100nM) insulin stimulation to determine insulin-stimulated insulin signaling. As insulin resistance is associated with mitochondrial dysfunction we also hypothesized that chronic hyperinsulinemia would decrease mitochondrial content and subsequently decrease fatty acid oxidation.

Following the 4-day hyperinsulinemic incubation insulin signaling was not down-regulated in either group as measured by Akt and AS160 phosphorylation. Rather, AS160 phosphorylation was increased in the LN, but not OB following chronic hyperinsulinema. Complete oxidation of fatty acids was significantly reduced and incomplete oxidation was significantly higher in OB compared to LN independent of insulin incubation. Mitochondrial content in myotubes was not different between the groups. These data suggest that lean insulin sensitive myocytes respond to chronic hyperinsulinemia by increasing AS160 phosphorylation. This response is not observed in cells from obese indicating that perhaps insulin sensitive myocytes exhibit a flexibility in response to hyperinsulinemia that is not present in insulin resistant myotubes suggesting that hyperinsulinemia-induced insulin resistance may not act through Akt and AS160.

Together, these studies suggest that RYGB reverses hyperinsulinemia in as little as one-week following surgery, in spite of continued insulin resistance, indicating the regulation of insulin by fasting glucose is altered following RYGB. Clinically, preserving insulin sensitivity prior to RYGB appears to be important, as patients that exhibit greater

insulin sensitivity prior to RYGB experience lower FPG post-RYGB. Additionally, cells from lean, insulin sensitive individuals were capable of responding to physiological hyperinsulinemia by increasing insulin signaling in response to an acute insulin stimulus whereas the cells from obese, insulin resistant individuals demonstrate an inability to respond to hyperinsulinemia.

Potential Mechanisms For Amelioration of Type 2 Diabetes Following Bariatric Surgery

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Melissa Anne Reed

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Potential Mechanisms For Amelioration of Type 2 Diabetes Following Bariatric Surgery

By:

Melissa Anne Reed

Approved By:

Co - Director of Dissertation

_____
G. Lynis Dohm, PhD

Co - Director of Dissertation

_____
Timothy P. Gavin, PhD

Committee Member

_____
Peter A. Farrell, PhD

Committee Member

_____
Joseph A. Houmard, PhD

Chair of the Department of Kinesiology

_____
Stacey R. Altman, JD

Dean of the College of Health
and Human Performance

_____
Glen G. Gilbert, PhD

Dean of the Graduate School

_____
Paul Gemperline, PhD

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aPKC: atypical protein kinase C

ADP: adenosine diphosphate

AIRg: acute insulin response to glucose

Akt: protein Kinase B

AMPK: 5'-adenosine monophosphate-activated protein kinase

ANOVA: analysis of variance

AS160: Akt substrate of 160 kda

ASM: acid-soluble metabolite

ATP: adenosine triphosphate

AU: arbitrary units

AUC: area under the curve

BCA: bicinchoninic acid

BMI: body mass index (kg/m^2)

BOLD: Bariatric Outcomes Longitudinal Database

BPD: biliopancreatic diversion

BPD-DS: biliopancreatic diversion with duodenal switch

BSA: bovine serum albumin

β-NADH: nicotinamide adenine dinucleotide

β-HAD: β-hydroxyacyl coenzyme A dehydrogenase

CO_2: carbon dioxide

CoA: coenzyme A

COX-IV: cytochrome C oxidase, isoform IV

CSA: citrate synthase activity

DNA: deoxyribonucleic acid

DM: human skeletal muscle differentiation media

DMEM: Dulbecco's Modified Eagle's Serum

DMSO: dimethyl sulfoxide

DTNB: 6,6'–dinitro–3,3'–dithiodibenzoic acid

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

FAO: fatty acid oxidation

FBS: fetal bovine serum

FPG: fasting plasma glucose

GLP-1: glucagon-like peptide 1

GM: human skeletal muscle growth media

GLUT-4: glucose transporter 4

HbA1c: glycosylated hemoglobin

HEPES: 4- (2-hydroethyl)-1-piperazineethenesulfonic acid

HOMA-B: homeostasis model assessment – pancreatic β cell function

HOMA-IR: homeostasis model assessment – insulin resistance

HOMA-S: homeostasis model assessment – insulin sensitivity

HSMC: human skeletal muscle cell

IMCL: intramyocellular lipid

IMTG: intramyocellular triglyceride

IR: insulin receptor

IRS-1: insulin receptor substrate 1

IVGTT: intravenous glucose tolerance test

Lean: BMI < 25 kg/m^2

MMT: mixed meal test

Morbid Obesity: BMI > 40 kg/m^2; also referred to as extreme obesity

mtDNA: mitochondrial deoxyribonucleic acid

NEFA: non-esterified fatty acid

NR: non-responder

O$_2$: Oxygen

Obesity: BMI > 29.9 kg/m^2

Overweight: BMI < 24.9 kg/m^2, BMI > 30 kg/m^2

PDK: phosphoinositide dependent kinase 1

PI3K: phosphoinositide-3-kinase

PPARγ: peroxisome proliferator-activated receptor gamma

R: responder

RYGB: Roux-en-Y gastric bypass surgery

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

S$_I$: insulin sensitivity index

SEM: standard error of the mean

T2DM: Type 2 diabetes mellitus

TZD: thiazolidinedione

CHAPTER 1: Introduction

Prevalence, pathophysiology and treatment of T2DM

The prevalence of obesity and T2DM is increasing rapidly, reaching epidemic

proportions. This is due to many factors including an overall increasing sedentary

lifestyle, caloric overconsumption, and an extended lifespan. It is estimated that in the

year 2000 approximately 150 million people worldwide suffered from T2DM and that

number is expected to increase to 221 million individuals by 2010, indicating a nearly

45% increase (Zimmet, Alberti, & Shaw, 2001). It is clear that both obesity and T2DM

are two of the greatest public health concerns in the upcoming decades.

T2DM is characterized by glucose intolerance (DeFronzo, 2004), insulin

resistance, pancreatic dysfunction and a state of chronic inflammation (Wellen &

Hotamisligil, 2005). T2DM is a multi organ and system disorder and is the most

common cause of renal disease, blindness and limb amputations. In addition, the

diagnosis of T2DM significantly increases the risk for cardiovascular disease and

coronary artery disease. T2DM is responsible for significant health care costs as well.

An estimated $153 billion annually are spent treating T2DM and related complications

(International Diabetes Federation, 2007).

There are a number of treatment options available for T2DM such as lifestyle

modifications involving changing dietary patterns and increasing physical activity as it

has been shown that decreasing caloric intake and increasing regular exercise induces

an increase in insulin sensitivity (Dixon et al., 2008). If these conventional mechanisms

are unable to maintain euglycemia, pharmaceutical therapy and eventually insulin may

be necessary to add to the treatment regimen to restore glucose homeostasis. Common

pharmaceuticals employed to treat T2DM and in turn increase insulin sensitivity are thiazolidinedione's (TZD's) that act on increasing the activity of peroxisome-proliferating activator receptor gamma (PPARγ) (Jay & Ren, 2007) (Towler & Hardie, 2007) and metformin which activates AMP-associated protein kinase (AMPK) and stimulates fatty acid transport into the mitochondria for subsequent oxidation (Ferre, Azzout-Marniche, & Foufelle, 2003; McGarry, 2002).

Metformin also increases insulin sensitivity by reducing hepatic glucose output and increasing peripheral skeletal muscle glucose uptake (Hundal & Inzucchi, 2003). Additionally, recent research suggests that metformin may act in reducing whole body inflammation as measured by C-Reactive protein (CRP), vascular cell adhesion molecule -1 (VCAM–1) and macrophage migration inhibitory factor (Dandona et al., 2004; Morin-Papunen et al., 2003). This makes metformin an attractive therapy for obese T2DM patients as they commonly experience impaired insulin signaling and decreased fatty acid oxidation.

Surgical Treatment for T2DM.

Roux-en-Y gastric bypass (RYGB) appears to be the most successful treatment for T2DM as it has been proven to provide rapid and durable resolution of T2DM (Pories et al., 1995). RYGB, developed for the treatment of morbid obesity, involves gastric restriction, bypassing of the proximal small intestine and rerouting of nutrients to the distal small intestine (Figure 1). One of the surprising benefits of RYGB, shown as early as 1984, is the durable remission of T2DM (Flickinger et al., 1984). The exact mechanisms of this reversal remain largely unknown. The main theories of T2DM reversal following RYGB are substantial weight loss, caloric restriction and/or bypassing

of the proximal small intestine. Studies have shown that T2DM is resolved in patients as early as days after surgery when significant weight loss has not occurred (Pories et al., 1995). In addition, it has been documented that caloric restriction does not result in similar improvements in glucose homeostasis as RYGB when comparing patients one-month post RYGB with a cohort that experienced calorically induced, but similar weight loss (Laferrere et al., 2008). This supports the hypothesis that weight loss alone may not be responsible for the reversal of T2DM and that bypass of the proximal small intestine during RYGB may contribute to the reversal of T2DM.

Figure 1. Roux-en-Y gastric bypass (RYGBP) includes the creation of a small gastric pouch while the jejunum is divided 30–50 cm distal to the ligament of Treitz. The distal limb of the jejunum is then anastomosed to the small gastric pouch and a jejuno–jejunostomy is performed 50 to 150 cm

Insulin sensitivity and bariatric surgery.

Skeletal muscle is responsible for approximately 70-80% of glucose disposal and is also the primary site for peripheral insulin resistance (Shulman et al., 1990). A reduction in skeletal muscle insulin sensitivity and an increase in insulin resistance are hallmark defects that occurs in T2DM (Corcoran, Lamon-Fava, & Fielding, 2007). Similarly, a reduction in skeletal muscle insulin sensitivity appears to be a leading factor in the development of T2DM (Petersen & Shulman, 2006).

Skeletal muscle insulin signaling begins with the binding of insulin and subsequent autophosphorylation of the insulin receptor (IR) and cascade of events that culminates with the translocation of glucose transporter 4 (GLUT4), which is responsible

for glucose transport into skeletal muscle cells (Figure 2). Once the insulin receptor is activated, tyrosine residues on insulin receptor substrate (IRS) are phosphorylated and phosphoinositide-3-kinase (PI3K) is activated which then stimulates phosphoinositide dependent kinase 1 (PDK1). PDK1 subsequently activates atypical protein kinase C (aPKC) and Akt (protein kinase B). Activation of aPKC then promotes both translocation of GLUT4 to the cell membrane and akt phosphorylation. Akt phosphorylation activates akt
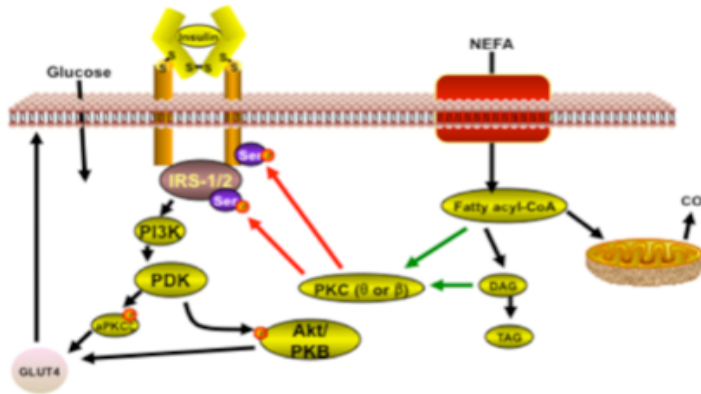
Figure 2. Insulin signaling cascade, involvement of NEFA's, DAG's and Fatty acyl-CoA's and subsequent activation of PKC's (adapted from Dohm)

substrate 160 (AS160), which also promotes GLUT4 translocation.

Insulin stimulated glucose transport is decreased in patients with T2DM (Dohm et al., 1988). There are numerous studies that confirm both decreased tyrosine kinase activity on the insulin receptor in obese and T2DM skeletal muscle (Caro et al., 1987; Itani, Zhou, Pories, MacDonald, & Dohm, 2000) and increased serine phosphorylation of IRS-1-Ser[312] which interferes with and down-regulates the insulin signaling cascade (Dohm et al., 1988; Zhou, Dolan, & Dohm, 1999).

T2DM is rapidly reversed following bariatric surgery (Pories et al., 1995) and skeletal muscle insulin sensitivity increases significantly as well (Friedman et al., 1992). More recently, there have been conflicting reports on the recovery of insulin sensitivity following RYGB with studies finding increases within weeks following surgery

(Wickremesekera, Miller, Naotunne, Knowles, & Stubbs, 2005) and others not finding improvements in insulin sensitivity until significant weight loss occurs (Campos et al., 2010). The homeostasis model assessment of insulin resistance (HOMA), which is a surrogate indicator of insulin sensitivity, primarily hepatic insulin sensitivity because it is calculated from fasting glucose and insulin values improves dramatically within the first month following RYGB indicating that insulin resistance is improved long before significant weight changes (Ferrannini & Mingrone, 2009; Wickremesekera et al., 2005).

It is possible that there are two or more distinct mechanisms that contribute to the reversal of T2DM following RYGB. Many studies have reported improvements in HOMA following RYGB (Ferrannini & Mingrone, 2009; Lima et al.; Wickremesekera et al., 2005) and this is likely due to decreased hepatic glucose output and decreased fasting insulin levels following RYGB (Bikman et al., 2008; Friedman et al., 1992).

Insulin Secretion and bariatric surgery

As T2DM progresses there is a decrease of β-cell sensitivity to glucose which appears to be independent of insulin resistance (Ferrannini et al., 2005). While the restoration of insulin sensitivity following bariatric surgery is widely accepted, less is known regarding the response of insulin secretion to bariatric surgery. Though severe obesity is associated with insulin resistance, it appears that β cell function may be preserved (Camastra et al., 2005). Long term results from studies indicate that first phase insulin secretion (in response to an IVGTT) one year post-surgery is improved (Polyzogopoulou, Kalfarentzos, Vagenakis, & Alexandrides, 2003) and that by two years post-surgery, β cell glucose sensitivity recovers (Camastra et al., 2005). There is little

data available regarding the response of insulin secretion shortly following bariatric surgery.

One mechanism by which insulin secretion is proposed to improve following bariatric surgery is through increases in glucagon-like peptide 1 (GLP1) post-surgery. GLP-1 is an incretin hormone secreted by L cells throughout the gut although predominantly in the distal small bowel, in response to nutrient delivery (Theodorakis et al., 2006). GLP-1 accounts for approximately 50% of nutrient stimulated insulin production (Preitner et al., 2004) and it acts to both increase β cells mass (Stoffers et al., 2000) and prevent β cell apoptosis (Li et al., 2003). GLP-1 response is impaired in both obesity and T2DM (Muscelli et al., 2008) but improves dramatically in T2DM patients following bariatric surgery (Laferrere et al., 2008). Potential mechanisms for the post-surgery increase in GLP-1 include the exclusion of nutrients from the foregut, often termed the "foregut hypothesis" (Rubino & Marescaux, 2004) or a rapid delivery of nutrients to the L cells in the distal small bowel, referred to as the "hindgut" hypothesis (Strader et al., 2005).

Lipid accumulation and duration of diabetes in skeletal muscle insulin resistance.

A potential mechanism contributing to skeletal muscle insulin resistance in obese and T2DM individuals is an increase in fatty acids and fatty acid intermediates. Studies have confirmed that skeletal muscles from obese individuals display a high concentration of intramyocellular lipid accumulation (IMCL) (Malenfant et al., 2001) yet still tend to utilize glucose as the preferred substrate for ATP production (Kelley, Goodpaster, Wing, & Simoneau, 1999). The accumulation of skeletal muscle lipids is closely associated with defects in skeletal muscle insulin signaling (McGarry, 2002).

Insulin resistance in skeletal muscle is associated with both reduced lipid oxidation and also increased lipid accumulation in patients with T2DM (Kelley & Simoneau, 1994). Additionally, lipid accumulation in skeletal muscle and insulin resistance are inversely correlated (Pan et al., 1997). It has been shown that reduced lipid oxidation in the skeletal muscle of T2DM patients is carried over into cell culture such that reduced skeletal muscle lipid oxidation present in diabetics in vivo is retained in human skeletal muscle myotubes from T2DM patients (Gaster, Rustan, Aas, & Beck-Nielsen, 2004). This indicates that skeletal muscle myotubes from T2DM patients may have inherited defects in fatty acid metabolism.

Studies have also shown that there are defects in fatty acid metabolism (Simoneau & Kelley, 1997) and decreased mitochondrial fatty acid oxidation (He, Watkins, & Kelley, 2001) in skeletal muscle of insulin resistant and T2DM individuals. Increased lipid accumulation and reduced fatty acid oxidation may result in increased fatty acid availability in the skeletal muscle of T2DM patients. One of the consequences of increased fatty acid availability is the increase in intracellular intermediates such as LCFA-CoA's, DAG's and ceramides which can potentially result in increased skeletal muscle insulin resistance and decreased glucose metabolism (Kraegen & Cooney, 2008).

Skeletal Muscle Mitochondria and Insulin Resistance

The role of mitochondria in insulin resistance is quite controversial. Skeletal muscle mitochondria are the site for fatty acid oxidation. Decreased fatty acid oxidation coupled with increased glycolytic activity have been linked to insulin resistance (Simoneau & Kelley, 1997) in obese individuals. Also, decreased mitochondrial volume

has been associated with insulin resistance as well (Kelley, He, Menshikova, & Ritov, 2002). The skeletal muscle of obese individuals has been shown to exhibit decreased oxidative enzyme capacity and smaller mitochondria in addition to reduced electron transport when compared with their lean counterparts (Kelley et al., 2002; Simoneau, Veerkamp, Turcotte, & Kelley, 1999). However, it remains unclear if these differences between lean and obese are due to mitochondrial dysfunction, a decrease in mitochondrial content, or both, in obese compared to lean individuals.

Additionally, there are studies that have shown situations where mitochondrial function and content are not related to insulin sensitivity, particularly with regards to T2DM individuals. Recently, a large clinical trial demonstrated that mitochondrial capacity is not related to insulin action in T2DM (Bajpeyi et al., 2011) and other work has shown that in rats, the implementation of a high fat diet induced insulin resistance but also increased mitochondria in skeletal muscle (Hancock et al., 2008). Further, a report concluded that animals that suffer from mitochondrial dysfunction and reduced oxidative phosphorylation do not exhibit insulin resistance (Pospisilik et al., 2007).

RYGB and T2DM Remission

Little is known regarding defects that may occur in insulin signaling pathways and substrate metabolism as diabetes progresses. As T2DM progresses it gets increasingly more difficult to treat and this appears to be consistent in surgical situations as well (Buchwald et al., 2004; Buchwald et al., 2009; Schauer et al., 2003). We have found following RYGB that there are some patients that do not experience diabetes resolution, which is consistent with other reports that approximately 15-25% of T2DM RYGB patients do not have diabetes resolution (Buchwald et al., 2004; Buchwald et al., 2009).

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One of the main factors in this lack of remission appears to be duration of diabetes (Renard, 2009). Schauer, et. al (2003) found that 95% of patients who had T2DM for less than 5 years experienced complete reversal following RYGB while only 54% of those patients that had the disease for greater than 10 years experienced complete reversal of diabetes (Schauer et al., 2003). This is agreement with other studies where the duration of diabetes was inversely correlated with diabetes resolution (Smith, Hinojosa, Reavis, & Nguyen, 2008).

Central Hypothesis

It is the main hypothesis of this proposal that bypass of the proximal small intestine significantly contributes to rapid T2DM resolution in response to RYGB in part through increases in skeletal muscle insulin sensitivity. We will also investigate if there are any pre-RYGB factors that can predict post-RYGB remission and lastly if chronic hyperinsulinemia decreases insulin signaling, mitochondrial content and fatty acid oxidation in lean insulin sensitive individuals and obese insulin resistant individuals. In order to examine these hypotheses we have developed the following Specific Aims. Specific Aim 1: To investigate the role of proximal small intestine bypass and weight loss in insulin sensitivity improvements following gastric bypass surgery in T2DM. It has been documented that RYGB performed in morbidly obese patients results in a reversal in T2DM in approximately 75-85% of cases. This reversal occurs before significant weight loss suggesting that proximal small intestine bypass may contribute to this reversal. In humans, we investigated the effects of proximal small intestine bypass and weight loss following gastric bypass surgery on insulin sensitivity by the

following: 1) Determining if insulin sensitivity is improved 1 week following gastric bypass when weight loss is minimal and 2) Determining if insulin sensitivity is improved to a greater extent 3 months following gastric bypass when weight loss is significant. As a follow-up to this study, specific aim 2 was developed which was To determine if pre-RYGB insulin sensitivity and/or insulin secretion is associated with post-RYGB T2DM remission. Little is known regarding the approximately 20% of type 2 diabetic patients that do not experience T2DM improvement following RYGB. Some factors that consistently appear to affect T2DM remission following RYGB include duration and severity of the disease. In addition, reports have indicated that glycosylated hemoglobin (HbA1c), fasting plasma glucose and body mass index (BMI) may also contribute to outcomes of T2DM remissions following RYGB. We investigated the effects of pre-RYGB insulin sensitivity and insulin secretion on post-RYGB T2DM remission in an attempt to determine if either insulin sensitivity and/or insulin secretion can predict post-RYGB T2DM remission. Lastly, in specific aim 3 we investigated if chronic hyperinsulinemia decreases skeletal muscle insulin signaling, mitochondrial content and fatty acid oxidation in cells from lean insulin sensitive and obese insulin resistant individuals. It has been observed that long-term diabetics have a greater resistance to diabetes resolution following bariatric surgery as opposed to shorter duration diabetics, potentially due to chronic exposure to hyperinsulinemia. Our pilot data suggested that decreased skeletal muscle insulin sensitivity in long duration diabetics is associated with poor T2DM resolution. We investigated if skeletal muscle from lean insulin sensitive and obese insulin resistant individuals exposed to chronic hyperinsulinemia exhibit decreases in insulin signaling,

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mitochondrial content and fatty acid oxidation. In doing this we assessed if: 1)

skeletal muscle insulin signaling is decreased following chronic hyperinsulinemia in

lean insulin sensitive and obese insulin resistant individuals and 2) if skeletal muscle

mitochondrial content and fatty acid oxidation is decreased following chronic

hyperinsulinemia in lean insulin sensitive and obese insulin resistant individuals.

CHAPTER 2: Roux-en-Y Gastric Bypass Corrects Hyperinsulinemia

Implications for the Remission of Type 2 Diabetes

Melissa A. Reed[1], Walter J. Pories[2], William Chapman[2], John Pender[2], Rita Bowden[2], Hisham Barakat[3], Timothy P. Gavin[1], Tom Green[3], Ed Tapscott[4], Donghai Zheng[1, 4], Nigel Shankley[5], Lynn Yieh[5], David Polidori[5], Steven P. Piccoli[6], Leona Brenner-Gati[7], and G. Lynis Dohm[4]

[1]Departments of Exercise and Sport Science, [2]Surgery, [3]Internal Medicine and [4]Physiology, East Carolina University, Greenville NC

[5]Johnson and Johnson Pharmaceutical Research & Development, L.L.C., San Diego, CA

[6]Johnson and Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ

[7]Johnson and Johnson Pharmaceutical Services, Inc., L.L.C., New Brunswick, NJ

Address for Correspondence: G. Lynis Dohm, Ph.D.

Department of Physiology

Brody Medical School

East Carolina University

600 Moye Blvd.

Greenville, NC 27834

Phone: 252-744-2682

Fax: 252-744-3460

E-mail: dohmg@ecu.edu

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Abstract

Roux-en-Y gastric bypass (RYGB) has been shown to induce a rapid and durable reversal of Type 2 diabetes. The aim of the study was to investigate a possible mechanism for the remission of type 2 diabetes following RYGB. A cross sectional, non-randomized, controlled study was conducted. Surgery patients were studied prior to RYGB and 1 week and 3 months post-surgery. This study was conducted at the Brody School of Medicine, East Carolina University. Subjects were recruited into three groups: 1) lean [body mass index (BMI) < 25 kg/m^2; n = 9]; 2) severely obese Type 2 diabetic patients (BMI > 35 kg/m^2; n = 9); and 3) severely obese non-diabetic patients (BMI > 35 kg/m^2; n = 9). The intervention utilized was RYGB. One week after RYGB, diabetes was resolved in spite of continued insulin resistance (S_I approximately 50% of lean controls) and reduced insulin secretion during an IVGTT (AIRg approximately 50% of lean controls). Fasting insulin decreased and was no different from lean control in spite of continued elevated glucose in the type 2 diabetic patients compared to lean. After RYGB, fasting insulin decreases to levels like those of lean control subjects and diabetes is reversed. This leads us to propose that 1) exclusion of food from the foregut corrects hyperinsulinemia and 2) fasting insulin is dissociated from the influence of fasting glucose, insulin resistance and BMI. The mechanism(s) for reversal of diabetes in the face of reduced insulin remains a paradox.

INTRODUCTION

Our group was the first to report, in 1980, that the Roux-en-Y gastric bypass (RYGB), a surgical procedure used to treat severe obesity involving gastric restriction and bypass of the proximal small intestine, results in rapid and sustainable reversal of type 2 diabetes (Pories, MacDonald, Flickinger et al., 1992). Recently, our meta-analysis examining 621 studies confirmed that 80% of patients experience complete type 2 diabetes (T2DM) reversal following RYGB (Buchwald et al., 2009) and we have found this improvement to be sustained for at least fourteen years (Pories et al., 1995). In addition to the reversal of diabetes we also discovered that RYGB corrects hyperinsulinemia and restores insulin sensitivity in non-diabetic patients (Bikman et al., 2008; Pories, MacDonald, Morgan et al., 1992). Most important, these changes were not merely a correction of abnormal laboratory values; the operation also reduced the mortality of the severely obese patients by 78% (MacDonald et al., 1997).

Although patients lose weight following RYGB the mechanism(s) for immediate diabetes reversal are not likely solely due to weight loss for the following reasons. 1) Patients become non-diabetic within days after the surgery, before significant weight loss (Pories et al., 1995). 2) Neither calorically induced weight loss nor adjustable gastric banding, the purely restrictive bariatric surgical procedure report similar diabetes reversal rates (Laferrere et al., 2008). 3) In a study comparing RYGB and gastric banding diabetes was resolved within one week following RYGB, a rapid improvement not seen in the gastric banding cohort (Kashyap et al., 2010). 4) The diabetes reversal

rate for gastric banding is 55% and that reversal generally does not occur until significant weight loss is achieved (Buchwald et al., 2009).

We are interested in the mechanisms that are responsible for the reversal of type 2 diabetes following gastric bypass surgery in human subjects. Type 2 diabetes is traditionally characterized by insulin resistance, eventual pancreatic cell dysfunction, decreased insulin secretion and hyperglycemia (DeFronzo, 2004).

The purpose of this study was to explore the relationship of the rapid and durable remission of type 2 diabetes following RYGB to changes in insulin secretion and insulin sensitivity. We utilized a mixed meal test to determine the role of the gut in regulating insulin secretion and insulin sensitivity related improvements. In addition to clinical and laboratory assessment of the course of these patients, we administered an insulin modified intravenous glucose tolerance test to exclude the gut and determine gastric bypass related improvements in insulin secretion and insulin sensitivity.

MATERIALS AND METHODS

A total of 27 women were studied: Nine severely obese non-diabetic and nine severely obese type 2 diabetic women were studied before and after Roux-en-Y gastric bypass surgery. Trials were performed approximately 1 week prior to surgery and 1 week and 3 months post surgery. For comparison, nine lean females who did not undergo RYGB were also studied.

Subjects

Lean (n = 9; BMI < 25 kg/m^2), severely obese non-diabetic (n = 9; BMI > 40 kg/m^2) and severely obese type 2 diabetic (n = 9; BMI > 40 kg/m^2). The study was limited to Caucasian women to exclude the influences of gender and race. All subjects

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were sedentary and performed normal activity with no specific exercise program. T2DM patients were instructed to stop their medications 48 hours prior to the trials that were performed. Patients taking thiazolidinedione (TZD) medications were excluded from the study. Subjects were informed of the protocol and signed an informed consent document. The protocol and consent form were approved by the East Carolina University and Pitt County Memorial Hospital Institutional Review Boards.

Roux-en-Y gastric bypass surgery

The gastric bypass surgery was performed as described previously (Pories, MacDonald, Flickinger et al., 1992). In brief, Roux-en-Y gastric bypass surgery consists of creation of a proximal gastric pouch of 30 cc through the partition of the stomach, a Roux-en-Y gastrojejunostomy with an alimentary limb of 100 cm and a bilio-pancreatic limb of 60 cm. Two weeks prior to surgery, patients were put on a diet of Optifast© 800 (Novartis Nutrition Group; Vevey, Switzerland) Ready to Drink Shakes which consists of 35% protein, 15% fat and 50% carbohydrates for a total of 800 kcal/day. Patients were instructed to consume 5 shakes per day for the 14 days prior to surgery. For the first week following surgery patients were given Bariatric Advantage Meal Replacement (Bariatric Advantage, Irvine, CA), which consists of 150-160 kcal; protein 54%; fat 8%; carbohydrate 23% and instructed to consume 1 meal replacement per day. All of the care was provided in a Bariatric Surgery Center of Excellence certified by the American Society for Metabolic and Bariatric Surgery; each of the subjects, except for the lean comparison group, was entered into the BOLD national database.

Mixed Meal Test (MMT)

A standardized liquid meal, HiCal (150 kcal; protein, 17%; fat, 43%;

carbohydrate, 40%) (Abbott Laboratories, Abbott Park, IL), was given after an overnight

fast. A catheter was placed in an antecubital vein and blood was drawn at 10, 20, 30,

60, 90, 120, and 180 minutes relative to the start of the meal. Samples were treated

with a DPP-IV Inhibitor (Millipore, Billerica, MA) and protease inhibitor cocktail

(Complete Mini Tabs, Roche, Indianapolis, IN) immediately following the blood draws to

prevent degradation of GLP-1. The samples were then centrifuged and plasma was

transferred and frozen at –80 °C for the later determination of glucose, insulin, c-peptide

and GLP-1. Glucose was determined by oxidation reaction (YSI model 2300 Stat Plus;

Yellow Springs Instruments, Yellow Springs, OH). Insulin, c-peptide and GLP-1 were

quantified utilizing an enzyme linked immunosorbent assay (Millipore, St. Charles, MO,

USA). To assess how insulin secretion changes with plasma glucose, a model based

method was used to assess beta-cell function based on a method developed by Mari et

al (Mari, Tura, Gastaldelli, & Ferrannini, 2002) that has been used successfully to study

effects of other treatments (Mari et al., 2007; Mari et al., 2005). To do this, we first used

deconvolution analysis (Van Cauter, Mestrez, Sturis, & Polonsky, 1992) to calculate the

insulin secretion rate profile and then used linear regression to determine the

relationship between plasma glucose concentrations and insulin secretion rate. The

slope of the relationship between plasma glucose and insulin secretion rate (in

(pmol/min/m2)/(mg/dl)) is denoted as the glucose-sensitivity for insulin secretion.

Insulin clearance was calculated as the AUC for insulin secretion (in pmol/min/m^2)

divided by the AUC of plasma insulin (in pmol/L) (Polonsky et al., 1994). Incremental

area under the curve (AUC) was calculated using the pre-meal values as baseline.

Intravenous Glucose Tolerance Test (IVGTT)

Initial blood samples were drawn following an overnight fast. Following the fasting blood samples, glucose (50%) was injected into a catheter placed in an antecubital vein at a dose of 0.3g/kg body mass. Insulin, at a dose of 0.025 U/kg body mass was injected at min 20. Blood samples were obtained at min 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180. Samples were centrifuged and plasma was aliquoted to storage tubes and frozen at –80° C for the subsequent determination of insulin and glucose. Insulin was determined by immunoassay (Access Immunoassay System; BeckmanCoulter, Fullerton, CA) and glucose was determined by oxidation reaction (YSI model 2300 Stat Plus; Yellow Springs Instruments, Yellow Springs, OH). Insulin sensitivity (S_I) index and beta cell function (acute insulin response to glucose, AIRg) were assessed utilizing minimal modeling analysis (MinMod Millennium, Los Angeles, CA) (Bergman et al., 1985). The disposition index (DI) was calculated as the product of S_I and AIRg. The homeostatic model assessment of insulin resistance (HOMA - IR), which is a measurement of insulin resistance, was calculated from fasting glucose and insulin values (fasting glucose mg/dL * fasting insulin µU/ml/405).

Statistics

All data are shown as mean $\pm$ SE. Pre-surgery comparisons between the groups were made using one-way ANOVA. Comparisons were made between type 2 diabetic and obese non-diabetic groups from pre-surgery, 1 week and 3 months post-surgery using a mixed plot factorial two-way ANOVA (Condition x Time) followed by Bonferroni *post hoc* analyses where appropriate.

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*RESULTS*

Subjects

Subject characteristics are reported in Table 1. There were no differences in age between the diabetic and non-diabetic groups but the diabetic group was significantly (P < 0.05) older than the lean comparison group. Before surgery both obese type 2 diabetic and obese non-diabetic patients had greater body weight and BMI than lean, but there were no differences between diabetic and non-diabetic obese groups. There was a significant effect of surgery over time on both body weight and BMI (P < 0.05), however weight was not different between diabetic and non-diabetic groups at either 1 week or 3 months post-surgery.

Fasting Metabolites

Fasting glucose was consistently higher in the type 2 diabetic patients at all times and there was a main effect of surgery to lower glucose in both groups at 1 week and at 3 months post-surgery (P < 0.05) (Fig. 3A). Pre-surgery fasting insulin was significantly higher (P < 0.05) in both the diabetic and non-diabetic groups compared to 1 week and 3 months post surgery (Fig. 3B). Pre-surgery fasting c-peptide was significantly higher (P < 0.05) in both the diabetic and non-diabetic groups compared to 1 week and 3 months post surgery (Fig. 3C). There was a significant time effect for fasting GLP-1, however post hoc analysis was unable to identify differences between time points (Fig. 3D).

Mixed meal test

In both the diabetic and non-diabetic groups it appears the kinetics of glucose and insulin change following RYGB. Compared to pre-surgery, the 1-week and 3 month

post-surgery peak values of glucose and insulin in response to a meal test were shifted from 60 minutes to about 30 minutes (Figs 4A and 4B). Both the diabetic and non-diabetic groups experienced a more robust insulin response from baseline to peak following the mixed meal compared to pre-surgery. In the non-diabetic group, the peak insulin response following a meal was increased approximately 20% following surgery (Fig. 4B) whereas the peak insulin following a meal was unchanged in the patients with type 2 diabetes (Fig. 4B). Similarly, c-peptide area under the curve was significantly increased ($P < 0.05$) in the non-diabetic group following surgery and remained unchanged in the patients with type 2 diabetes (data not shown). GLP-1 area under the curve in both the diabetic and non-diabetic groups significantly increased ($P < 0.05$) both 1 week and 3 months post-surgery (Fig. 4C).

Insulin Sensitivity

There were no differences in HOMA-IR or IVGTT-assessed insulin sensitivity between the obese non-diabetic and lean control groups. As morbidly obese individuals are typically very insulin resistant, we believe there were no differences due to the caloric restriction (800 kcal/day for two weeks prior to surgery) of the pre-surgery diet in the obese non-diabetics. In a previous study, we found the S_I of morbidly obese patients was only about 20% that of lean controls when they were not under dietary restriction (Bikman et al., 2008). The diabetic group was on the same restricted diet and remained extremely hyperglycemic, hyperinsulinemic and insulin resistant. Pre-surgery HOMA-IR was significantly higher ($P < 0.05$) in the diabetic group compared to all other groups at all time points (Fig. 5A). Three months post-surgery the diabetic group's HOMA-IR was 1.64 $\pm$ 0.54 and approached the lean control HOMA-IR value of

1.51 $\pm$ 0.60.   This was due to significant decreases in both fasting insulin and glucose

three months post-surgery, but at 1 week post-surgery was due primarily to a the

significant decrease in fasting insulin. Prior to surgery, S_I was significantly lower (P <

0.05) in the diabetic group compared to the lean group (Fig 5B) but not different when

compared with the obese non-diabetic group. One week following surgery, S_I increased

two-fold in the diabetic group but was still only approximately 50% of the lean group.

Three months post-surgery the diabetic group remained approximately 40% as insulin

sensitive as the lean controls. There were no changes in the S_I of the obese non-

diabetic group from pre to 1 week or 3 months post surgery.

Insulin Secretion

The acute insulin response to glucose (AIRg) was significantly lower (P < 0.05) in

the obese diabetic group compared to the obese non-diabetic group pre-surgery (Fig.

5C). Three months post-surgery in the diabetic group the AIRg was significantly

increased (P < 0.05) (Fig. 5C). Interestingly, the AIRg in the obese group decreased 1

week and 3 months following surgery and the diabetic group AIRg significantly

increased yet both groups appear to normalize to lean control values at 3 months post-

surgery. At all time points, the disposition index was lower in the diabetic group as

compared to the obese non-diabetic group (P < 0.05) (Fig. 5D). Glucose sensitivity for

insulin secretion was lower at all time points in the diabetic group compared to the

obese group (P < 0.05) (Fig. 5E). The change (Δ) in plasma insulin from fasting to peak

(30 minutes) following the mixed meal test was 3-fold higher in type 2 diabetic patients 1

week following RYBG compared to pre-surgery (Fig 5F). At all time points, the change

in plasma insulin was lower in the diabetic group. Insulin clearance doubled in the type

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2 diabetic patients from pre-surgery to 3 months post-surgery (0.46 ± 0.07 L/min/m^2 pre-surgery vs. 0.93 ± 0.31 L/min/m^2 3 months post-surgery). There were no changes in insulin clearance in the obese non-diabetic group from pre to 1 week or 3 months post-surgery (Fig 3G).

DISCUSSION

Our initial purpose in performing this study was to examine changes in insulin secretion and insulin sensitivity to identify a potential mechanism(s) in the remission of type 2 diabetes. In type 2 diabetic patients we found that insulin sensitivity and secretion increased one-week following surgery (Figs 5A, B, C, and E) consistent with previously reported data from our group and others (Bikman et al., 2008; Guidone et al., 2006; Pories, 1992; Pories, MacDonald, Flickinger et al., 1992; Salinari et al., 2009). It does not seem likely that these changes are the mechanism(s) responsible for the amelioration of type 2 diabetes one-week post-surgery, for the following reasons. While insulin sensitivity more than doubles in patients with type 2 diabetes one week following surgery these patients are still profoundly insulin resistant with S_I only 50% of lean control subjects (Fig 5B). Likewise, insulin secretion following surgery is not normalized in patients with type 2 diabetes. In response to an IVGTT one week post-surgery, AIRg is clearly below both lean controls and obese non-diabetic patients (Fig 5C). Similarly, glucose sensitivity for insulin secretion following a meal is lower in diabetic patients than either the lean controls or obese non-diabetic patients (Fig 5E). Additionally, while the change in insulin concentration from basal to peak in response to a meal in diabetic patients one week after surgery is similar to lean subjects it is still lower than obese

non-diabetic patients (Fig 5F). Thus, changes in insulin sensitivity and insulin secretion do not seem sufficient to explain the remission of type 2 diabetes.

Of the metabolic changes that occur after gastric bypass surgery the most pronounced and consistent change is a decrease in fasting insulin (Fig 3B) (Bikman et al., 2008; Pories, MacDonald, Morgan et al., 1992). Plasma insulin was elevated in non-diabetic obese patients and even higher in diabetic patients prior to surgery, yet within days following surgery insulin values were within the range of normal lean control subjects (Kashyap et al., 2010; Pories, MacDonald, Flickinger et al., 1992). This raises the counterintuitive question whether a decrease in fasting plasma insulin precedes and contributes to the remission of type 2 diabetes. This concept would be compatible with the finding that basal-state hyperinsulinemia in healthy normoglycemic adults constitutes an independent risk factor for developing dysglycemia over 24 years (Dankner, Chetrit, Shanik, Raz, & Roth, 2009).

After surgery fasting insulin in diabetics was not different than lean controls, despite glucose being elevated by approximately 25% (Fig 6A). This suggests that in diabetic patients glucose is not driving the production of insulin in the fasting state following RYGB. Fasting insulin is also normally elevated in insulin resistant individuals. Yet, one week post-surgery fasting insulin in our diabetic patients was normal, despite the fact that they were still obese and profoundly insulin resistant (S_I was only 50% of lean controls). In a similar fashion, fasting insulin is usually elevated with increasing BMI but our patients did not have any change in insulin between 1 week and 3 months, despite returning to a regular diet and having a significant decrease in BMI. These changes of the relationships of fasting insulin to plasma glucose, insulin resistance and

BMI are also demonstrated in our previously reported data of non-diabetic post-surgery patients studied in a weight stable condition (Bikman et al., 2008). Bikman et al. (Bikman et al., 2008) showed that for non-surgery control subjects fasting insulin was highly correlated with BMI, but the gastric bypass patients had insulin as low as lean individuals and this did not change with increasing BMI. The same changes in the relationships of fasting insulin to plasma glucose and insulin sensitivity were found in that study but the data is not shown in a way to make the relationships apparent. In Figure 6 we have redrawn the data that was previously published (Bikman et al., 2008). Fasting plasma insulin was lower for gastric bypass patients than controls at any concentration of fasting glucose (Fig 6A). Likewise, the relationship between fasting insulin and insulin sensitivity was shifted to the left in gastric bypass patients, demonstrating lower fasting insulin for any value of insulin sensitivity (Fig 6B). We believe these results suggest a paradigm shift in which the foregut plays a role in insulin regulation and by bypassing the proximal small intestine, such as in RYGB, it is possible to correct hyperinsulinemia and change the relationship between insulin and glucose, insulin resistance and BMI.

This led us to consider the significance of fasting insulin and the role it plays in the resolution of type 2 diabetes following surgery. The remission of type 2 diabetes can be a result of either increased glucose disposal and/or a decrease in glucose production. One measure of glucose disposal is area under the curve following a meal. Fig 6C shows there was no change in glucose AUC one-week after surgery suggesting that glucose disposal is not altered. In contrast, fasting glucose was significantly

decreased one-week post-surgery indicating perhaps the reduction in glucose production is a mechanism contributing to the amelioration of type 2 diabetes.

Patients with type 2 diabetes experience fasting hyperinsulinemia and the pancreas, already working to capacity, has an inadequate capacity to secrete the additional amount of insulin needed for glucose disposal (Fig. 6D pre-surgery). One week after surgery there is a robust increase in insulin after the meal, but this is a result of reduced fasting insulin with peak insulin being approximately the same before and after surgery (Fig 6D). These data suggest that one-week post-surgery fasting insulin is decreased and the pancreas now has a greater capacity for change to regulate glucose disposal.

The significant reduction in fasting insulin most likely has significant consequences for insulin action in peripheral tissues as well. In response to insulin, IRS-1 becomes phosphorylated on tyrosine and serine residues. Tyrosine phosphorylation is the signal for insulin action (e.g., increased glucose uptake by peripheral tissues), but serine phosphorylation (especially on serine 307/312) causes insulin resistance (Itani et al., 2000; Zhou et al., 1999). We find excessive phosphorylation of serine 312 on IRS-1 in muscle of obese patients before surgery but the level of IRS-1 serine phosphorylation in muscle of patients who have had gastric bypass surgery is equivalent to that of lean subjects and significantly less than of weight matched controls (Bikman et al., 2008). The mechanism for the increase in insulin sensitivity that we observe in diabetics a week after surgery may well be a result of the reduction in fasting insulin which allows for the appropriate pancreatic secretion of insulin in response to a physiological stimulus.

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Our data suggest that correction of hyperinsulinemia is a primary event in severely obese diabetic and non-diabetic patients when they undergo RYGB. This leads us to the hypothesis that exclusion of food from the foregut corrects hyperinsulinemia and dissociates fasting insulin from the influences of fasting plasma glucose, insulin resistance and BMI. We recognize that it is counterintuitive that lowering fasting plasma insulin would precede and likely contribute to the remission of type 2 diabetes. However, if this is correct, a paradigm shift in how we treat type 2 diabetes may be to remove the stimulus causing hyperinsulinemia. Further, if hyperinsulinemia is a central abnormality of T2DM, what is the role of insulin therapy? We believe the stimulus that causes hyperinsulinemia is a signal coming from the foregut and research in this area is warranted.

Table 1. Patient Demographic Information: Significance = $p < 0.05$ ‡ significantly different from lean pre surgery, ‡‡ significantly different from obese pre surgery, * main effect of Time – time points significantly different from all other time points.

		Pre	1 Week	3 Months
Age (years)				
	Lean	34.9 + 5.1		
	Obese	37.4 + 2.6		
	Diabetic	49.1 + 2.0 ‡		
Weight (kg)				
	Lean	61.2 + 2.9		
	Obese	125.9 + 4.6 ‡	118.8 + 4.5 *	97.1 + 3.8 *
	Diabetic	127.6 + 6.4 ‡	118.1 + 7.7 *	97.4 + 5.7 *
BMI (kg/m^2)				
	Lean	22.9 + 0.9		
	Obese	44.8 + 1.1 ‡	42.3 + 1.0 *	34.3 + 1.0 *
	Diabetic	46.6 + 2.3 ‡	43.2 + 2.3 *	35.5 + 1.9 *
HbA1C				
	Lean			
	Obese	5.3 + 0.2		
	Diabetic	8.7 + 0.5 ‡‡		

Figure 3. Fasting glucose (A) (n = 9 lean, n = 8 obese and T2DM) insulin (B) (n = 8 lean, n = 8 obese and T2DM), C-peptide (C) (n = 9 lean and n = 8 obese and T2DM) and GLP-1 (D) (n = 9 lean, n = 8 obese and n = 6 T2DM) pre-surgery and 1 week and 3 months post-surgery. In D, there was a significant main effect of time but post-hoc analysis did not determine a difference. The mean $\pm$ SEM for the lean control group is represented by the solid and dashed lines. * significantly different from lean and obese pre-surgery, # significantly different from lean pre-surgery.

A.

Fasting Glucose

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.087
group: p = 0.006
time: p = 0.002 (pre vs. 1 week and 3 months)

Y-axis: Glucose (mg/dl), 0 to 200
X-axis: Obese, Diabetic

B.

Fasting Insulin

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.124
group: p = 0.730
time: p = 0.001 (pre vs. 1 week and 3 months)

Y-axis: Insulin (pmol), 0 to 300
X-axis: Obese, Diabetic

C.

Fasting C-peptide

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.470
group: p = 0.798
time: p = 0.001 (pre vs. 1 week and 3 months)

Y-axis: C-peptide (pmol), 0 to 1000
X-axis: Obese, Diabetic

D.

Fasting GLP-1

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.397
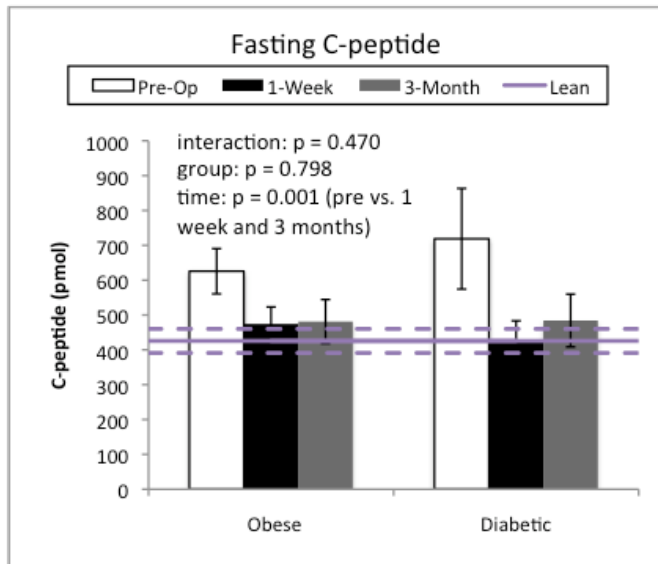group: p = 0.791
time: p = 0.016

Y-axis: GLP-1 (pmol), 0 to 25
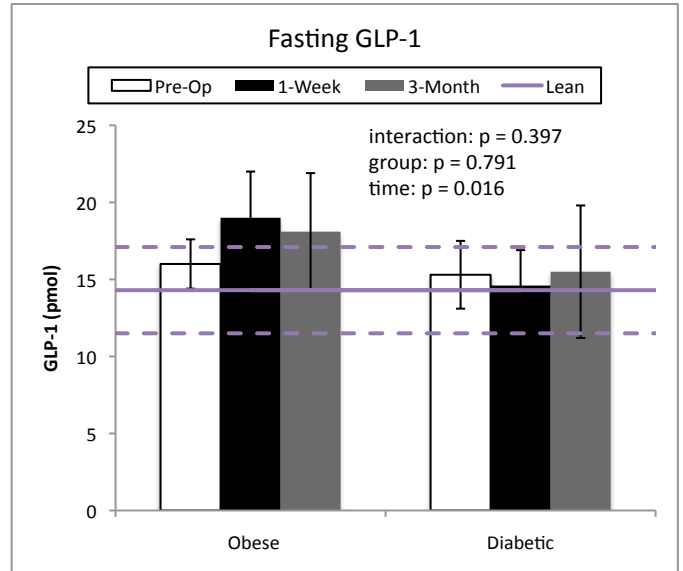X-axis: Obese, Diabetic

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Figure 4. Changes in glucose (A), insulin (B) and GLP-1 (C) in response to a mixed meal tolerance test in lean, obese (pre-surgery, 1-week post-surgery and 3 months post surgery) and diabetic (pre-surgery, 1-week post-surgery and 3 months post-surgery) subjects. * pre-surgery area under the curve significantly different than 1 week and 3 months post-surgery. For lean patients n = 9 in all data sets. Pre-surgery obese n = 9 with the exception of GLP-1, n = 6. 1 week post-surgery obese n = 9 for all except GLP-1, n = 8. 3 months post-surgery obese n = 8 with the exception of GLP-1, n = 7. For all measurements in the T2DM, n = 10 pre-surgery and 1 week post-surgery and n = 8 3 months post-surgery.

A.

Lean — Glucose (mg/dL) vs Time (minutes)

Obese — Glucose (mg/dL) vs Time (minutes)

Diabetic — Glucose (mg/dL) vs Time (minutes)

Legend: Pre, 1 Week, 3 Months

B.

Lean — Insulin (pM) vs Time (minutes)

Obese — Insulin (pM) vs Time (minutes)

Diabetic — Insulin (pM) vs Time (minutes)

Legend: Pre, 1 Week, 3 Months

C.

Lean — GLP1 (pM) vs Time (minutes)

Obese — GLP1 (pM) vs Time (minutes)

Diabetic — GLP1 (pM) vs Time (minutes)

Legend: Pre, 1 Week, 3 Months

Figure 5. HOMA- IR (A), S_I (B), AIRg (C), and Disposition Index (D) in lean, obese and diabetic patients pre surgery, 1-week and 3-months post surgery in response to an IVGTT. Glucose sensitivity (E), basal (0 time) to peak (30 minutes) change (Δ) in plasma insulin (F) and insulin clearance (G) in lean, obese and diabetic patients pre-surgery, 1-week and 3-months post-surgery in response to a mixed meal. * significantly different than lean and obese pre-surgery, # significantly different than lean pre-surgery and ** significantly different than obese pre-surgery.

A.

HOMA-IR

B.

Insulin Sensitvity

C.

AIRg

D.

Disposition Index

E.

Glucose Sensitivity for Insulin Secretion

Legend: Pre-Op, 1-Week, 3-Months, Lean

interaction: p = 0.573
group: p = 0.004
time: p = 0.066

Y-axis: Glucose Sensitivity for Insulin Secretion (pmol/ min/m²/mg/dl)

X-axis: Obese, Diabetic

F.

ΔInsulin $_{30-0}$

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.922
group: p = 0.002
time: p = 0.045 (pre different than 3 months)

Y-axis: Insulin (pmol)

X-axis: Obese, Diabetic

G.

Insulin Clearance

Legend: Pre-Op, 1-Week, 3-Month, Lean

interaction: p = 0.278
group: p = 0.279
time: p = 0.135

Y-axis: Insulin Clearance (L/min/m2)

X-axis: Obese, Diabetic

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Figure 6. The relationship of fasting insulin to fasting glucose (A) and insulin sensitivity (B) in normal subjects (open circles) and non-diabetic gastric bypass patients (closed squares) who have lost weight and are weight stable. Redrawn from data previously published (Bikman et al., 2008). Glucose area under the curve (AUC), fasting glucose and fasting insulin in type 2 diabetic patients pre-surgery and 1 week and 3 months post-surgery (C). Plasma insulin in response to a mixed meal in type 2 diabetic patients pre-surgery and 3 months post-surgery (D).

A.

B.

C.
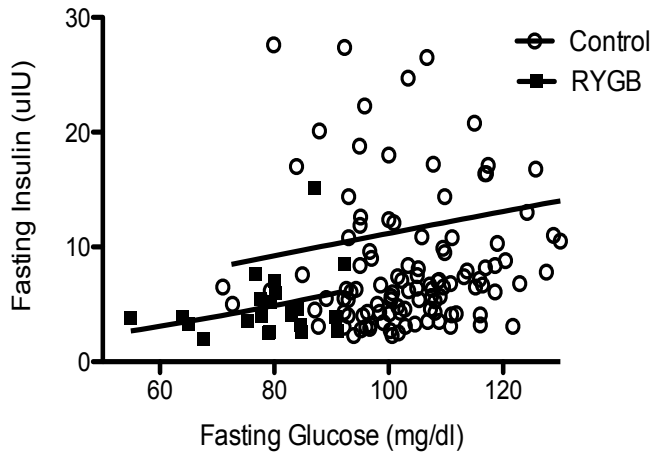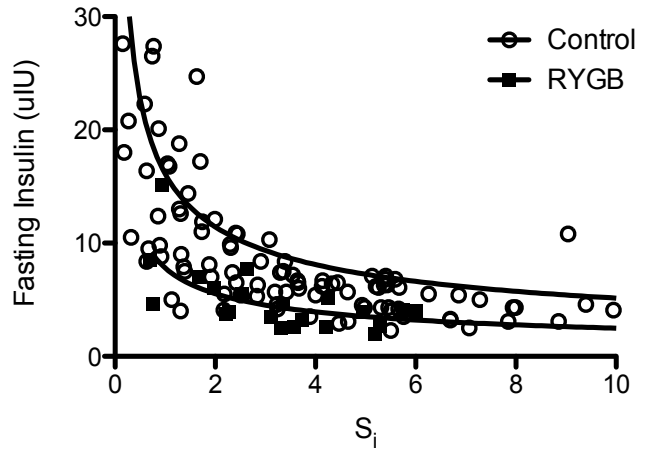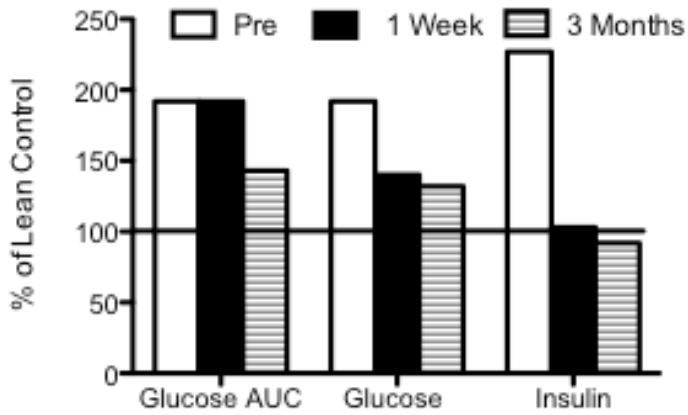
D. Diabetic
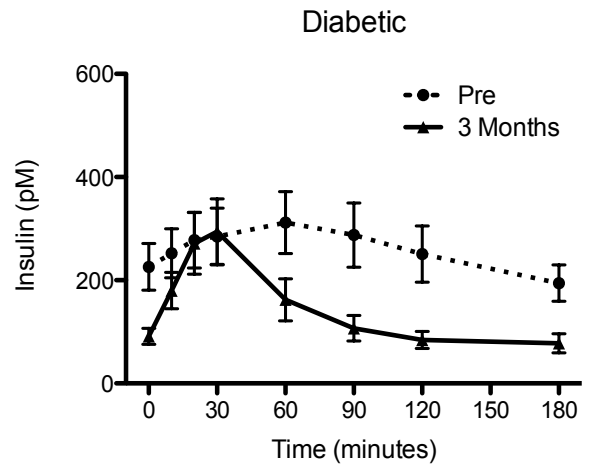
CHAPTER 3: Insulin Sensitivity Predicts Resolution of Type 2 Diabetes following
Gastric Bypass Surgery

Short Title: Gastric Bypass and Diabetes Resolution

Melissa A. Reed[1], Walter J. Pories[2], William Chapman[2], John Pender[2], Rita Bowden[2],

Hisham Barakat[3], Timothy P. Gavin[1], Tom Green[3], Ed Tapscott[4], Nigel Shankley[5], Lynn

Yieh[5], David Polidori[5], Steven P. Piccoli[6], Leona Brenner-Gati[7], and G. Lynis Dohm[4]

[1]Departments of Kinesiology, [2]Surgery, [3]Internal Medicine and [4]Physiology, East

Carolina University, Greenville NC

[5]Johnson and Johnson Pharmaceutical Research & Development, L.L.C., San Diego,

CA

[6]Johnson and Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ

[7]Johnson and Johnson Pharmaceutical Services, Inc., L.L.C., New Brunswick, NJ

Address for Correspondence: G. Lynis Dohm, Ph.D.

Department of Physiology

Brody Medical School

East Carolina University

600 Moye Blvd.

Greenville, NC 27834

Phone: 252-744-2682

Fax: 252-744-3460

E-mail: dohmg@ecu.edu

Key words: Gastric bypass, diabetes resolution, insulin sensitivity, insulin secretion

Abstract

Approximately 80% of Type 2 diabetics exhibit complete reversal of diabetes following gastric bypass surgery. The purpose of this study was to examine potential mechanisms that allow some patients to respond to gastric bypass while others do not. Roux-en-Y gastric bypass (RYGB) was performed in eight severely obese (BMI > 40) diabetic Caucasian women. Both an insulin-modified intravenous glucose tolerance test (IVGTT) and mixed meal tolerance test (MMTT) were performed prior to and 1 week and 3 months post surgery. At 3-months post-RYGB, three of the diabetic patients had complete diabetes resolution as defined by fasting plasma glucose (FPG) below 100 mg/dl without diabetic medication (Responders [R]: FPG 90.2 $\pm$ 6.5 mg/dl), three patients exhibited impaired fasting glucose (FPG: 106 $\pm$ 2 mg/dl) and two patients remained diabetic (Non responders [NR]: 174.2 $\pm$ 34.7 mg/dl). The pre-RYGB characteristics that were correlated with 3-mo post-RYGB FPG were duration of diabetes ($r = 0.7450$, $p = 0.0339$) and insulin sensitivity (S_I) obtained from the IVGTT ($r = -0.7799$, $p = 0.0386$). These data suggest that pre-RYGB insulin sensitivity may be a predictor of Type 2 diabetes resolution following RYGB.

INTRODUCTION

Roux-en-Y gastric bypass (RYGB) has been shown to be both a rapid and durable method for reversing type 2 diabetes mellitus in severely obese individuals (Pories et al., 1995). It has been reported that 80% of type 2 diabetics that undergo RYGB experience complete resolution of the disease following surgery (Buchwald et al., 2009). Possible mechanisms for the amelioration of type 2 diabetes following RYGB include restoration of insulin sensitivity and secretion, weight loss and changes in gut hormones due to the rearrangement of the gastrointestinal tract (Ferrannini & Mingrone, 2009; Rubino & Marescaux, 2004; Whitson et al., 2007).

Little is known regarding the approximately 20% of type 2 diabetic patients that do not respond to RYGB although it appears that duration and severity of the disease are two factors that contribute to the resolution of type 2 diabetes following surgery (Buchwald et al., 2004; Buchwald et al., 2009). Recently, reports have indicated that glycosylated hemoglobin (HbA1c), fasting plasma glucose, body mass index (BMI) and the presence of the co-morbidity hypertension may also affect diabetes resolution following RYGB (Hall, Pellen, Sedman, & Jain, 2010; Hayes, Hunt, Foo, Tychinskaya, & Stubbs, 2011).

We are interested in determining the mechanism(s) why the majority of T2DM patients improve their diabetes following RYGB while some do not. As T2DM is a progressive disease, clinically it is imperative to counsel patients on the best options for their treatment. If it is possible to determine what pre-surgery characteristics are favorable for remission of T2DM, clinicians will be better able to recommend appropriate treatment. To date, the majority of reports have identified pre-RYGB descriptive factors

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that are associated with the reversal of T2DM post-RYGB (Buchwald et al., 2004; Buchwald et al., 2009; Hall et al., 2010; Hayes et al., 2011). It has consistently been reported that both duration and severity of T2DM are associated with poor outcomes following RYGB, however, it is not known whether this is due to a decrease in pancreatic function, peripheral insulin sensitivity or a combination of the two. The purpose of this study was to elucidate potential mechanisms that could be predictive of T2DM resolution post-RYGB.

MATERIALS AND METHODS

Our original study was conducted in order to determine changes in insulin sensitivity and secretion one week and three months following RYGB in severely obese type 2 diabetic and non- diabetic women (Reed et al., 2011). We then examined our original cohort of participants and grouped them into one of three categories; responders, defined by fasting plasma glucose < 100 mg/dL three months post-RYGB without anti-diabetic medication; impaired, defined by fasting plasma glucose between 100 mg/dL and 124 mg/dL three months post-RYGB; or non-responders, defined by fasting plasma glucose > 125 mg/dL three months post-RYGB. Following the classification of the patients into one of three groups we then sought to identify the factors related to Type 2 diabetes amelioration following RYGB.

Subjects

This study included severely obese type 2 diabetic (n = 8) women. Subject characteristics are in Table 1. The study was limited to Caucasian women to exclude the influences of gender and race. All subjects were sedentary and performed normal activities of daily living with no specific exercise program. Patients were instructed to

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stop their medications 48 hours prior to the trials that were performed and patients

taking thiazolidinedione (TZD) medications were excluded from the study. Participants

were informed of the protocol and signed an informed consent document. The protocol

and consent form were approved by the East Carolina University and Pitt County

Memorial Hospital Institutional Review Board.

Roux-en-Y gastric bypass surgery

The gastric bypass surgery was performed as described previously (Pories,

MacDonald, Flickinger et al., 1992). Briefly, RYGB consists of the creation of a proximal

gastric pouch of 30 cc through the partition of the stomach, a Roux-en-Y

gastrojejunostomy with an alimentary limb of 100 cm and a bilio-pancreatic limb of 60

cm. Two weeks prior to surgery, patients were put on a diet of Optifast© 800 (Novartis

Nutrition Group; Vevey, Switzerland) Ready to Drink Shakes which consists of 35%

protein, 15% fat and 50% carbohydrates for a total of 800 kcal/day. Patients were

instructed to consume 5 shakes per day for the 14 days prior to surgery. For the first

week following surgery patients were given Bariatric Advantage Meal Replacement

(Bariatric Advantage, Irvine, CA), which consists of 150-160 kcal; protein 54%; fat 8%;

carbohydrate 23% and instructed to consume 1 meal replacement per day. All of the

care was provided in a Bariatric Surgery Center of Excellence certified by the American

Society for Metabolic and Bariatric Surgery and each subject was entered into the

Bariatric Outcomes Longitudinal Database (BOLD) national database.

Mixed Meal Test (MMT)

A standardized liquid meal, HiCal (150 kcal; protein, 17%; fat, 43%;

carbohydrate, 40%) (Abbott Laboratories, Abbott Park, IL), was given after an overnight

fast and patients were instructed to consume the meal within 10 minutes. A catheter

was placed in an antecubital vein and blood was drawn prior to the meal and at 10, 20,

30, 60, 90, 120, and 180 minutes relative to the start of the meal. Samples were treated

with a protease inhibitor cocktail (Complete Mini Tabs, Roche, Indianapolis, IN)

immediately following the blood draws. The samples were centrifuged and plasma was

transferred and frozen at −80 °C for the later determination of glucose and insulin.

Glucose was determined by oxidation reaction (YSI model 2300 Stat Plus; Yellow

Springs Instruments, Yellow Springs, OH) and insulin was quantified utilizing an enzyme

linked immunosorbent assay (Millipore, St. Charles, MO, USA).

Intravenous Glucose Tolerance Test (IVGTT)

Initial blood samples were drawn following an overnight fast. Following the

fasting blood samples, glucose was injected into a catheter placed in an antecubital vein

at a dose of 0.3g/kg body mass. Insulin, at a dose of 0.025 U/kg body mass was

injected at min 20. Blood samples were obtained at min 2, 3, 4, 5, 6, 8, 10, 12, 14, 16,

19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180. Samples were

centrifuged and plasma was aliquoted to storage tubes and frozen at −80° C for the

subsequent determination of insulin and glucose. Insulin was determined by

immunoassay (Access Immunoassay System; BeckmanCoulter, Fullerton, CA) and

glucose was determined by oxidation reaction (YSI model 2300 Stat Plus; Yellow

Springs Instruments, Yellow Springs, OH). Insulin sensitivity (S_I) index and beta cell

function (acute insulin response to glucose, AIRg) were assessed utilizing minimal

modeling analysis (MinMod Millennium, Los Angeles, CA) (Bergman et al., 1985). The

homeostatic model assessment of insulin resistance (HOMA - IR), which is a

measurement of insulin resistance, was calculated from fasting glucose and insulin

values (fasting glucose mg/dL * fasting insulin μU/ml/405)(Emoto et al., 1999).

Statistics

Data are shown as the both absolute and relative change from pre to 3-mo post-RYGB. Linear regression was preformed to investigate associations between fasting

blood glucose three months post-RYGB and 1.) S_I pre-surgery, 2.) duration of diabetes

pre-RYGB, 3.) BMI pre-RYGB and 4.) HbA1c pre-RYGB. A priori we chose the pre-RYGB variables duration of diabetes (Schauer et al., 2003), BMI (Hayes et al., 2011),

and HbA1c (Hall et al., 2010) based on previous reports that have found associations

between these factors and remission of T2DM. Recently, glucose disposition index, a

product of insulin sensitivity and beta cell function, has also been reported to influence

reversal of T2DM following RYGB (Perugini & Malkani, 2011). However, insulin

sensitivity and beta cell function have not been examined separately as potential

predictors of T2DM remission following RYGB. We examined these factors when

determining associations between both S_I pre-RYGB and AIRg (as a measure of beta

cell function) pre-RYGB and fasting blood glucose three months post-RYGB. This was

done to determine potentially predictive factors in the resolution of Type 2 diabetes

following RYGB.

RESULTS

Following RYGB, we found that the pre-RYGB factors significantly correlated with

3-mo post-RYGB FPG (as a measure of diabetes resolution) were insulin sensitivity ($r =$

-0.7799, p=0.0386) and duration of diabetes ($r = 0.7450$, p=0.0339) (Figures 7A and

1B). There was no correlation between 3-mo post-RYGB fasting glucose and pre-RYGB

BMI, HbA1c, or AIRg (Figures 7C, 1D and 1E).

Three months post-RYGB, three of the patients had complete diabetes resolution

with fasting plasma glucose < 100 mg/dL and no diabetic medication usage as the

criteria for resolution (Responders [R]: FPG=90.2±6.5 mg/dl), three patients exhibited

impaired fasting glucose (FPG=106±2 mg/dl) two remained diabetic (Non responders

[NR]: FPG=174.2±34.7 mg/dl) (Table 2). Pre-RYGB FPG and glucose AUC and insulin

secretion from the MMT were similar between the R and NR and post-RYGB weight

loss was approximately 20% for both the R and NR.

Fasting insulin was 70% higher in the R compared to the NR pre-RYGB but 3-mo

post surgery fasting insulin decreased by 81% in the R and by 36% in the NR (Table 2).

Insulin secretion in response to a meal was increased in both groups as the R exhibited

a 20% increase and the NR had an 86% increase three months post-surgery (Table 2).

Similarly, the AIRg for both groups increased from pre to 3-mo post-surgery with the R

increasing by 82% and the NR increasing by 62% (Table 2). The responders

consistently exhibited higher S_I pre and post-RYGB and while S_I increased 50% in NR it

was still only 25% that of R at 3-mo post-RYGB (Table 2). HOMA-IR decreased in the

R by 91% and 70% (Table 2).

DISCUSSION

The central finding in this study was uncovering the positive correlation between

insulin sensitivity pre-RYGB and fasting blood glucose three months post-RYGB. This

indicates the potential predictive value of insulin sensitivity in regards to diabetes

resolution following RYGB. We also identified duration of T2DM is a negative predictor

of T2DM resolution following RYGB which is in agreement with other recent literature (Pories, MacDonald, Morgan et al., 1992; Schauer et al., 2003). In contrast to other studies, we did not see a correlation between FPG three months post-RYGB (as a measure of diabetes resolution) and pre-RYGB BMI or HbA1c (Hayes et al., 2011; Schauer et al., 2003).

The American Diabetes Association reports that approximately 20 million Americans are afflicted with T2DM and this number is expected to increase to approximately 40 million by 2050 ("Economic costs of diabetes in the U.S. In 2007," 2008). Patients with T2DM are at an increased risk for both macro and microvascular complications such as heart disease, nephropathy and retinopathy and T2DM is the fifth leading cause of mortality ("Economic costs of diabetes in the U.S. In 2007," 2008). Maintaining optimal glycemic control is imperative in managing and treating patients with T2DM. Currently, an HbA1c < 7% is considered optimal because it is associated with decreased microvascular complications and optimal fasting blood glucose is between 70-130 mg/dl ("Standards of medical care in diabetes--2011,"). However, only approximately 37% of previously diagnosed T2DM patients achieved an HbA1c < 7% when utilizing a combination of pharmaceutical therapies including oral hypoglycemics and insulin therapy (Saydah, Fradkin, & Cowie, 2004).

T2DM is a progressive disease and becomes increasingly difficult to control pharmaceutically over time. By contrast, T2DM is rapidly and durably reversed in the majority of patients who undergo RYGB. It has been reported that following RYGB approximately 80% of patients have complete resolution of T2DM, defined as no longer requiring anti-diabetic medication, and 86.6% exhibit either complete resolution of

T2DM or an improvement in T2DM (Buchwald et al., 2009). Moreover, we have reported diabetes resolution sustained for fourteen years indicating the long-term success of RYGB in ameliorating T2DM (Pories et al., 1995).

Although approximately 15% of T2DM patients do not experience any diabetes improvement following RYGB, there does not appear to be one mechanism that solely contributes to the lack of T2DM resolution in the current cohort. Based on our study it appears that insulin sensitivity prior to surgery could be of great predictive value in determining the course of treatment for T2DM patients considering RYGB. Remarkably, it has recently been reported that patients that undergo RYGB experience greater insulin sensitivity when compared to a weight matched group placed on a hypocaloric diet that has the same amount of weight loss (Plum et al., 2011). Additionally, we have previously reported that regardless of weight post-RYGB, both insulin sensitivity and glucose transport are increased in weight stable post-RYGB patients compared to their weight-matched non-RYGB counterparts (Bikman et al., 2008) indicating RYBG elicits a weight-independent mechanism increasing S_I. The novel finding of the increase in S_I in the R compared to the NR both pre and post-RYGB (Table 2) indicates a potential predictive factor of T2DM following RYGB.

Conversely, our study suggests that insulin secretion may not be a predictive factor in the resolution of T2DM following RYGB as the NR had similar if not potentially greater insulin secretion (AIRg) pre-operatively compared to the R (Table 2). These results are in contrast to the recent report in which β cell function appeared to be a potential predictor of T2DM resolution following RYGB (Perugini & Malkani, 2011). This possible discrepancy could be addressed by the different testing that was done in order

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to assess beta cell function. In our study, an insulin-modified IVGTT was performed

which gives a measure of endogenous insulin production in response to an IV glucose

bolus whereas Perugini, et. al assessed beta cell reserve utilizing HOMA-B (a measure

of beta cell function based on fasting insulin and glucose) and HOMA-S (a measure of

insulin sensitivity based on fasting insulin and glucose) (Perugini & Malkani, 2011). In

addition, we administered a liquid, oral mixed meal in order to determine insulin

secretion in response to a meal and consistent with the insulin modified IVGTT data it

appears the NR have as good of a response as the R (Table 2). This suggests that

pancreatic function is not the limiting factor in the remission of T2DM following RYGB as

the NR exhibit an 86% increase in insulin AUC following RYGB indicating that perhaps

there is β cell reserve in this cohort of T2DM patients.

In both groups, insulin resistance (HOMA-IR) decreases 1 week and 3 months

following RYGB (Table 2). This is due to the decrease in fasting glucose and insulin in

both groups (Table 2) in accordance with other reports (Pournaras et al., 2010; Umeda

et al., 2011). In R fasting glucose decreased by 55% and fasting insulin decreased by

81% 3-mo following RYGB. The NR experienced a modest decrease of 15% in fasting

glucose from pre to post-RYGB and a 36% decrease in fasting insulin from pre to 3-mo

post-RYGB. Interestingly, the R fasting insulin pre-RYGB was 55% higher than the NR

and 3-mo post RYGB the fasting insulin in the R was 17% less than the NR which

suggests that the endogenous production of insulin is not a problem in this cohort.

Rather, from this study it appears that pre-RYGB insulin sensitivity could be a potential

mechanism in the remission of T2DM following RYGB (Table 2 and Figure 7).

This study highlights the importance of maintaining peripheral insulin sensitivity. The predictive value of pre-RYGB insulin sensitivity could be of great value to clinicians in determining the appropriate treatment for patients. Measuring insulin sensitivity prior to surgery could offer insight as to the potential reversal of T2DM following surgery and thus influence the type of surgery chosen. The remission rates for more invasive malabsorptive procedures such as the bilio-pancreatic diversion (BPD) and bilio-pancreatic diversion with duodenal switch (BPD-DS) are approximately 95% (Buchwald et al., 2009). Those patients that have decreased insulin sensitivity may benefit from the BPD or BPD-DS and have greater improvement in their T2DM.

We acknowledge the small subset of patients utilized for this report. However, these data are the first to indicate insulin sensitivity as a pre-operative factor related to reversal of T2DM.   Clinically, being able to predict potential remission of T2DM based on pre-operative measures could be beneficial in determining course of treatment for patients seeking bariatric surgery primarily for T2DM management. Additionally, in determining potential predictive factors of T2DM resolution it may be possible to discover the mechanism(s) ultimately responsible for the successful reversal of T2DM following RYGB.

Table 2. Anthropometric parameters, fasting plasma hormone levels and IVGTT and

MMT values for T2DM responders and non responders.

		Pre-RYGB	3 months post-RYGB	Δ from pre to post-RYGB	% change from pre to post-RYGB
Age (years)					
	Responders	47.3 $\pm$ 2.2			
	Non Responders	54.4 $\pm$ 4.0			
Weight (kg)					
	Responders	125.3 $\pm$ 5.5	97.3 $\pm$ 7.7	- 28.0	23% ↓
	Non Responders	139.5 $\pm$ 15.5	111.5 $\pm$ 17.5	- 28.0	20% ↓
BMI (kg/m^2)					
	Responders	46.7 $\pm$ 0.4	36.2 $\pm$ 1.8	- 10.5	23% ↓
	Non Responders	52.8 $\pm$ 3.2	41.7 $\pm$ 4.0	- 11.1	21% ↓
HbA1c (%)					
	Responders	9.1 $\pm$ 0.9			
	Non Responders	8.3 $\pm$ 1.3			
Fasting Glucose (mg/dl)	Responders	199.0 $\pm$ 35.1	90.2 $\pm$ 6.5	- 108.8	55% ↓
	Non Responders	205.6 $\pm$ 96.4	174.2 $\pm$ 34.7	- 31.4	15% ↓
Fasting Insulin (pmol/l)	Responders	332.7 $\pm$ 113.8	98.1 $\pm$ 15.1	- 234.6	81% ↓
	Non Responders	184.7 $\pm$ 15.0	118.0 $\pm$ 2.5	- 66.7	36% ↓
HOMA - IR	Responders	10.7 $\pm$ 2.0	1.2 $\pm$ 0.3	- 9.5	91% ↓
	Non Responders	16.4 $\pm$ 5.6	4.9 $\pm$ 0.3	- 11.5	70% ↓
S_I (min^{-1}/μU/ml)	Responders	1.1 $\pm$ 0.2	1.6 $\pm$ 1.0	+ 0.5	32% ↑
	Non Responders	0.2 $\pm$ 0.2	0.4 $\pm$ 0.2	+ 0.2	50% ↑
AIRg (μU/ml)	Responders	53.6 $\pm$ 29.1	293.4 $\pm$ 146.6	+ 239.8	82% ↑
	Non Responders	82.6 $\pm$ 104.9	217.9 $\pm$ 154.1	+ 135.3	62% ↑
MMT Glucose AUC	Responders	2,926.0 $\pm$ 812.2	1,820.0 $\pm$ 576.4	- 1,106.0	48% ↓
	Non Responders	2,619.2 $\pm$ 1,900.9	3,506.0 $\pm$ 628.0	+ 886.8	26% ↑
MMT Insulin AUC	Responders	6,373.7 $\pm$ 1,550.7	7,951.0 $\pm$ 2,501.8	+ 1,577.3	20% ↑
	Non Responders	4,011.5 $\pm$ 149.5	16,519.0 $\pm$ 5,188.0	+ 12,507.5	86% ↑

Figure 7. Correlations between FPG three months post-surgery and pre-surgery insulin

sensitivity (A) duration of diabetes (B), BMI (C), HbA1c (D) and AIRg (E).
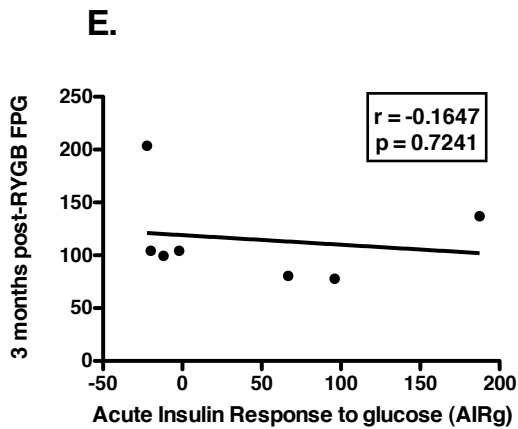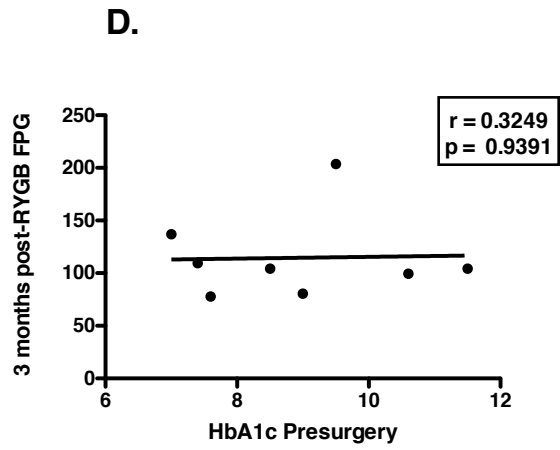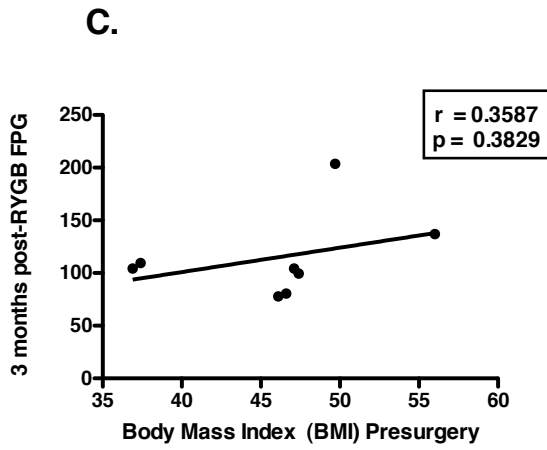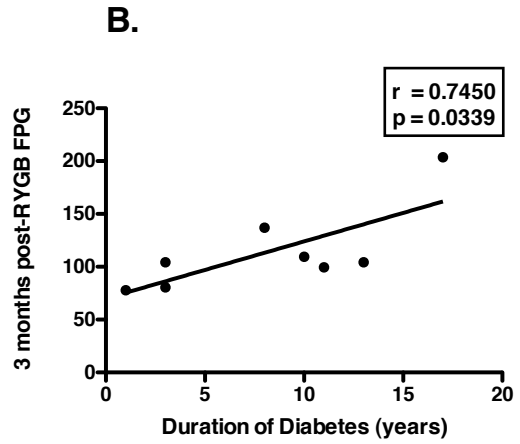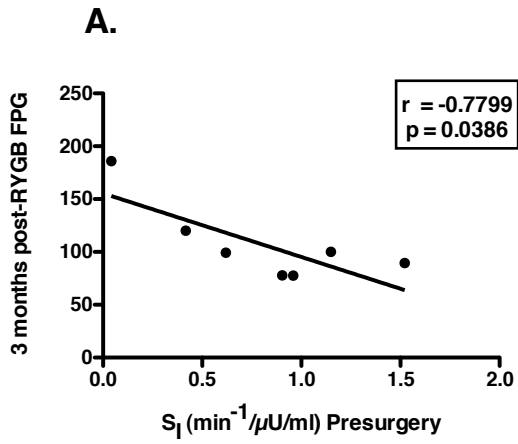
CHAPTER 4: Effect of Chronic Hyperinsulinemia on Insulin Signaling and Mitochondria

in Lean and Obese Human Myotubes

Short Title: Insulin Incubation and Insulin Signaling

Melissa A. Reed[1], Jill Maples[1], Todd Weber[1], Walter J. Pories[2], Joseph A. Houmard[1],

G. Lynis Dohm[3] and Timothy P. Gavin[1]

[1]Departments of Kinesiology, [2]Surgery and [3]Physiology, East Carolina University,

Greenville NC

Address for Correspondence: Timothy P. Gavin, Ph.D.

Department of Kinesiology

Human Performance Laboratory

East Carolina University

363 Ward Sports Medicine Bldg.

Greenville, NC 27848

Phone: 242-737-4684

Fax: 242-737-4689

E-mail: gavint@ecu.edu

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Abstract

Skeletal muscle is the principle site for insulin stimulated glucose uptake and primary human skeletal muscle cell cultures have been shown to be an effective system to elucidate potential mechanisms that contribute to insulin resistance. The purpose of this study was to determine if exposure to chronic hyperinsulinemia would down-regulate the insulin signaling pathway in skeletal muscle myocytes from lean insulin sensitive and obese insulin resistant individuals. This study also examined the effects of chronic hyperinsulinemia on mitochondrial content and fatty acid oxidation. Insulin signaling, mitochondrial content and fatty acid oxidation were measured in myotubes pooled from lean insulin sensitive and obese insulin resistant individuals following a 4 day incubation of either low (80 pM) insulin or high (5000 pM)) insulin. Insulin signaling was not down-regulated due to chronic hyperinsulinemia in either group. Rather, AS160 was increased in the lean group after chronic hyperinsulinemia. Complete oxidation of FA was significantly reduced and incomplete oxidation was significantly higher in the obese group compared to the lean group independent of insulin incubation. In the current study, mitochondrial content was not different between the lean and obese groups for either insulin condition. Chronic hyperinsulinemia increases insulin signaling in lean insulin sensitive myocytes compared to obese suggesting the obese exhibit a blunted response. Also, chronic hyperinsulinemia did not affect fatty acid oxidation or mitochondrial content in the lean or obese group but the obese group experienced dysregulated fatty acid metabolism independent of insulin suggesting an inherent defect in the obese compared to the lean group.

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INTRODUCTION

Skeletal muscle is the main site of insulin-mediated glucose uptake and is also the principal site for peripheral insulin resistance (Shulman et al., 1990). Insulin resistance and obesity related metabolic disorders, such as Type 2 diabetes, are associated with metabolic perturbations including mitochondrial dysfunction, inappropriate fatty acid metabolism and decreased insulin signaling (Bikman et al., 2010; Kelley & Goodpaster, 1999; Kim, Hickner, Cortright, Dohm, & Houmard, 2000; Ritov et al., 2005).

Recently, we reported that Roux-en-Y gastric bypass (RYGB) reverses hyperinsulinemia in severely obese non-diabetic and Type 2 diabetic (T2DM) women within one-week of surgery even though fasting glucose levels were not completely normalized to lean control levels (Reed et al., 2011). The reversal of hyperinsulinemia following RYGB is clinically relevant because hyperinsulinemia and metabolic disease are implicated with increases in cardiovascular disease and all-cause mortality (Mottillo et al., 2010).

Hyperinsulinemia may also be detrimental for skeletal muscle insulin sensitivity. Insulin can independently cause insulin resistance, whether administered exogenously (Samuelsson et al., 2006) or through an insulin infusion (Rizza, Mandarino, Genest, Baker, & Gerich, 1985) implicating hyperinsulinemia as a potential cause of peripheral insulin resistance. In primary skeletal muscle cells isolated from both lean and T2DM individuals, hyperinsulinemia decreases glucose transport (Ciaraldi, Abrams, Nikoulina, Mudaliar, & Henry, 1995). These data indicate that perhaps independent of blood

glucose, hyperinsulinemia could lead to a down-regulation of the insulin signaling pathway.

Severely obese individuals have decreased rates of complete fatty acid oxidation (Bell et al., 2010; Consitt et al., 2010), and increased incomplete fatty acid oxidation has been associated with insulin resistance (Koves et al., 2008). Decreased fatty acid oxidation in obese individuals has been reported at the whole body level (Guesbeck et al., 2001), in muscle homogenates (Kim et al., 2000) and in primary human skeletal muscle cell culture (Bell et al., 2010). Further, in primary human skeletal muscle cell culture, our laboratory reported that exposure to lipid decreased fatty acid oxidation and concomitantly decreased insulin sensitivity in myotubes from lean individuals to levels similar to cells from obese indicating that lipid incubation can cause the lean cells to exhibit responses that are similar to the obese cells (Bell et al., 2010).

The relationship between skeletal muscle mitochondria and insulin resistance is complex and contradictory. Skeletal muscle mitochondria are the primary site for fatty acid oxidation and have been implicated in the pathogenesis of insulin resistance as decreased oxidative capacity relative to glycolytic capacity has been reported in obese insulin resistant women (Simoneau, Colberg, Thaete, & Kelley, 1995). Additionally, it is has been shown that obesity is associated with decreased mitochondrial content (Consitt et al., 2010). Conversely, reports have also shown that skeletal muscle mitochondria content increases in high fat fed insulin resistant rats (Hancock et al., 2008). Further, a recent clinical trial has demonstrated that mitochondrial capacity and insulin action, while related in an insulin resistant population, are not associated in individuals with Type 2 Diabetes that underwent a hyperinsulinemic-euglycemic clamp

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(Bajpeyi et al., 2011). It is not known how hyperinsulinemia directly affects skeletal muscle mitochondrial content and fatty acid oxidation.

In the present study we investigated if chronic exposure to hyperinsulinemia alters insulin signaling, mitochondrial content and fatty acid oxidation. In doing this, we have attempted to recapitulate physiological hyperinsulinemia in primary myotubes. It was hypothesized that chronic exposure to hyperinsulinemia would down-regulate insulin signaling in myotubes from both lean and obese. Further, we hypothesized that mitochondrial content and fatty acid oxidation would be depressed in the cells from obese insulin resistant individuals compared to cells from the lean insulin sensitive individuals based on previous literature from our laboratory (Consitt et al., 2010).

MATERIALS AND METHODS

Subjects

Lean insulin sensitive (n=4) (LN) and severely obese insulin resistant (n=4) (OB) Caucasian women were recruited for participation in this study. Insulin resistance was determined by the homeostatic model assessment of insulin resistance (HOMA - IR), which was calculated from fasting glucose and insulin values (fasting glucose mg/dL * fasting insulin µU/ml/404) (Emoto et al., 1999). All subjects were considered sedentary and in good health after completion of a medical history questionnaire. Participants were informed of potential risks associated with the study and signed an informed consent document before the study began. The protocol and consent form were approved by the East Carolina University Institutional Review Board.

Primary Human Skeletal Muscle Culture

Following a 12-h overnight fast, approximately 50-100 mg of skeletal muscle from the vastus lateralis of subjects was obtained by percutaneous biopsy (Evans, Phinney, & Young, 1982).   The isolation and culturing of human primary skeletal muscle cells from biopsies was performed as previously described (Muoio et al., 2002). In brief, approximately 30 – 50 mg of muscle sample was transferred to cold low-glucose Dulbecco's modified Eagle's medium [DMEM; media and media supplements were purchased from GIBCO (Invitrogen, Carlsbad, CA)] and all visible connective and adipose tissue was removed before culture.   Satellite cells were isolated by trypsin digestion, pre-plated for 1–3 h in 3.0 ml of growth media (GM) [low-glucose DMEM supplemented with 10% FBS, 0.5 mg/ml BSA, 0.4 mg/ml fetuin, 20 ng/ml human epidermal growth factor, 0.39 µg/ml dexamethasone, and 40 µg/ml gentamicin/amphotericin B (Lonza, Walkersville, MD)] on an uncoated T-25 tissue culture flask (Fisher Scientific, Waltham, MA) in order to remove fibroblasts, and then transferred to a type I collagen-coated T-25 flask for myoblast adherement. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. After reaching ~80% confluence, myoblasts were trypsinized and transferred to a type I collagen-coated T-75 flask. After again reaching ~80% confluence, myoblasts from lean and obese subjects were separately pooled by adding roughly 100×10^3 cells/sample in a lean or obese cell pool, as previously reported (Bikman et al., 2010). After the pooled cells reached ~80% confluence, myoblasts were then subcultured onto six- and 24-well type I collagen-coated plates at densities of 80 and 20×10^3 cells per well, respectively. After achieving ~90% confluence, differentiation was induced by replacing GM with low-serum differentiation media (DMEM, 2% heat-inactivated horse serum, 0.5 mg/ml BSA,

0.5 mg/ml fetuin, and 50 µg/ml gentamicin/amphotericin B). Media was changed every

day and on day 3 of differentiation, media was supplemented with low insulin (80 pmol/l)

or high insulin (5,000 pmol/l). All human myotube cellular experiments were performed

on day 7 of differentiation. Therefore, all isolated cells were exposed to 4 days of low or

high (hyperinsulinemia) insulin.

Fatty Acid Oxidation

On day 7, fatty acid oxidation assays were performed after a 3-h incubation in sealed

24-well plates at 37° C in differentiation media, 12.4 mm HEPES, 0.5% BSA, 1 mm

carnitine, 250 µm sodium oleate and 1 µCi/ml [1-^{14}C] oleate (Sigma-Aldrich, St. Louis,

MO). After the incubation period, the medium was transferred to new plates and

assayed for the collection of $^{14}CO_2$ production (complete oleate oxidation), which was

quantified via liquid scintillation counting using 4 ml of Uniscint BD (National

Diagnostics, Atlanta, GA). Incomplete oxidative products (acid-soluble metabolites,

ASM's) were also measured as previously described (Muoio et al., 2002). The results

are reported as a ratio between incomplete (acid-soluble metabolites) and complete (1-

$^{14}CO_2$) radiolabeled products. Following the fatty acid oxidation assay, cells were

washed twice with ice-cold PBS and harvested in 600 µl 0.05% sodium dodecyl sulfate

(SDS) and cell lysates were stored at -80 C for subsequent protein determination.

Insulin signal transduction via Western Blotting

Insulin signal transduction and mitochondrial content were determined in cultured

muscle cells from lean and obese individuals following a 4d incubation of low insulin or

high insulin. For insulin signaling, following the 4d incubation, cells were serum starved

for 5 h, acutely insulin stimulated (100 nm porcine insulin) (Sigma-Aldrich) for 15 min,

and immediately harvested in 100 µl of ice-cold lysis buffer containing 50 mM HEPES, 100 mM sodium fluoride, 50 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 10 mM EDTA, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO) per well, and sonicated for 5 s, followed by a 10 minute spin at 20,000g at 4°C and individual protein concentrations from the cell lysates were determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Cellular protein (10 µg) was separated by SDS-PAGE using Tris·HCl gels, transferred to nitrocellulose membranes, and then probed overnight for either pan-akt (Santa Cruz Biotechnology, Santa Cruz, CA) phospho akt (Thr[308]), phospho AS160 (Thr[642]), or pan AS160 all from Cell Signaling Technologies (Beverly, MA) and diluted in 5% BSA using Tris-buffered saline (TBST) with 0.1% Tween. Phosphoproteins were normalized to total protein for both Akt and AS160 quantification. Following incubation with primary antibodies, all blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase activity was assessed with enhanced chemiluminescence solution (Thermo Scientific, Rockford, IL) and developed utilizing ImageLab (BioRad).

Mitochondrial COXIV and Citrate Synthase

Cytochrome C oxidase (COX)IV and citrate synthase were measured from cells incubated in insulin for 4 days but without the acute 15 minute 100nM insulin stimulation. For mitochondrial measurements cells were harvested from 6-well plates in ice-cold buffer containing 100mM KH_2PO_4, 2mM EDTA, 0.1% Triton X-100, pH 7.2 and total protein was assessed using the BCA protein assay (Pierce, Rockford, IL) and COXIV was probed by following the technique described above. The COXIV antibody

was purchased from Cell Signaling Technologies (Beverly, MA) and normalized to actin as a loading control. For the citrate synthase assay, 10µg protein were loaded to all sample wells and citrate synthase activity was determined using a commercially available kit from Sigma (Sigma-Aldrich). Citrate synthase activity was determined by measuring the formation of 5-thio-2-nitrobenzoic acid spectrophoetrically at 412 nm.

Statistics

Data are presented as Means ± SE. Data were analyzed using a 2x2 (group x [insulin]) mixed plot factorial ANOVA. After a significant F ratio, post hoc analysis was performed using the Bonferroni test. Linear regression was performed to determine the association between citrate synthase activity and COXIV. Significance was set at $P \leq$ 0.05.

RESULTS

Subjects

There was no significant difference between lean and obese groups for age (lean; 28.3 $\pm$ 2.1 vs. obese 33.5 $\pm$ 4.3). By design, groups were significantly different in BMI (lean; 23.0 $\pm$ 1.7 kg/m^2 vs. obese; 41.4 $\pm$ 3.8 kg/m^2) and HOMA-IR (used as an indicator of insulin resistance) (lean; 1.3 $\pm$ 0.3 vs. obese; 5.8 $\pm$ 1.1).

Insulin Stimulated Insulin Signaling

Obesity is associated with impaired insulin induced signaling and lipid overload can induce similar impairments in insulin-induced signaling in lean, even in primary cell culture (Bell et al., 2010). We questioned if hyperinsulinemia would impair insulin-induced signaling in primary skeletal muscle cells isolated from lean individuals to levels observed in obese. Figure 8 depicts the Akt and AS160 response to acute stimulation

in lean and obese following chronic incubation with low or high physiological levels of insulin. There was a trend (p=0.11) for lower insulin-induced Akt phosphorylation in OB compared to LN regardless of insulin incubation conditions consistent with cells from obese being insulin resistant compared to leans (Fig. 8A). There was no effect of hyperinsulinemia on insulin-induced Akt phosphorylation. However, hyperinsulinemia significantly increased insulin-induced AS160 phosphorylation in LN, but not OB (Fig. 8B). Thus, in human primary skeletal muscle cells from lean individuals hyperinsulinemia increases insulin signaling and this response is attenuated in obese.

Fatty Acid Oxidation (FAO)

Muscle (in vivo or primary skeletal muscle cell culture) from obese individuals demonstrates impaired FAO (Bell et al., 2010; Guesbeck et al., 2001). Figure 9 shows FAO from LN and OB following chronic hyperinsulinemia. Complete FAO (Fig. 9A) was lower and ASM/CO_2 (Fig. 9C) was higher in OB compared to LN. There was no effect of hyperinsulinemia on complete FAO, total FAO or ASM/CO_2. As the ASM/CO_2 ratio is often referred to as a measurement of FAO efficiency, these results offer further evidence that severely obese individuals exhibit less efficient FAO compared to lean individuals (Bell et al., 2010; Consitt et al., 2010; Kim et al., 2000).

Citrate Synthase Activity (CSA) and COXIV

As reduced mitochondrial content has been implicated in obesity related insulin resistance and to identify a potential mechanism for reduced FAO in cells from obese, we measured COXIV and CSA as indexes of mitochondrial content (Fig. 10). There was no difference in CSA (Fig. 10A) or COXIV content (Fig. 10B) between the LN and OB. There was no effect of hyperinsulinemia on either CSA or COXIV in the LN or OB.

Linear regression reveals a significant correlation between mitochondrial content measured by CSA and COXIV (Fig. 10C) supporting the use of these measures for mitochondrial content. These results suggest that differences in FAO between LN and OB do not appear to be due to differences in mitochondrial content in the current experiments.

DISCUSSION

Our initial hypothesis was that exposure to chronic hyperinsulinemia would down-regulate insulin signaling in both lean and obese primary human cell cultures. While there was no decrease in insulin signaling in either group, the LN group responded to the acute insulin stimulation by increasing AS160 phosphorylation while the OB group did not exhibit a similar response. These results suggest that the LN group retains flexibility allowing up-regulation of insulin signaling in response to hyperinsulinemia while the OB group does not experience a similar response.

The current report is in conflict with previous reports that hyperinsulinemia can induce insulin resistance in vivo (Del Prato et al., 1994; Iozzo et al., 2001; Rizza et al., 1985) and in myotubes (Ciaraldi et al., 1995). One possible explanation for these results is that in the current study a physiological concentration of hyperinsulinemia (approximately 5,000 pM) was utilized whereas prior reports used pharmacologic concentrations (approximately 30 µM) (Ciaraldi et al., 1995). Also, Rizza et al. reported that glucose utilization decreases following a 40h continuous exposure to hyperinsulinemia thus suggesting that hyperinsulinemia induces whole body insulin resistance (Rizza et al., 1985). However, it is possible this hyperinsulinemia-induced insulin resistance is due to a specific reduction in glycogen synthesis and is not a result

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of the down regulation the IRS signaling cascade that is responsible for insulin-induced GLUT4 translocation to t-tubule sarcolemma. It has been documented that 48-72h of euglycemic hyperinsulinemia decreases whole body glucose uptake and this is predominantly due to a reduction in glycogen formation (Del Prato et al., 1994). Follow up studies elucidated that 24h of physiological hyperinsulinemia induces insulin resistance by decreasing insulin induced glycogen synthase in skeletal muscle, yet GLUT4 protein content was unchanged (Iozzo et al., 2001). Therefore, it is possible that physiological hyperinsulinemia induces insulin resistance by decreasing whole body glucose utilization but this may be independent of the insulin mediated insulin signaling cascade.

In support of this divergent response to physiological hyperinsulinemia, AS160 phosphorylation in skeletal muscle, when exposed to physiological hyperinsulinemia, is increased in normal control subjects while T2DM subjects exhibit reduced AS160 phosphorylation compared to lean (Karlsson et al., 2005). Also, it is possible that AS160 remained phosphorylated following the 5 hour insulin starvation, similar to phosphorylation observed post exercise 2.5 hours (Sriwijitkamol et al., 2007) and 14 hours following exercise (Frosig et al., 2007).   While AS160 is rapidly dephosphorylated following the removal of rat skeletal muscle from insulin, the long term effects of insulin exposure are not known at the cellular level (Sharma, Arias, & Cartee, 2010). Additionally, Hoy et. al., (Hoy et al., 2009) demonstrated that physiological levels (~300 mU/l) of insulin added during a lipid infusion in rats resulted in an increase in skeletal muscle insulin resistance but that increase was independent of the insulin signaling pathway as no changes in Akt or AS160 were observed. Similarly, it has been reported

in rats that glucose infusion can result in skeletal muscle insulin resistance without dysregulation of the insulin signaling pathway due to decreases in glucose uptake and glycogen synthesis, but no changes in Akt or AS160 phosphorylation (Hoy et al., 2007). These data suggest that factors other than differences in insulin signaling in the metabolism of lipids or glucose could contribute to hyperinsulinemia-induced insulin resistance.

There was a disconnect in the insulin signaling pathway from Akt to AS160 where the LN group did not experience a significant increase in Akt phosphorylation in response to insulin induced insulin stimulation but did in AS160 following hyperinsulinemia incubation. It is possible there are alternate substrates other than Akt that phosphorylate AS160 which is consistent with a report from our lab that exhibited a similar disconnect between Akt and AS160 in response to lipid induced insulin resistance (Bikman et al., 2010).   Another possibility for the disconnect between Akt and AS160 is that with a 15-minute acute supraphysiological stimulation we have missed maximal pAkt^{Thr308} signaling in the myotubes but have reported maximal pAS160^{Thr642} signaling. Five, 10 and 15 minutes have been reported to induce maximal pAkt^{Thr308} stimulation in mouse skeletal muscle (Witczak et al., 2010) but the exact time required for similar stimulation in primary human skeletal muscle cell culture is not known, indicating perhaps pAkt^{Thr308} stimulation was already decreasing while downstream pAS160^{Thr642} was maximally phosphorylated. Also in the report by Bikman et. al., the myotubes from lean were able to up-regulate AS160 phosphorylation in response to a 16 hour lipid exposure while myotubes form obese did not (Bikman et al., 2010). Similar to those findings, in the current study, the OB group experienced a

decreased ability to respond to the hyperinsulinemic challenge compared to the LN group. These data suggest the muscle from obese, insulin resistant exhibit an impaired response in insulin-induced insulin signaling compared to muscle from lean, insulin sensitive.

Additionally, we observed that complete FAO was decreased in the OB group independent of insulin concentration. Reduced FAO in cells from obese is consistent with studies that have reported depressed FAO in skeletal muscle homogenates (Kim et al., 2000), intact muscle strips (Hulver et al., 2003), primary human skeletal muscle cell culture (Bell et al., 2010), during lipid infusions (Thyfault et al., 2004) and via indirect calorimetry (Guesbeck et al., 2001). Also, the OB group exhibit increased incomplete:complete FAO indicating the muscle from obese have higher levels of incomplete oxidation and thus less efficient fatty acid oxidation. This was observed during both normal and hyperinsulinemia suggesting that chronic hyperinsulinemia does not affect fatty acid oxidation, rather that muscle from obese experiences a persistent decrease in oxidation compared to muscle from lean.

Reduced mitochondrial content has been implicated as a likely cause of reduced FAO in obesity and is associated with insulin resistance (Consitt et al., 2010; Morino et al., 2005). Mitochondrial content was determined in order to account for potential differences in FAO and there were no differences in either CSA or COXIV protein between the LN and OB groups, which suggests that the decrease in complete FAO is not due to decreased mitochondrial content in the OB group. Also, we found no differences in mitochondrial content between groups at either insulin concentration indicating that hyperinsulinemia does not affect mitochondrial content. These results

suggest muscle mitochondrial content may not be different between lean and obese and that reduced fatty acid oxidation may be due to mitochondrial dysfunction (Kelley et al., 2002; Koves et al., 2008).

In summary, it appears that when challenged with hyperinsuliemia, muscle from obese exhibit decreased flexibility to hyperinsulinemia compared to muscle from lean in which hyperinsulinemia increases AS160 phosphorylation. This appears to be independent of mitochondrial content as both citrate synthase and COXIV were unchanged between the LN and OB groups for both insulin conditions. Additionally, though complete fatty acid oxidation was decreased and the ratio of incomplete:complete FAO was increased in the OB group this was independent of insulin and appears to be a defect inherent in the OB group. These results suggest that hyperinsulinemia-induced insulin resistance in skeletal muscle is not due to decreased insulin signaling but rather to other pathways involved in glucose transport and disposal.

Figure 8. The effects of 4d of low (80 pM) or high (5000 pM) insulin on insulin-stimulated insulin signaling in pooled lean (open bars) and obese (filled bars) myotubes from human participants. Myotubes were probed for p-AKT (A) and p-AS160 (B), AU (Arbitrary Units). * Significant interaction with post-hoc testing indicating significantly different from all other time points (p<0.05).

A.

L-Low   O-Low   L-High   O-High

p-Akt^{THR308}

Total Akt

B.

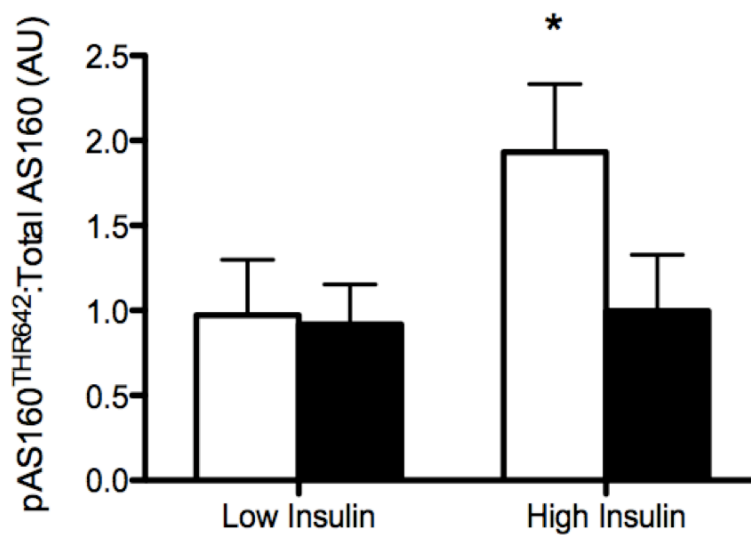L-Low   O-Low   L-High   O-High
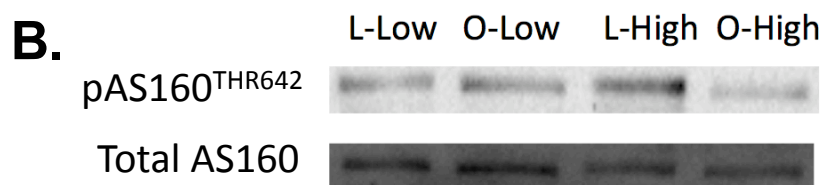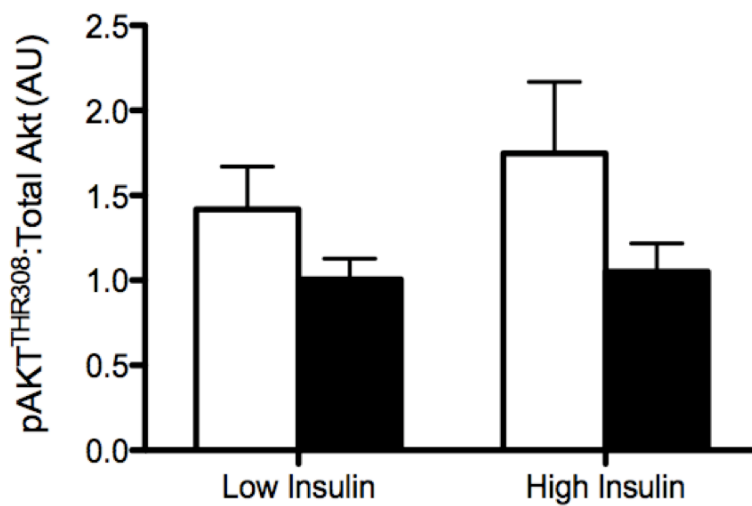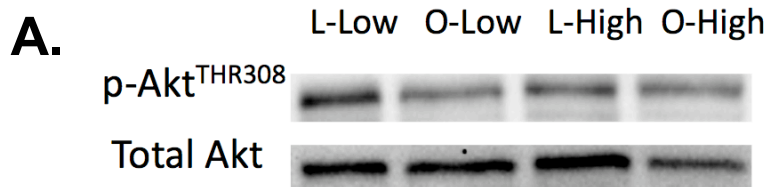
pAS160^{THR642}

Total AS160

Figure 9. The effects of 4d of low (80 pM) or high (5000 pM) insulin on fatty acid

oxidation in pooled lean (open bars) and obese (filled bars) myotubes from human

participants. Complete FAO was measured from ^{14}C-labeled incorporation into CO_2 (A).

Total FAO (B) was calculated as the sum of ^{14}C-labeled incorporation into CO_2 and ^{14}C-

labeled incorporation into acid-soluble metabolites, which is a measurement of

incomplete FAO. FAO efficiency was calculated as the ratio of ASM to complete FAO,

represented as ASM/CO_2 (C). * Significant main effect of BMI ($p<0.05$)

A.

B.

C.
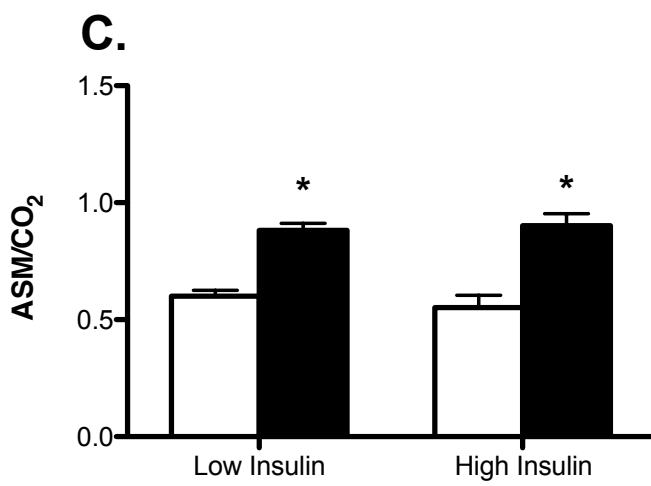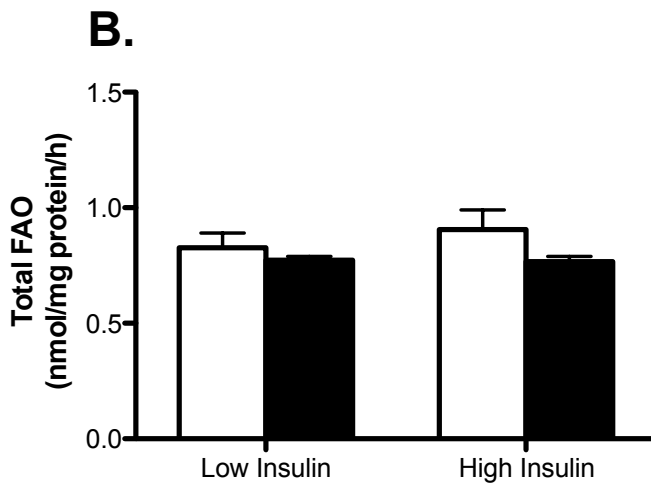
Figure 10. The effects of 4d of low (80 pM) or high (5000 pM) insulin on mitochondrial content in pooled lean (open bars) and obese (filled bars) myotubes from human participants. Myotubes were measure for citrate synthase activity (A) and probed for COXIV (B), AU (Arbitrary Units). Additionally, a correlation was performed between COXIV and citrate synthase activity (C).

A.

B.

C.

CHAPTER 5: Integrated Discussion

The development of T2DM is characterized by insulin resistance, chronic hyperinsulinemia and, over time, pancreatic β cell failur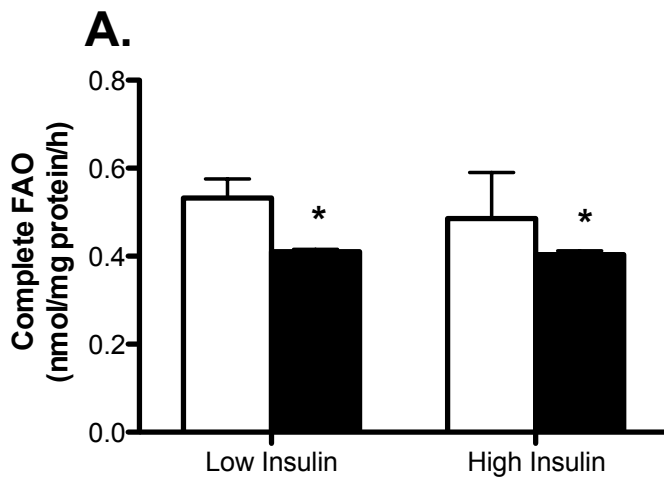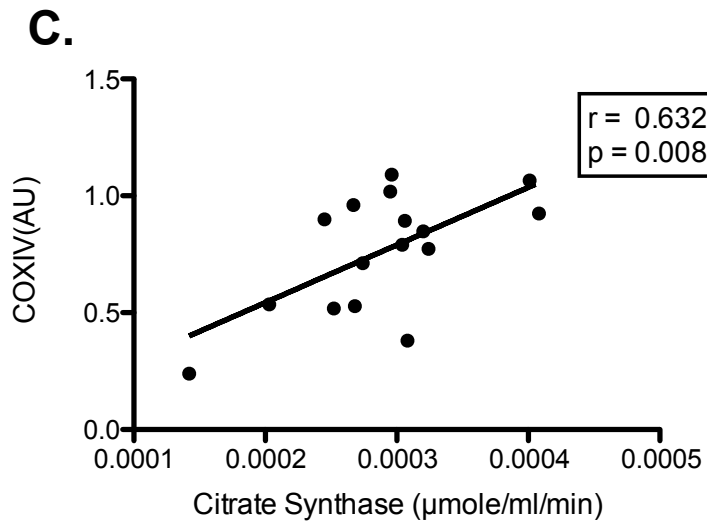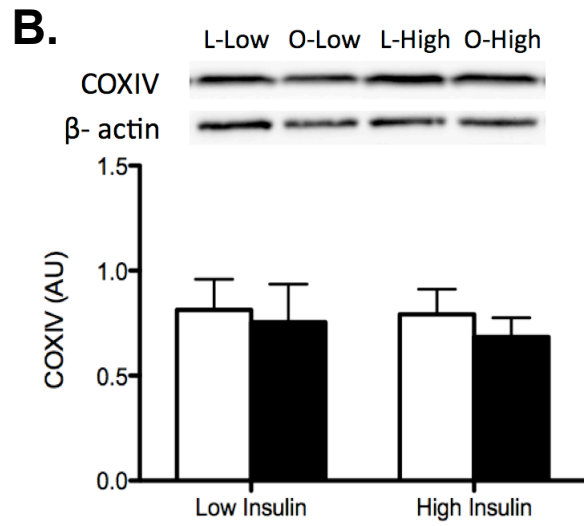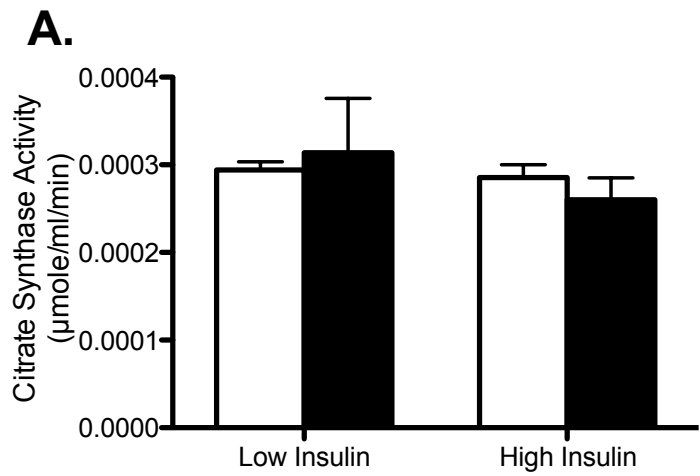e (DeFronzo, 2004). Specifically, skeletal muscle in T2DM exhibits insulin resistance, decreased substrate oxidation and mitochondrial dysfunction (Kelley et al., 2002; Ritov et al., 2005). Yet, the exact mechanism(s) responsible for the development of T2DM are not clearly elucidated. While T2DM becomes progressively difficult to manage pharmacologically, RYGB has proven to ameliorate T2DM in approximately 80% of T2DM patients and this reversal is durable, with patients as long as 14 years post-RYGB reporting successful continued remission of T2DM (Flickinger et al., 1984). These improvements are likely a result of improved insulin sensitivity and insulin secretion following RYGB but exact mechanisms for the reversal of T2DM remain unknown.

In Chapter 2 (Study One), we observed a significant decrease in fasting insulin in T2DM patients to levels similar to lean controls by one week following RYGB even though fasting glucose, while reduced, remained elevated. This finding indicates that RYGB alters insulin regulation such that fasting insulin is dissociated from glucose and suggests that post-RYGB fasting insulin may be regulated by mechanisms other than glucose. Future work in this area is needed to determine the exact mechanism by which fasting insulin is restored to normal following RYGB seemingly independent of fasting glucose.

Though there was great improvement of T2DM following RYGB in Chapter 2, our results were consistent with the literature in that approximately 20% of patients do not

exhibit T2DM remission following RYGB. Of the patients with T2DM, three patients experienced complete diabetes reversal (FPG < 100 mg/dL), three had impaired blood glucose (FPG 100-124 mg/dL), indicating some improvement in their T2DM and two patients that did not respond to RYGB and remained diabetic (FPG > 125 mg/dL) following RYGB. Therefore, approximately 25% of patients did not experience remission of diabetes following RYGB. There has been no specific mechanism defined that can explain unsuccessful resolution of T2DM following RYGB. Previously, several factors have been associated with T2DM resolution following RYGB including disease severity (as indicated by the use multiple pharmacological therapies and/or exogenous insulin), length of the disease, inadequate control of blood glucose (as indicated by a high HbA1c), low BMI and the presence of hypertension (Hall et al., 2010; Hayes et al., 2011). However, it was not known mechanistically what may contribute to T2DM resolution. We questioned if insulin sensitivity and insulin secretion pre-RYGB are predictive of post-RYGB diabetes remission. It was the overall goal of Study Two (Chapter 3) to determine potential mechanisms responsible for the amelioration of T2DM following RYGB.

In Chapter 3 we reported that pre-RYGB insulin sensitivity appears to be a predictor in the resolution of T2DM post-RYGB. Insulin sensitivity was correlated with FPG 3-months post-RYGB and patients with the greatest insulin sensitivity prior to RYGB experienced greater diabetes remission 3-months post-RYGB (defined as FPG >100mg/dl and no diabetic medication) compared to those patients that had low insulin sensitivity prior to RYGB. Also, duration of T2DM prior to surgery was negatively correlated with T2DM remission following RYGB, consistent with previous reports.

There was no association between pre-RYGB insulin secretion and T2DM reversal following RYGB suggesting that differences in insulin sensitivity between patients, but not insulin secretion are important for T2DM remission following RYGB.

Together, with the results from Chapter 2 indicating that the return of insulin to normal is an early and potentially important in RYGB-induced T2DM resolution and Chapter 3 that pre-RYGB insulin sensitivity is associated with remission of T2DM success following RYGB, we questioned if hyperinsulinemia could produce insulin resistance. Specifically, it was hypothesized that hyperinsulinemia would lower insulin-induced insulin signaling (Akt and AS160) in myotubes from both lean insulin sensitive (LN) and obese insulin resistant (OB) individuals. As insulin resistance is also associated with depressed fatty acid oxidation and mitochondrial dysfunction, we hypothesized that chronic hyperinsulinemia would lower fatty acid oxidation and mitochondrial content.

In Chapter 4, it was reported that while hyperinsulinemia did not induce insulin resistance in either LN or OB, LN and OB experienced divergent insulin signaling responses. In response to chronic hyperinsulinemia, LN responded by increasing AS160 phosphorylation whereas OB did no suggesting OB have a blunted response to chronic hyperinsulinemia. Given that hyperinsulinemia is known to induce insulin resistance, these results suggest that insulin resistance may not occur through decreased insulin signaling and implicates other mechanisms responsible for hyperinsulinemia-induced insulin resistance.

Also noted in Chapter 4, there were no differences in mitochondrial content between LN and OB for either insulin concentration. Even though there were no

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observed differences in mitochondrial content, incomplete fatty acid oxidation was increased and complete fatty acid oxidation was decreased in the OB compared to LN. This finding suggests that in the current study mitochondrial content may not be different between lean and obese, but that obese may not be as efficient in oxidizing fatty acids compared to leans.

In summary, it appears that RYGB may contribute to the amelioration of T2DM by reducing hyperinsulinemia and perhaps removing a currently unknown stimulus for insulin production. Further research is necessary to elucidate the exact mechanism by which RYGB acts to reverse hyperinsulinemia in T2DM patients within one-week of RYGB. This finding, along with the novel discovery that pre-RYGB insulin sensitivity is predictive of remission of T2DM post-RYGB have significant implications for clinical management of T2DM that may need to focus on the importance of maintaining insulin sensitivity. Finally, the finding that there is a divergent response in AS160 phosphorylation in response to hyperinsulinemia in cells from lean insulin sensitive and obese insulin resistant individuals suggests that hyperinsulinemia-induced insulin resistance occurs through pathways other than those through AS160 and warrants further exploration.

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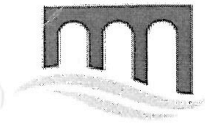
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University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building• 600 Moye Boulevard • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • www.ecu.edu/irb

TO: Walter Pories, MD, Department of Surgery, BSOM, ECU

FROM: UMCIRB

DATE: February 3, 2011

RE: Expedited Continuing Review of a Research Study

TITLE: "The Role of Incretins in Type 2 Diabetes"

UMCIRB #06-0135

The above referenced research study was initially reviewed and approved by the convened UMCIRB on 2/22/06. This research study has undergone a subsequent continuing review using expedited review on 11/16/10. This research study is eligible for expedited review because it is a continuing review of research previously approved by the convened IRB as follows:(a) where (i) the research is permanently closed to the enrollment of new subjects; (ii) all subjects have completed all research-related interventions; and (iii) the research remains active only for long-term follow-up of subjects; or (b) where no subjects have been enrolled and no additional risks have been identified; or(c) where the remaining research activities are limited to data analysis. The Chairperson (or designee) deemed this **Johnson & Johnson Pharmaceuticals/McNeil Nutritionals, LLC** sponsored study **no more than minimal risk** requiring a continuing review in **12 months**. 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.

The above referenced research study has been given approval for the period of **11/16/10** to **11/15/11**. The approval includes the following items:
- Continuing Review Form (dated 11/3/10)
- Protocol (version 10 dated 2/12/08)
- Informed consent: Bariatric patients (version 4 dated 2/12/08)
- Informed consent: Lean control (version 5 dated 2/12/08)

The Chairperson (or designee) does not have a conflict of interest on this study.

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418
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