

Abstract

Effect of Duodenal-jejunal Bypass on Skeletal Muscle Insulin Signaling in Goto-Kakizaki Rats

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Gastric bypass surgery (RYGBP) for the treatment of obesity has proven to clinically reverse type 2 diabetes mellitus. RYGBP involves both gastric reduction and bypass of the proximal small intestine. Duodenal-jejunal Bypass (DJB) is a surgical procedure that bypasses the proximal small intestine without gastric reduction and has been shown to improve oral glucose tolerance in Goto-Kakizaki (GK) rats, a non-obese animal model of T2DM. We hypothesized that DJB may improve oral glucose tolerance in GK rats by improving insulin signaling in skeletal muscle, the main depot for insulin stimulated glucose uptake. DJB was performed on male 10-12 week old GK rats (GK-DJB), and sham operations were performed on GK rats (GK-Sham). Insulin stimulated IRS-1, phospho-serine 307 of IRS-1, Akt, and phospho-Akt were determined using Western blot. Phospho-Akt was significantly higher in GK-DJB when compared to GK-Sham in soleus and tended to be higher in gastrocnemius ($p=0.107$). Akt was significantly higher in GK-DJB when compared to GK-Sham in gastrocnemius and tended to be higher in soleus ($p=0.074$). Phospho-serine 307 of IRS-1 and total IRS-1 were not different between GK-DJB and GK-Sham in gastrocnemius. In conclusion,

bypassing the proximal small intestine (DJB) improves components of skeletal muscle insulin signaling in GK rats, providing mechanistic evidence for the improvement in oral glucose tolerance associated with DJB.

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Kakizaki Rats

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INTRODUCTION

Type 2 diabetes is an epidemic that affects millions of people in the United States and worldwide. The disease is characterized by insulin resistance and the inability of the pancreas to secrete a sufficient amount of insulin needed to prevent hyperglycemia.

Although there are a number of treatment options available to attenuate the disease, the progression of pancreatic insufficiency becomes too great to prevent hyperglycemia. As type 2 diabetes worsens, the development of comorbidities such as cardiovascular disease becomes prevalent. Cardiovascular disease is the number one killer of Americans each year.

Interestingly, bariatric surgery for the treatment of morbid obesity has proven to have a secondary effect on type 2 diabetes. Operations such as Roux-En-Y Gastric Bypass (RYGBP) and Biliopancreatic Diversion (BPD) have proven to reverse type 2 diabetes in 80 – 100% of diabetic patients that undergo these operations. Bypassing of the proximal small intestine is a commonality between RYGBP and BPD and could play a role in the reversal of type 2 diabetes following surgery. Bypassing of the proximal small intestine can improve the type 2 diabetic condition without subsequent weight loss in non-obese diabetic rats. Bypassing of the proximal small intestine may lead to increased skeletal muscle insulin sensitivity, which is impaired in type 2 diabetes. Further investigation studying the role of bypassing the proximal small intestine on the reversal of type 2 diabetes could lead to a better understanding of the mechanisms that lead to the disease.

REVIEW OF LITERATURE

Prevalence and Economic Impact

The prevalence of type 2 diabetes mellitus (T2DM) has increased dramatically over the past few decades. From 1980 to 2005, the incidence of T2DM in the United States has tripled (Center for Disease Control, 2007). Worldwide, the disease has reached epidemic proportions; in 2003, 194 million people suffered from T2DM. This number is expected to reach 333 million by 2025 (International Diabetes Federation, 2007). The economic effects of diabetes are also very alarming. Annually, the direct costs associated with T2DM exceed \$153 billion dollars; when indirect costs are taken into account, the costs exceed \$286 billion (International Diabetes Federation, 2007).

Characteristics of T2DM

T2DM is the result of two major problems: insulin resistance and pancreatic insufficiency. Insulin resistance is characterized as a reduction in the ability of insulin sensitive tissues such as skeletal muscle and liver to respond to a given amount of insulin. The result is decreased glucose disposal by skeletal muscle and decreased insulin inhibition of glucose production by the liver. Pancreatic insufficiency is characterized by the inability of the pancreas to produce a sufficient amount of insulin to maintain normal glycemia. Both insulin resistance and pancreatic insufficiency can lead to above normal blood glucose levels, known as hyperglycemia. Hyperglycemic conditions can lead to numerous complications such as cardiovascular disease, retinopathy, and kidney disease. Cardiovascular disease results in the bulk of costs associated with T2DM.

Cardiovascular disease is the number one killer of people in the United States; each year, approximately 700,000 people die from cardiovascular disease (Center for Disease Control, 2007).

Treatment Options

There are a number of interventions used to clinically treat T2DM. The American Diabetes Association Executive Summary for the treatment of diabetes indicates that the first line of treatment involves a lifestyle modification that includes increasing physical activity and dietary changes. “People with diabetes should be advised to perform at least 150 min/week of moderate-intensity aerobic physical activity (50–70% of maximum heart rate)...combined with reducing calories and reducing intake of dietary fat (American Diabetes Association, 2008).” It has been universally accepted that regular exercise and a decrease in calorie intake have positive effects on T2DM. Regular exercise has been shown to improve blood glucose control, and higher levels of exercise intensity have been associated with even greater improvements in glucose control (Dixon et al., 2008; Knowler et al., 2002; Pan et al., 1997; Tuomilehto et al., 2001).

As the disease progresses, the Executive Summary suggests that drug therapies must be used. Metformin is typically the first line drug of choice for the treatment of T2DM. Metformin acts to decrease glucose output from the liver by down regulating gluconeogenesis; therefore, fasting glucose levels are reduced, acting to prevent hyperglycemia. Metformin also improves glycemia by acting to increase insulin sensitivity in peripheral tissues such as skeletal muscle while decreasing absorption of

glucose in the gastrointestinal tract (Bailey and Turner, 1996). As T2DM progresses, pancreatic function worsens resulting in an insufficient amount of insulin being produced. When insulin sensitizing drugs and lifestyle modification are no longer sufficiently effective in treating the disease, glycemic control is lost (DeFronzo, 1992). Insulin injections must be prescribed to make up for the inadequate production of insulin by the pancreas. With further progression of the disease, increased amounts of insulin must be used to treat the patient.

The most effective treatment for obese T2DM patients is bariatric surgery. Bariatric surgery was designed to treat morbid obesity, but surprisingly had a secondary effect on T2DM. As early as 1984, Flickinger et al. observed that out of 12 T2DM patients that underwent gastric bypass, only one required drug therapy post-operatively (Flickinger, 1984).

Presently, the standard patient requirement for bariatric surgery issued by the National Institute of Health is a body mass index (body mass index (BMI) = kg/m^2) ≥ 40 kg/m^2 or a BMI ≥ 35 kg/m^2 with comorbidities such as cardiovascular disease or diabetes (National Institute of Health, 1998). Among the most frequently performed bariatric surgeries is Roux-en Y gastric bypass (RYGBP, Figure 1A). RYGBP includes gastric restriction and bypass of the proximal small intestine. A systematic review and meta-analysis of bariatric surgery by Buchwald et al. revealed that RYGBP reversed T2DM in 84% of patients and resulted in approximately 100 lbs of body weight loss at one year post operatively. Reversing T2DM is clinically defined as the ability of a patient to be independent of all anti-diabetic medications while maintaining glycemic control (fasting blood glucose < 126 mg/dL) (Buchwald et al., 2004).

Biliopancreatic Diversion (BPD, Figure 1B) is a second bariatric surgical procedure similar to RYGBP in that it involves gastric restriction but with a greater amount of proximal small intestine bypass. BPD reverses T2DM in 98% of patients and results in a body weight loss of approximately 100 lbs (Buchwald et al., 2004). Gastric banding (LAGB) is a third bariatric surgical procedure that involves gastric restriction by placing an adjustable band around the cardiac region of the stomach; however, there is no bypassing of the proximal small intestine. Gastric banding reverses T2DM in 48% of patients and the resultant weight loss one-year post operatively is approximately 65 lbs (Buchwald et al., 2004). Despite the various lifestyle modifications and drug treatments available, bariatric surgery proves to be the most beneficial treatment for T2DM. In particular, bariatric surgical procedures that involve bypassing the small intestine have proven to be the most efficacious treatment for T2DM.

Mechanisms for Diabetes Reversal following Bariatric Surgery

The mechanisms by which RYGBP, BPD, and Gastric Banding reverse T2DM are not fully understood. There have been three proposed mechanisms for the reversal of T2DM following RYGBP: weight loss, food restriction and bypassing of the proximal small intestine. Weight loss occurs due to the reduced size of the stomach and malabsorption. Weight loss achieved following surgery has been suggested to reverse diabetes; LAGB, which involves only gastric reduction, has been shown to reverse T2DM but to a lesser extent than that of RYGBP and BPD (Buchwald et al., 2004). It is

believed that LAGB reverses T2DM solely due to weight loss due to the fact that there is no bypassing of the proximal small intestine.

Pories et al. observed that diabetes reversal takes place within a matter of days following RYGBP long before significant weight loss has occurred (Pories et al., 1995). Preliminary data from our laboratory demonstrates a significant reduction in fasting blood glucose within one week following RYGBP, with a modest body weight loss of 2 kg.

A study by Hickey et al. supports the hypothesis that weight loss does not solely account for the anti-diabetic effect of bariatric surgery (Hickey et al., 1998). In this study, RYGBP was performed on six patients (surgical group). After being weight stable for at least six months, the surgical group was compared to another non-surgical group of individuals that had not undergone RYGBP but were matched for current age, weight, fat mass, fat free mass, percent fat, BMI, waist circumference, and aerobic capacity. The non-surgical group was also weight stable. Compared to non-surgical weight matched controls, fasting plasma glucose and fasting plasma insulin levels were significantly lower in the RYGBP group, and insulin sensitivity measured by minimal model was higher (Hickey et al., 1998).

The fact that reversal of T2DM takes place before significant weight loss has occurred suggests that weight loss is not the only factor contributing to reversal of diabetes following RYGBP. A recent study by Laferrere et al. sought to discover whether caloric restriction plays a role in the reversal of diabetes following RYGBP. In this study, calorically restricted female subjects were matched to RYGBP surgical

patients for sex, age, body weight, BMI, and diabetes duration and control. GLP-1 levels and insulin levels were increased in the surgical group when compared to calorically restricted subjects that lost the same amount of weight as the surgical group. This study suggests that the early improvement of diabetes following RYGBP is the result of the surgical procedure and not the result of weight loss induced by caloric restriction (Laferrere et al., 2008).

Pories et al. were the first to hypothesize that RYGBP improved the T2DM condition by changes in endocrine mechanisms possibly associated with changes in the proximal small intestine after surgery (Pories et al., 1995). This “foregut hypothesis” suggests that an unknown mechanism associated with the proximal small intestine results in the reversal of T2DM following RYGBP and BPD, both of which bypass the proximal small intestine. The foregut hypothesis is also supported by the fact that Gastric Banding, which does not involve bypassing the proximal small intestine, does not achieve a rate of diabetes reversal similar to RYGBP and BPD. To further strengthen the hypothesis, BPD bypasses a greater portion of the proximal small intestine than that of RYGBP, and BPD has a greater rate of T2DM reversal when compared to that of RYGBP (Buchwald et al., 2004).

Foregut Hypothesis – Bypassing the Proximal Small Intestine

To investigate the foregut hypothesis proposed by Pories et al., Rubino et al. studied the effects of duodenal-jejunal exclusion on Goto-Kakizaki (GK) rats (Rubino and Marescaux, 2004). GK rats are a lean animal model of type 2 diabetes.

Characteristics of GK rats include: fasting hyperglycemia, impaired secretion of insulin in response to glucose, and peripheral insulin resistance. Males develop type 2 diabetes at approximately 14-16 weeks of age. Late complications include retinopathy, microangiopathy, neuropathy and nephropathy (Taconic, 2008).

Duodenal-jejunal bypass surgery (DJB, Figure 2) is a procedure in which the proximal intestine (foregut) is bypassed while leaving stomach volume unchanged. DJB is very similar to RYGBP; however, DJB does not include gastric restriction. In a study by Rubino and Marescaux, GK rats randomly underwent one of the following: DJB surgery, sham operation, food restriction, or no intervention (Wistar rats were used as controls) (Rubino and Marescaux, 2004). Transections and enterotomies were performed on sham operated GK rats to mirror those that were performed in DJB operated GK rats. Transections and enterotomies were then reanastomosed in the sham operated GK rats to leave in place a normal anatomical gastrointestinal tract.

Fasting glucose levels were significantly reduced in DJB operated animals versus sham operated. DJB significantly improved glucose tolerance compared to sham operated and food restricted GK rats. Thirty-six weeks following surgery, glucose tolerance was still significantly improved in DJB versus sham operated GK rats. DJB operated rats showed significantly less weight loss than food restricted GK rats suggesting that weight loss is not the sole reason for the improvement of glucose tolerance following DJB (Rubino and Marescaux, 2004).

In this same study by Rubino and Marescaux, another group of GK rats randomly underwent one of the following: DJB, rosiglitazone treatment, or no intervention.

Rosiglitazone is an anti-diabetic drug that increases insulin sensitivity in liver, muscle, and adipose tissue via activation of the peroxisome proliferator-activated receptor γ (PPAR γ). DJB and rosiglitazone both improved glucose tolerance; however, DJB improved glucose tolerance to a greater extent than rosiglitazone as shown by a decreased glucose area under the curve in response to oral glucose tolerance (Rubino and Marescaux, 2004).

Cohen et al. studied the effects of laparoscopic DJB surgery in humans in two cases. Criteria for surgery was 20-30 years of age, BMI 22-34, and a history of T2DM for less than or equal to 10 years. Both patients were discharged from the hospital 48 hours following surgery. At discharge patient 1 had their oral agent withdrawn and insulin dosage decreased from 22 to 13 U. Patient 2 was discharged without any anti-diabetic medications. Fasting glucose, plasma insulin, and HbA1c levels were normalized without significant changes in BMI after 9 months following surgery (Cohen et al., 2007).

Hindgut Hypothesis – Rapid Delivery of Nutrients to the Distal Small Intestine

A later study by Rubino et al., sought to discover whether the rapid resolution of diabetes after DJB was due to the enhanced delivery of nutrients to the distal intestine and increased secretion of hindgut signals that improve glycemia or because of altered signals from the excluded segment of proximal intestine (Rubino et al., 2006). To test the “hindgut hypothesis,” Rubino et al. studied the effects of Gastrojejunostomy on GK rats.

Gastrojejunostomy is a surgery that delivers nutrients to the distal intestine without excluding the proximal intestine (Rubino et al., 2006).

In this study, GK rats randomly underwent one of the following: DJB surgery, GJ surgery, sham operation, or no intervention (Wistar rats, a non-diabetic control was used for no intervention). DJB GK rats showed significantly better glucose tolerance than all other GK groups without significant weight loss when compared to other surgical groups. The GJ procedure had no impact on glucose tolerance; however, after GJ rats were re-operated on to convert to DJB, glucose tolerance was significantly improved. DJB rats also underwent another surgery to convert them to GJ in order to test whether or not a reversal in glycemic improvements would be observed. This surgery had a high mortality rate; however, the two rats that survived the conversion surgery from DJB to GJ showed a reversal of glycemic improvements (Rubino et al., 2006).

Effects of Bariatric Surgery on Insulin Sensitivity

Bariatric surgery has proven to have a profound effect on insulin sensitivity. One week following BPD, Guidone et al. observed a 2-fold increase in insulin sensitivity in obese patients as determined by euglycemic-hyperinsulinemic clamp when compared to pre-surgical values. Total insulin output and fasting plasma insulin levels were also decreased by approximately 2-fold, confirming the increase in insulin sensitivity (Guidone et al., 2006). Hickey et al. showed a similar improvement in insulin sensitivity as determined by minimal model. Patients who underwent RYGBP enjoyed a 2-fold increase in insulin sensitivity when compared to non-surgical controls (Hickey et al.,

1998). These studies by Guidone et al. and Hickey et al. suggest that bypassing of the foregut alone might have a positive effect on insulin sensitivity; in particular, skeletal muscle insulin sensitivity may be improved due to the fact that 80-90% of insulin stimulated glucose uptake occurs in skeletal muscle tissue.

Insulin Signaling in Skeletal Muscle

Activation of the insulin receptors located on the plasma membrane of skeletal muscle cells leads to a cascade of intracellular events that ultimately results in the facilitated diffusion of glucose into the intracellular compartment where it can be used for energy production. There are many proteins responsible for the regulation of the insulin stimulated uptake of glucose in skeletal muscle (Figure 3); however, several key proteins have been universally accepted as being essential to the cascade of molecular events leading to glucose disposal in skeletal muscle. These proteins include: insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and the glucose transporter, GLUT4. The activation of these proteins, respectively, leads to GLUT4 exocytosis from the intracellular compartment to the plasma membrane. GLUT4 is responsible for the transport of glucose from the plasma membrane to the intracellular compartment (Thong et al., 2005). A defect in the insulin cascade has been shown to lead to insulin resistance and type 2 diabetes (Karlsson et al., 2005).

Tamemoto et al. showed that Tyrosine phosphorylation of IRS-1 leads to normal insulin stimulated glucose disposal by subsequent activation of PI3K. PI3K stimulates the phosphorylation of Akt and further activation of the glucose transporter, GLUT4

(Tamemoto et al., 1994); however, decreased Tyrosine phosphorylation of IRS-1 is observed with an increase in Serine 312 (307 in rats) phosphorylation of IRS-1 (Hotamisligil et al., 1996). Subsequently, an increase in Serine 312 phosphorylation results in decreased insulin sensitivity by reducing insulin stimulated glucose uptake.

Downstream defects in the insulin signaling cascade can also lead to decreased insulin sensitivity. Akt knockout mice have been shown to develop severe type 2 diabetes (Yang et al., 2004). With normal IRS-1 and PI3K activity, these Akt knockout mice exhibit decreased insulin sensitivity. Supporting these findings, Cozzone et al. observed that phosphorylation of Serine 473 on Akt was significantly lower in myotubes of type 2 diabetic patients when compared to healthy control participants (Cozzone et al., 2008).

Summary

Type 2 diabetes is becoming more prevalent in the United States and worldwide. T2DM is a progressive disease that worsens due to an increase in both insulin resistance and pancreatic insufficiency over time, resulting in hyperglycemia. Lifestyle modifications, medications, and surgery are effective at treating the disease; however, surgery, specifically surgery that bypasses the proximal small intestine, proves to have the greatest rate of diabetes reversal and increase in insulin sensitivity. DJB is an experimental surgical procedure that involves bypassing the proximal small intestine without gastric restriction. Studies have shown that DJB improves glucose tolerance in a non-obese animal model of type 2 diabetes and humans. DJB may improve type 2

diabetes by inducing positive changes in skeletal muscle insulin sensitivity. A better understanding of the mechanisms by which DJB improves glucose tolerance could lead to improved treatment options for type 2 diabetes.

Hypothesis

DJB will improve skeletal muscle insulin signaling in a non-obese animal model of type 2 diabetes.

MATERIALS AND METHODS

Rats

Male Goto-Kakizaki (GK) rats 10-12 weeks of age were used (Taconic of North America). All rats were kept in a controlled setting at 22° C and on a 12:12 hour light:dark cycle and fed a standard chow and water ad libitum. After one week of acclimatization in the facility, GK rats were randomly assigned to one of two surgical groups: Duodenal Jejunal Bypass operation (GK-DJB) or Sham operation (GK-Sham) (see below). DJB and Sham operations were performed as previously described by Rubino and Marescaux (Rubino and Marescaux, 2004).

Pre-Operative Care

Eighteen hours prior to surgery, food was removed to avoid the presence of substance in the gastrointestinal tract, and wire grates were placed in cages to elevate the rat from the bottom surface of the cage to avoid consumption of fecal matter. Thirty minutes prior to surgery, an intramuscular injection of Ceftriaxone (3rd generation cephalosporin antibiotic) was given prophylactically at a dosage of 0.25 g/kg. Rats were anesthetized using a mixture of 2% isoflurane and 1.5 L O₂/minute, titrated as needed for proper level of anesthesia. Immediately following administration of general anesthesia, the surgical site was shaved, cleaned using a 2% chlorohexidine solution, and paralube was placed in the eyes to prevent drying. Prior to the initial incision, an analgesic (morphine at 10 mg/kg) was administered subcutaneously. Rats were kept on a water-circulated heating pad throughout the duration of the surgery.

Duodenal-jejunal Bypass Operation

The duodenum and proximal jejunum were bypassed, and the volume of the stomach was not altered. The duodenum was transected just distal to the pylorus, and closed off at its proximal end (closest to stomach). The jejunum was transected 10 cm distal to the ligament of Treitz; the distal of the two limbs was connected to the pylorus, creating the gastrojejunal anastomosis; the proximal of the two limbs carrying the biliopancreatic juices was connected 25 cm distal to the ligament of Treitz (in the context of the original continuity of small bowel), creating the jejunojejunal anastomosis. All anastomoses were created using 6-0 resorbable suture. The length of the bypass with DJB was proportional to the proximal small intestinal exclusion in human RYGBP.

Sham Operation

Sham operations were performed by transecting the jejunum 10 cm distal to the ligament of Treitz and by separating the duodenum from the pylorus. Both transections were then reanastomosed, restoring the normal anatomical structure of the gastrointestinal tract.

Post-Operative Care

Immediately following surgery, rats were placed in a warming, oxygenated chamber at approximately 30° C and 1.5 L O₂/minute. Animals were then given access to liquid food supplementation (Boost[®]) for up to two days following surgery and water

ad libitum. Three hours following surgery, rats were given a second subcutaneous injection of analgesia (morphine at 10 mg/kg). One day and up to seven days following surgery, animals were given a non-steroidal anti-inflammatory analgesic as needed (Meloxicam, 5 mg/kg). Three days following surgery, standard chow was introduced to the rats *ad libitum* and liquid food supplementation was removed. The Institutional Animal Care and Use Committee of East Carolina University approved all procedures.

Insulin-Glucose Injections

Approximately two weeks following surgery, rats were fasted for 12 hours. All rats received an intraperitoneal injection of insulin (0.36 mg/kg BW) and glucose (1.32 g/kg BW). Ten minutes following injections, the first 10 animals were euthanized with 70% carbon dioxide/30% oxygen for one minute, followed by 100% carbon dioxide for five minutes and cervical dislocation and exsanguination. The remaining animals were euthanized by cervical dislocation and exsanguination following sedation under 2-3 % isoflurane. Immediately following death, left and right gastrocnemius and soleus muscles were excised and stored at -80° Celsius.

Muscle Powdering

Gastrocnemius and soleus samples were removed from -80° C storage freezer and immediately placed into liquid nitrogen. A stainless steel mortar and pestle were removed from -80° C storage freezer and placed on dry ice. A small amount of liquid

nitrogen was added to the mortar. The muscle sample was placed in the mortar and covered with the pestle. The pestle was hammered until muscle was sufficiently powdered. Liquid nitrogen was added in small amounts as needed to prevent muscle from thawing. A spatula was used to transfer powdered muscle from mortar to scintillation vial. Scintillation vials were placed in liquid nitrogen.

Muscle Homogenization and Preparation

Homogenization buffer was made using 20mL of lysis buffer (50 mM Hepes, 10 mM EDTA, 100 mM NaF, and 50 mM Na pyrophosphate), 0.037g sodium orthovanadate (MW 183.91), 100 μ L protease inhibitor, 200 μ L phosphatase cocktail I, and 200 μ L phosphatase cocktail II. Homogenization tubes were prepared and labeled for each sample, and 1 mL of homogenization buffer were added to each tube. Tubes were placed on ice at 4° Celsius. Approximately 50 mg of each powdered muscle sample was added to homogenization tubes. Samples were homogenized one at a time with tubes placed in ice water, 3 x 15 seconds with ultraturax on setting blue with 20 seconds rest between each homogenization. The homogenizer was washed with distilled deionized water and homogenization buffer between samples and then dried. 50 μ L of 20% detergent [Triton (1% final)] were added to each sample and vortexed on setting 5 for 3 seconds. Each sample was sonicated on ice for 10 seconds at a setting of 2.5. Following sonication, samples were transferred to 1.7 mL microcentrifuge tube and allowed to rotate at 4° C for 2 hours. Tubes were then centrifuged at 14,000 rpm for 15 minutes at 4° C. Supernatant

was collected using a small transfer pipette and placed in a separate 1.7 mL microcentrifuge tube. Samples were stored at -80° C.

BCA procedure and Western Blot

Standards were run in triplicate using BSA at concentrations of 0, 0.125, 0.250, 0.500, 0.750, 1.000, 1.500 and 2.000 µg/ µL. Samples were diluted with distilled deionized water by a factor of 7 (10 µL of sample). Samples + 190 µL of BCA reagent were added to each well in triplicate and incubated for 30 minutes at 37° C. The plate was cooled to 20° C and read at 540 nm.

Equal amounts of solubilized proteins (50 µg) were resolved using SDS-PAGE and transferred to nitrocellulose membrane. A 7.5 % gel was run at 100 V for approximately 30 minutes and at 150 V for an additional 60 minutes. Membrane was blocked with 5 % milk in Tris buffered saline with 10 % Tween (TBST) buffer (10% Tween) and probed with primary antibody; antibodies specific for phospho-Akt (product # SC-7985-R; Santa Cruz, USA), total Akt (product # sc-8312; Santa Cruz, USA), and IRS1 Serine 307 phosphorylation (product # 07-247; Millipore, USA) raised from rabbits were used. Incubation with the primary antibody was followed by the anti-rabbit secondary antibody (product # sc-2004; Santa Cruz, USA). Enhanced chemiluminescent detection was used (product # 34080; ThermoScientific, USA). Film was exposed, and protein levels were quantified using computerized densitometry (Gel-Pro Analyzer® software).

Statistical Analysis

A student's t-test (Sham vs. DJB) and 2-way ANOVA (Time (Pre- and Post-Operative) × Surgical treatment) were used. Significance was established at $p < 0.05$.

RESULTS

Pre and Post Operative Weights

Body weights were measured in male GK-Sham (n=12) and GK-DJB (n=11) rats prior to surgical treatment and 2 weeks post-operatively. Body weights are presented in Figure 4. There were no differences in body weight between GK-Sham and GK-DJB pre-operatively (GK-Sham pre-op: 335.8 ± 5.5 vs. GK-DJB pre-op: 334.2 ± 8.1) and post-operatively (GK-Sham post-op: 330.4 ± 6.3 vs. GK-DJB post-op: 327.5 ± 6.0).

Insulin Signaling: Gastrocnemius

Phosphorylation of Akt tended ($p=0.107$) to be higher in GK-DJB when compared to GK-Sham (1.21 ± 0.21 and 0.81 ± 0.12 , respectively; Figure 5). Akt protein content was higher ($p=0.039$) in GK-DJB when compared to GK-Sham (1.10 ± 0.08 and 0.91 ± 0.04 , respectively; Figure 5). Phosphorylation of Akt corrected for total Akt (pAkt/Akt) was not different in GK-DJB when compared to GK-Sham (1.04 ± 0.13 and 0.86 ± 0.11 , respectively; Figure 5). Phosphorylation of serine 307 of IRS-1 was not different in GK-DJB when compared to GK-Sham (41675 ± 13472 and 46491 ± 9549 , respectively; Figure 6). IRS-1 protein content was not different in GK-DJB when compared to GK-Sham (2.54 ± 0.27 and 2.53 ± 0.40 , respectively; Figure 6). Phosphorylation of serine 307 corrected for total IRS-1 was not different in GK-DJB when compared to GK-Sham (17659 ± 5841 and 21701 ± 5645 , respectively; Figure 6).

Insulin Signaling: Soleus

Phosphorylation of Akt was significantly higher ($p=0.007$) in GK-DJB when compared to GK-Sham (1.16 ± 0.09 and 0.86 ± 0.05 , respectively; Figure 7). Akt protein content tended to be higher ($p=0.074$) in GK-DJB when compared to GK-Sham (1.13 ± 0.10 and 0.87 ± 0.10 , respectively; Figure 7). Phosphorylation of Akt corrected for total Akt (pAkt/Akt) was not different in GK-DJB when compared to GK-Sham (1.08 ± 0.11 and 1.18 ± 0.24 , respectively; Figure 7).

Figure 1

A) Roux-en-Y Gastric Bypass (RYGBP) – creation of a small gastric pouch while the jejunum is transected 30 to 50 cm distal to the ligament of Treitz. The distal limb of the jejunum is then anastomosed to the gastric pouch (Gastrojejunostomy). A jejuno-jejunoanastomosis is performed 50 to 150 cm distal from the Gastrojejunostomy.

B) Biliopancreatic Diversion (BPD) – includes resection of the distal stomach and includes diversion of the biliopancreatic secretions to the terminal ileum, 50 to 100 cm proximal to the ileo-cecal valve (adapted from Rubino et. al., 2004).

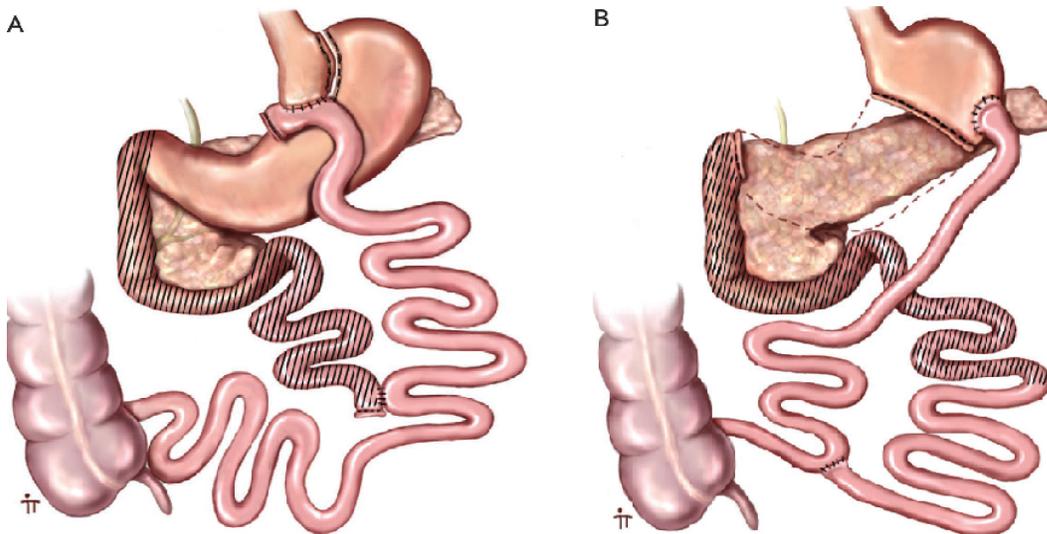


Figure 2

Duodenal-jejunal Bypass (DJB) – the duodenum was separated from the stomach, and the jejunum was transected 8 cm distal to the ligament of Treitz. The distal of the two limbs was connected to the stomach, and the proximal of the two limbs was anastomosed, end-to-side, to the bowel 12 cm distal to the jejunal transection (adapted from Rubino et. al., 2004).

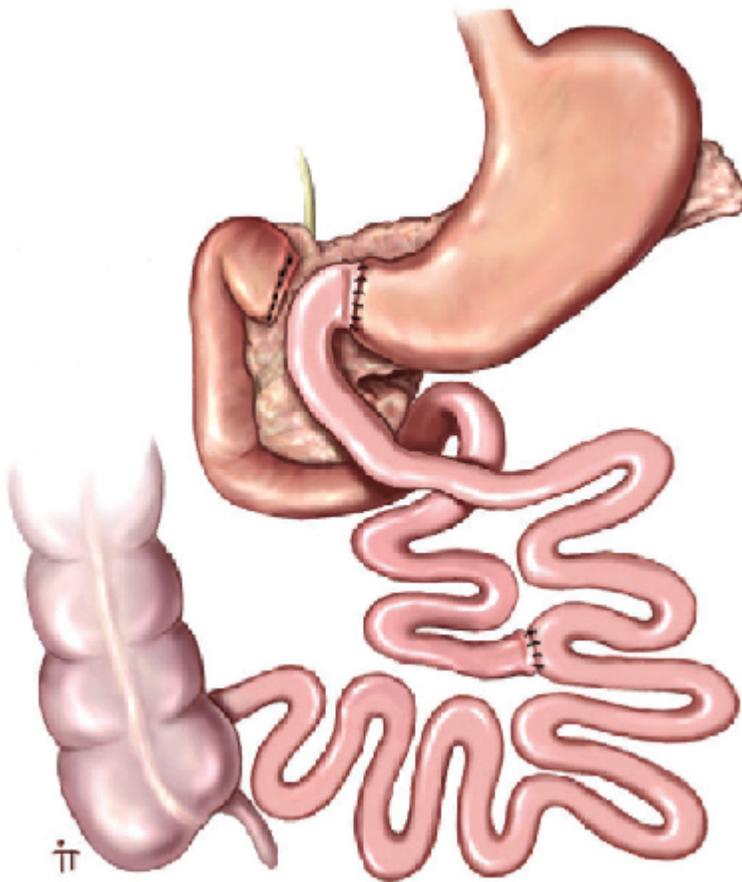


Figure 3

Insulin stimulated cascade of molecular events leading to glucose uptake. Insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K), phosphoinositide dependent kinase (PDK), protein kinase B (Akt), glucose transporter (GLUT4).

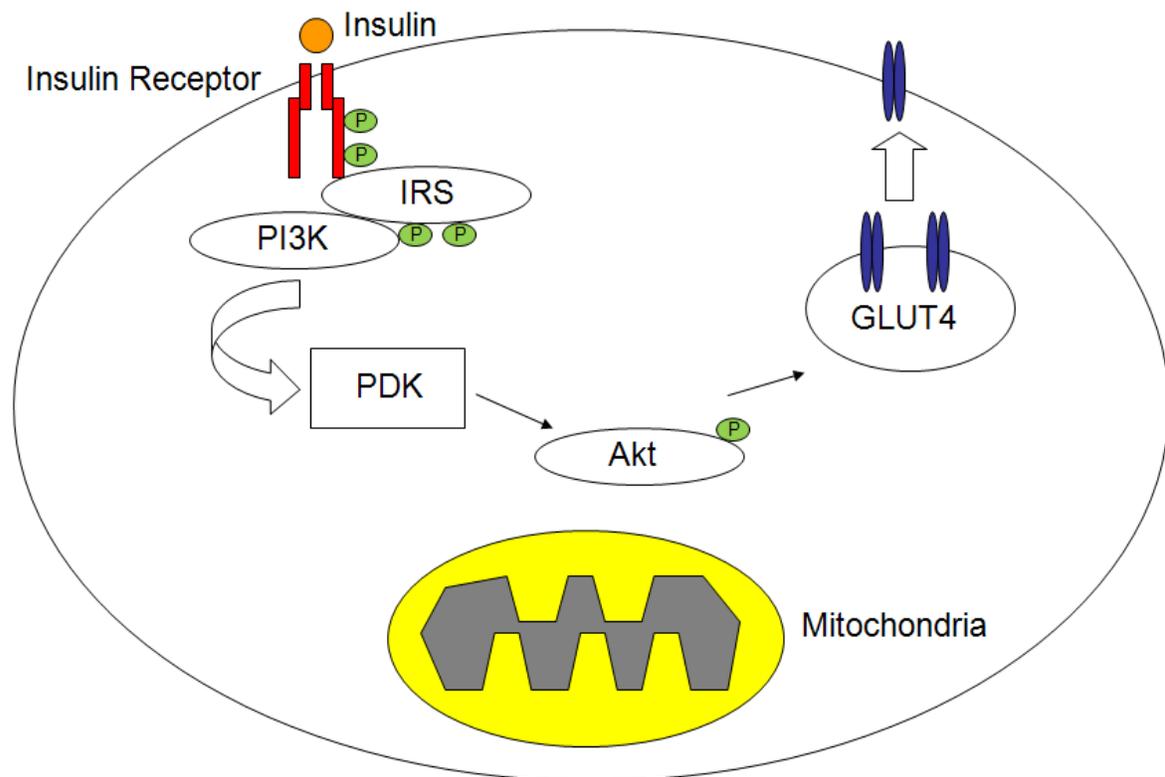


Figure 4

Rat weights pre-op and 2 weeks post-op of Sham (n=12) and DJB (n=11). There is no statistical significance between groups pre and 2 weeks post surgery.

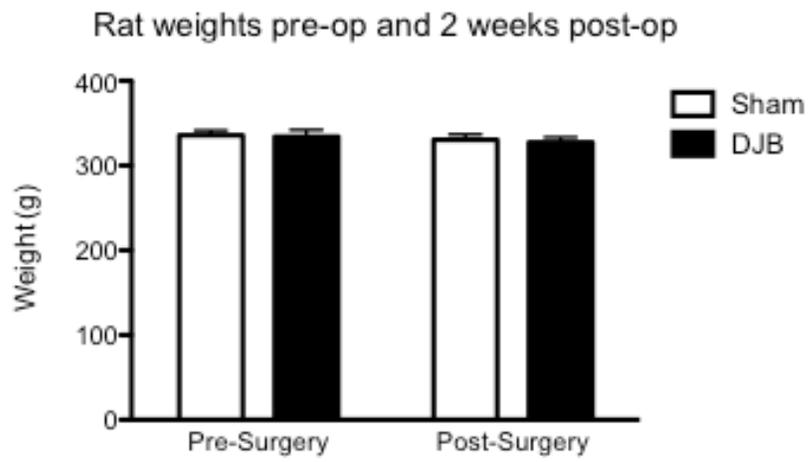


Figure 5

Phosphorylation of Akt in GK-Sham (n=12) and GK-DJB (n=11) in gastrocnemius muscle. Phosphorylation of Akt tended to be higher in GK-DJB when compared to GK-Sham but was not statistically significant. Akt protein content in GK-Sham (n=12) and GK-DJB (n=11) in gastrocnemius muscle. Akt protein content was higher in GK-DJB when compared to GK-Sham ($p=0.039$). Phosphorylation of Akt corrected for total Akt (pAkt/Akt) in GK-Sham (n=12) and GK-DJB (n=11) in gastrocnemius muscle. pAkt/Akt was not different in GK-DJB when compared to GK-Sham.

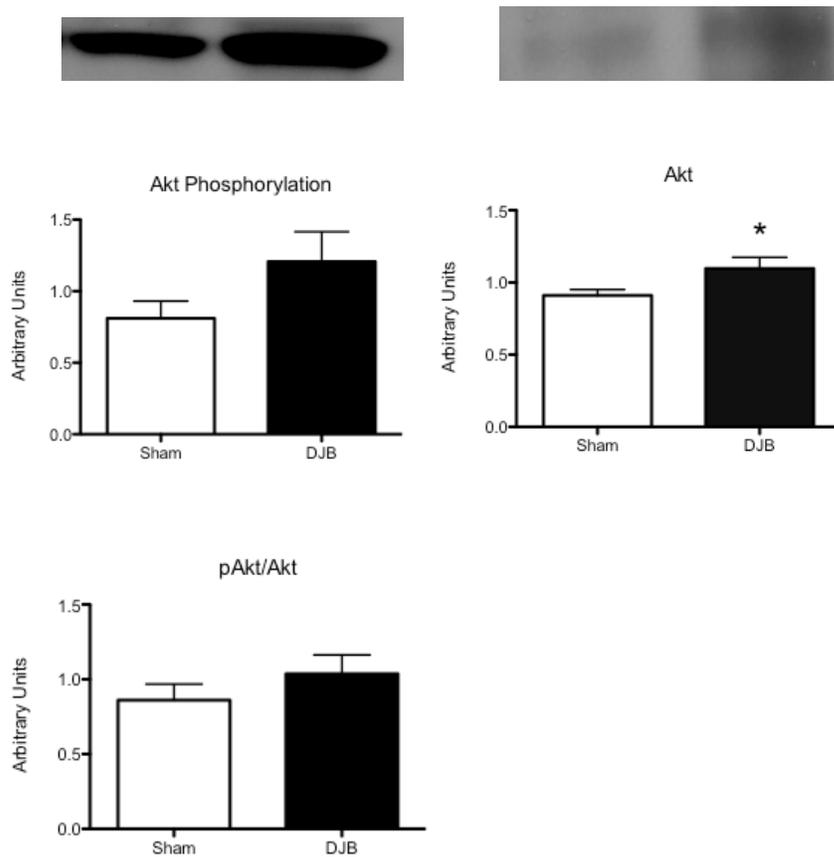


Figure 6

Phosphorylation of serine 307 of IRS-1 in GK-Sham (n=6) and GK-DJB (n=5) in gastrocnemius muscle. Phosphorylation of serine 307 of IRS-1 was not different in GK-DJB when compared to GK-Sham. IRS-1 protein content GK-Sham (n=6) and GK-DJB (n=5) in gastrocnemius muscle. IRS-1 was not different in GK-DJB when compared to GK-Sham. Phosphorylation of serine 307 corrected for total IRS-1 (Pser307/IRS-1) in GK-Sham (n=6) and GK-DJB (n=5) in gastrocnemius muscle. Pser307/IRS-1 was not different in GK-DJB when compared to GK-Sham.

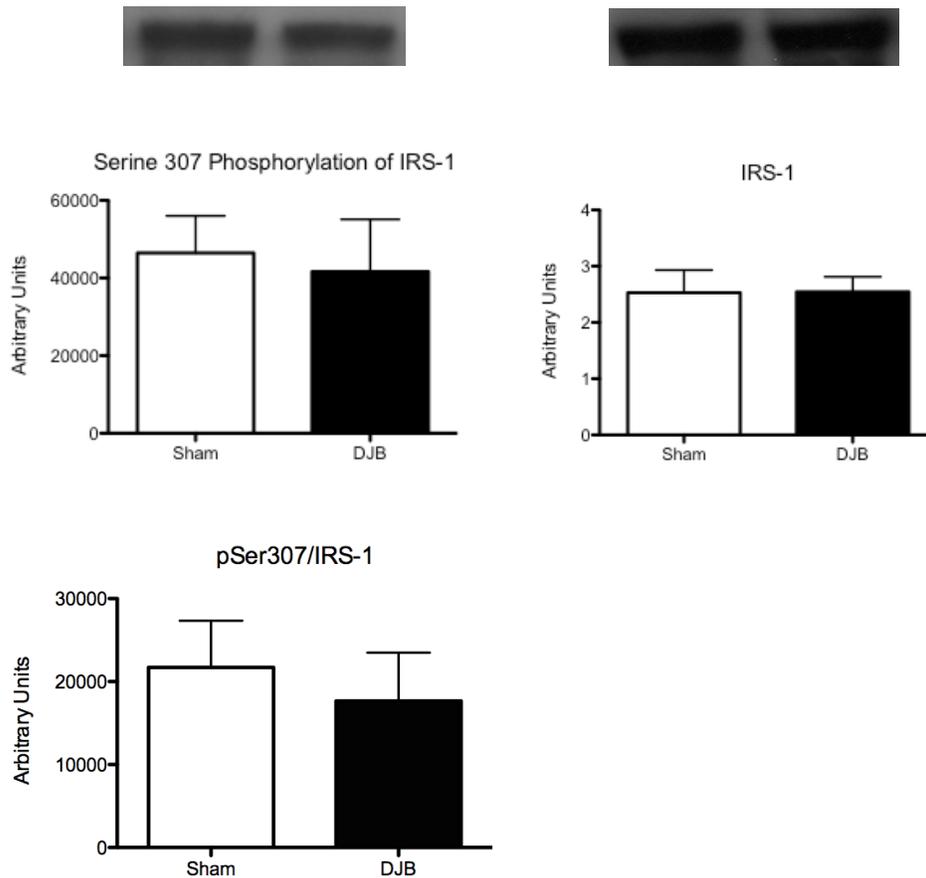


Figure 7

Phosphorylation of Akt in GK-Sham (n=10) and GK-DJB (n=10) in soleus muscle.

Phosphorylation of Akt was significantly higher in GK-DJB when compared to GK-

Sham (p=0.007). Akt protein content in GK-Sham (n=10) and GK-DJB (n=10) in soleus

muscle. Akt protein content tended to be higher in GK-DJB when compared to GK-

Sham (p=0.074). Phosphorylation of Akt corrected for total Akt (pAkt/Akt) in GK-Sham

(n=10) and GK-DJB (n=10) in soleus muscle. pAkt/Akt was not different in GK-DJB

when compared to GK-Sham.

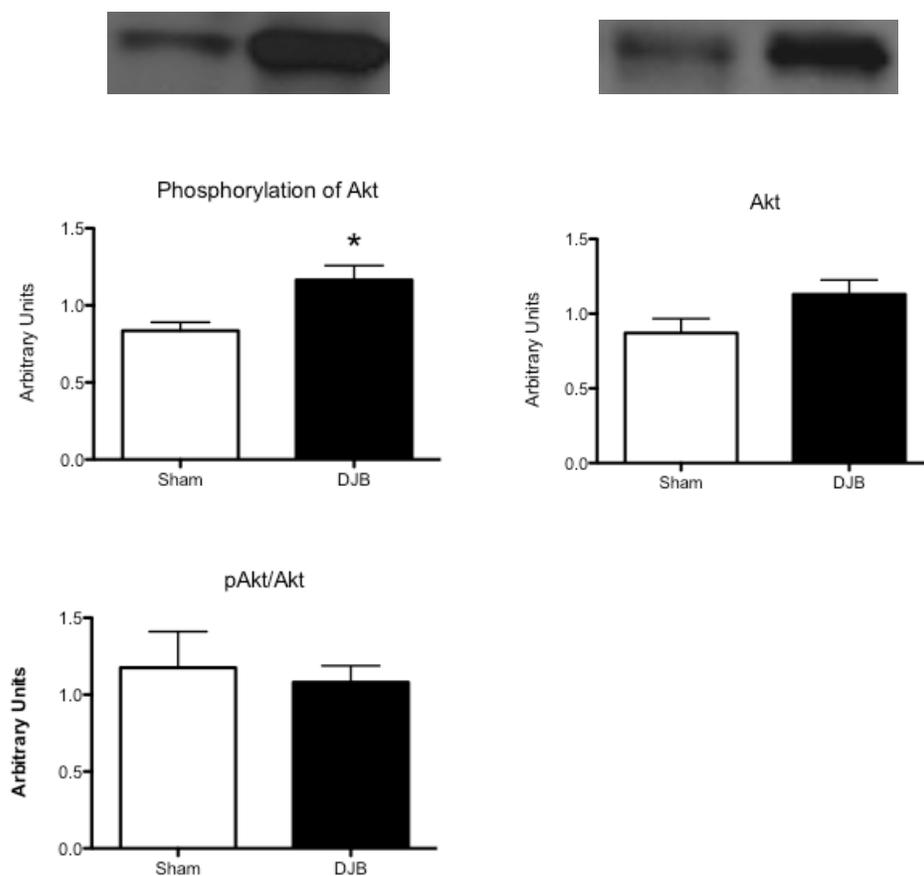
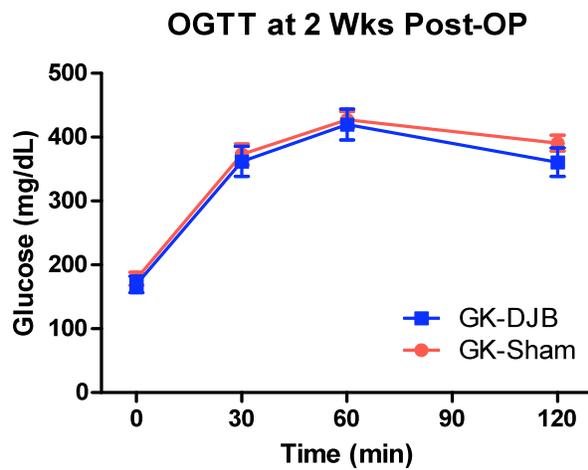


Figure 8

Oral glucose tolerance in GK-Sham (n=12) versus GK-DJB (n=11) rats 2 weeks following surgery. There is no statistical difference between GK-Sham and GK-DJB oral glucose tolerance.



DISCUSSION

Main Findings

Similar to Roux-En-Y Gastric Bypass (RYGBP) (Bikman et al., 2008), Duodenal-jejunal Bypass (DJB) tended to improve insulin signaling in skeletal muscle. In particular, insulin-stimulated Akt phosphorylation tended to be higher in GK-DJB rats when compared to GK-Sham in both gastrocnemius and soleus muscle. Interestingly, the improvement observed in insulin signaling following DJB occurs rapidly, within two weeks, without weight loss. In contrast to RYGBP, DJB does not alter serine 307 phosphorylation of IRS-1, an upstream inhibitory component of insulin signaling.

Improvement of glycemia following DJB

Pories et al. were the first to hypothesize that RYGBP improved T2DM by changes in endocrine mechanisms possibly associated with changes in the proximal small intestine after surgery (Pories et al., 1995). Recently, Rubino et al. showed that DJB can improve glycemia and oral glucose tolerance, without weight loss, in Goto-Kakizaki (GK) rats (Rubino and Marescaux, 2004), suggesting that the improvement in glucose tolerance following DJB may be due to mechanisms involved in the proximal small intestine. The improvement in glycemia following DJB strengthens the foregut hypothesis: bypassing the proximal small intestine may be responsible for the improvement in glycemia following surgery. Unlike the study conducted by Rubino and Marescaux, the present study did not observe a difference in oral glucose tolerance in GK-DJB vs. DK-Sham.

DJB Effects on Downstream Insulin Signaling

The current work hypothesized that the improvement in glycemia following DJB might be due to an improvement in skeletal muscle insulin signaling. Skeletal muscle is responsible for the majority of insulin stimulated glucose uptake suggesting that an improvement in insulin signaling could lead to an improvement in glycemia and oral glucose tolerance. Contrary to the study performed by Rubino et al., the current study did not show an increase in glucose tolerance (Figure 8). This may have been due to the low endogenous insulin secretion in the GK rats; however, the improvement observed in insulin signaling may have been due to the dosage of insulin administered, which may have circumvented the low endogenous insulin secretion. The current study showed a significant increase in phosphorylation of Akt in soleus ($p=0.007$) muscle, and phosphorylation of Akt in gastrocnemius muscle tended to be higher ($p=0.107$). Phosphorylation of Akt in skeletal muscle is a key downstream event in insulin signaling (Tamemoto et al., 1994). The phosphorylation of Akt indirectly leads to the relocation of GLUT4 to the sarcolemma allowing for the entry of glucose into cell (Tamemoto et al., 1994). Interestingly, the increase observed in Akt phosphorylation occurred concomitantly with an increase in total Akt protein content. Akt was significantly higher in gastrocnemius muscle and tended ($p=0.074$) to be higher in soleus muscle. Due to the increase in total Akt protein content, phosphorylation of Akt corrected for total Akt content was not significantly different in GK-DJB when compared to GK-Sham. Previously, Park et al. showed that Akt mRNA may be increased following RYGBP, suggesting that total Akt protein expression may be increased (Park et al., 2006). These data support the notion that bypassing the proximal small intestine may result in an

increase in Akt protein expression, though it is not possible to separate the effects of intestinal bypass from weight loss in these patients.

These data suggest that the improvement in oral glucose tolerance following DJB may be due to an increase in insulin stimulated glucose disposal in skeletal muscle. The increase in Akt phosphorylation as a result of increased Akt content suggests that an increase in the activation of upstream insulin signaling proteins may not be responsible for the improvement in oral glucose tolerance following DJB. The increased phosphorylation of Akt observed in GK-DJB may be due to the fact that there is more intramyocellular Akt available for phosphorylation following surgery and that the upstream signaling proteins responsible for the phosphorylation and activation of Akt were not rate limiting prior to surgery.

DJB Effects on Upstream Insulin Signaling

Phosphorylation of serine 307 of IRS-1 has been shown to be associated with insulin resistance and T2DM (Hotamisligil et al., 1996). Phosphorylation of serine 307 of IRS-1 has been shown to be decreased following RYGBP in T2DM patients (Bikman et al., 2008). In contrast to RYGBP, DJB had no effect on serine 307 phosphorylation of IRS-1 nor was total IRS-1 content altered. These data suggest that the increase in phosphorylation of Akt is not due to improved insulin signaling caused by a reduction in serine 307 phosphorylation following DJB; rather, these data support the proposition that the increase in phosphorylation of Akt may be due to the fact that there is simply a greater amount of intramyocellular Akt available for phosphorylation.

Regulation of Akt

Mammalian target of rapamycin (mTOR) is an important serine/threonine kinase that like PDK1 phosphorylates Akt and leads to the activation of Akt (Figure 8). Interestingly, mTOR is upregulated by Akt in a positive feedback mechanism; therefore, mTOR acts as both an upstream and downstream regulator of Akt. mTOR also has additional control on protein synthesis. When activated, mTOR phosphorylates 4E-binding protein (4E-BP), an important factor in translational control of protein synthesis. When 4E-BP is hypophosphorylated, translation of particular proteins is inhibited. Following 4E-BP phosphorylation by mTOR, activation of eIF4E occurs allowing protein synthesis to occur. mTOR may be increased following DJB allowing for increased phosphorylation of Akt and an increase in Akt protein content due to its effects on protein synthesis.

The increase in Akt protein content following DJB may also be due to reductions in the mechanisms that lead to the degradation of Akt. Increased intracellular levels of ceramide have been shown to increase degradation of Akt (Martin et al., 2002). Additionally, increased ceramide levels have been shown to be associated with insulin resistant skeletal muscle further supporting its possible effects on Akt (Adams et al., 2004). DJB may increase Akt protein content by reducing the mechanism by which ceramide accumulates in skeletal muscle.

Clinical Implications

Approximately 52% of type 2 diabetic patients are not obese and would therefore not meet the criteria to receive RYGBP and receive its beneficial treatment for T2DM (Center for Disease Control and Prevention, 2007). DJB could be a beneficial surgical treatment for lean T2DM patients that would presently be ineligible for RYGBP. Recently, Cohen et al. and Ramos et al. have provided evidence that DJB can improve the type 2 diabetic condition in patients (Cohen et al., 2007 and Ramos et al., 2009). These studies showed that glycosylated hemoglobin was decreased within 6 months following DJB in T2DM patients with BMI < 30 kg/m².

Conventional treatments for type 2 diabetes such as diet and exercise have proven to be beneficial; however, these treatments rarely show long-term effectiveness (Paisey et al., 2002 and Pinkney et al., 2001). Additionally, oral anti-diabetic agents and insulin therapy have proven to be beneficial but not without limitations, side effects, and a reduction in quality of life (Evans and Krentz, 1999 and Liebl, 2002). DJB could offer a one time surgical treatment for T2DM in patients with a body mass index < 35 kg/m². With any surgery, DJB will involve the risk of mortality; however, the mortality risk associated with RYGBP in obese patients is 0.2% (Gentileschi et al., 2002). Due to the comorbidities associated with obese patients undergoing RYGBP, the risk of mortality associated with lean patients undergoing DJB may be substantially less.

Future Investigations

Future studies should be conducted in order to elucidate the mechanism for increased Akt and phosphorylation of Akt following DJB. Although serine 307

phosphorylation associated with IRS-1 was not different between GK-DJB and GK-Sham, tyrosine phosphorylation of IRS-1 may have been altered. Tyrosine phosphorylation of IRS-1 is a required event in insulin signaling (Tamemoto et al., 1994). The improvement in the phosphorylation of Akt may be due to an increase in tyrosine phosphorylation or a decrease in tyrosine phosphatase activity of IRS-1 following DJB. The increase in tyrosine phosphorylation of IRS-1 and decrease in tyrosine phosphatase activity associated with IRS-1 could lead to further activation of downstream insulin signaling proteins such as the phosphorylation of Akt; however, an improvement in tyrosine phosphorylation of IRS-1 could not explain the increase in total Akt protein content.

mTOR may also be increased following DJB which could lead to both phosphorylation of Akt and an increase in Akt. The increase in Akt may be due to increased protein synthesis of Akt through the mTOR pathway. An increase in mTOR following DJB could lead to increased translation of Akt mRNA. Measuring mTOR content following DJB could provide insight into the mechanism by which Akt is increased following DJB.

Summary

DJB improves downstream insulin signaling in skeletal muscle of GK rats, a non-obese animal model of T2DM. The improvement in insulin signaling occurs due to both an increase in the phosphorylation of Akt and an increase in total Akt protein content. Interestingly, the improvement in insulin signaling occurs without weight loss suggesting

that the mechanism for improvement may be due to exclusion of the proximal small intestine. Changes in the continuity of the gastrointestinal tract involved in DJB may alter cellular mechanisms associated with Akt phosphorylation and content. Further studies should be conducted in order to determine the mechanisms by which DJB improves downstream insulin signaling in skeletal muscle.

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APPENDIX



Animal Care and Use Committee

East Carolina University

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Greenville, NC 27834

252-744-2436 office • **252-744-2355** fax

April 30, 2007

Timothy Gavin, Ph.D.

Department of EXSS

388 Ward Sports Medicine Bldg.

East Carolina University

Dear Dr. Gavin:

Your Animal Use Protocol entitled, "Bariatric Surgery and Diabetes," (AUP #P040) was reviewed by this institution's Animal Care and Use Committee on 4/30/07. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies .

Sincerely,

A handwritten signature in black ink that reads "Robert G. Carroll, Ph.D."

Robert G. Carroll, Ph.D.

Chairman, Animal Care and Use Committee