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The Effects of Duodenal-jejunal Bypass on Glucose Homeostasis

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The Effects of Duodenal-jejunal Bypass on Glucose Homeostasis.

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In the Graduate Program of Pathobiology and Molecular Medicine of the College of Medicine

May 26th, 2010

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ABSTRACT

Roux-en-y gastric bypass (RYGB) is a bariatric surgery used for the treatment of morbid obesity that involves the creation of a small gastric pouch with partial gastric and proximal small bowel exclusion and expedited distal small bowel nutrient delivery. Evidence exists that RYGB is among the most effective bariatric procedures in treating type 2 diabetes with the rate of diabetes resolution well exceeding that which can be explained by weight loss. Although the mechanisms for the weight-independent restoration of euglycemia have yet to be established, the surgical diversion of nutrients away from the duodenum appears to play an important role. The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), are secreted from the gastrointestinal tract in response to enteral nutrients and augment glucose-mediated insulin secretion from the pancreas. We hypothesized in this thesis that an important aspect of the resolution of type 2 diabetes after RYGB involves altered incretin secretion after duodenal-jejunal bypass (DJB) with enhanced GLP-1 and decreased GIP secretion.

To test this hypothesis, we first needed to determine if DJB decreases gastrointestinal GIP secretion. We used lymphatic sampling to study nutrient-induced incretin secretion two weeks after DJB or Sham surgery in Wistar rats. We found that DJB did not improve glucose tolerance or alter meal-stimulated GIP secretion. The second aim tested the hypothesis that DJB increases GLP-1 secretion and thus improves glucose tolerance in type 2 diabetes. We first characterized the lymphatic incretin response to different nutrients in type 2 diabetic, Goto-Kakizaki (GK) rats. We found that GK rats have a defect in glucose-mediated secretion of both incretins to a glucose-
containing meal. We subsequently compared DJB and ileal interposition (IT) in GK rats to determine if duodenal bypass offers an independent mechanism to improve glucose tolerance. We found that both DJB and IT equally and modestly improved oral glucose tolerance suggesting a mechanism mediated by enhanced distal small bowel nutrient stimulation. DJB increased GLP-1 secretion, and systemic GLP-1 receptor antagonism reversed the small improvement noted in glucose tolerance. This study was the first to our knowledge to document a cause and effect relationship between duodenal bypass, an enhancement in GLP-1 receptor signaling, and the improvement in glucose tolerance. Finally, we tested the hypothesis that DJB improves insulin resistance independent of weight loss by performing DJB or Sham surgery in high-fat fed Wistar and Long-Evans rats. We found that DJB did not affect body weight or oral glucose tolerance. Formal tests of insulin sensitivity via a hyperinsulinemic-euglycemic clamp found no improvement in insulin resistance with DJB.

In summary, the magnificent reversal of glucose intolerance and insulin sensitivity seen in type 2 diabetic patients after RYGB can not be solely explained by duodenal bypass. This work supports that gastric exclusion of the neuro-endocrine stomach may be the most important contributing component beyond weight loss mediating a significant metabolic improvement after bariatric surgery. Further, animal and clinical studies are needed to determine how gastric exclusion or resection alters post-prandial glucose homeostasis.
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This thesis is based on the following papers, referred to in the text by their Roman numeral.

I. Nutrient-driven incretin secretion into intestinal lymph is different between diabetic Goto-Kakizaki rats and Wistar rats.
   Kindel TL, Yang Q, Yoder SM, Tso P.

II. Duodenal-jejunal exclusion improves glucose tolerance in the diabetic, Goto-Kakizaki rat by a GLP-1 receptor-mediated mechanism.
    Kindel TL, Yoder SM, Seeley RJ, D'Alessio DA, Tso P.

III. The effect of duodenal-jejunal bypass on glucose-dependent insulinotropic polypeptide secretion in Wistar rats.
    Kindel TL, Yoder SY, D'Alessio DA, Tso P.

IV. Bypassing the duodenum does not improve insulin resistance associated with diet-induced obesity in rodents.
    Kindel TL, Martins PJF, Yoder SM, Seeley RJ, D'Alessio DA, Obici S, Tso P.
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<th>Description</th>
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<tbody>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>DJB</td>
<td>duodenal-jejunal bypass</td>
</tr>
<tr>
<td>DPPIV</td>
<td>dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>Ex9</td>
<td>Exendin (9-39)</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>GIPR</td>
<td>glucose-dependent insulinotropic polypeptide receptor</td>
</tr>
<tr>
<td>GK</td>
<td>Goto-Kakizaki</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>IT</td>
<td>ileal interposition</td>
</tr>
<tr>
<td>K$_{ATP}$</td>
<td>ATP-dependent potassium</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PI-3</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide YY</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-y gastric bypass</td>
</tr>
<tr>
<td>SG</td>
<td>sleeve gastrectomy</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol response element binding protein</td>
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</table>
BACKGROUND

1. Glucose homeostasis

   Maintenance of whole-body glucose homeostasis is a process requiring the interaction of multiple tissues and organs with the coordinated and careful regulation of the following processes: intestinal glucose absorption, pancreatic β-cell secretion of insulin and α-cell secretion of glucagon, hepatic glucose production (either via glycogen breakdown and/or gluconeogenesis), and glucose uptake and disposal by skeletal muscle and adipose tissue [Molina 2006].

   1a. Pancreas. The pancreas is central to whole-body glucose regulation as documented by the development of brittle diabetes with total pancreatectomy. The pancreas is a mixed exocrine and endocrine gland. The endocrine pancreas is composed of the islets of Langerhans which are small, clustered, highly vascular endocrine cells which compose only 1-2% of the pancreatic mass [Molina 2006]. Islets are primarily composed of α-cells and β-cells, which secrete the counter-regulatory hormones glucagon and insulin, respectively, for the precise physiologic control of fasting and post-prandial glucose concentrations. The pancreas also secretes several other endocrine hormones including somatostatin, amylin and pancreatic polypeptide. The islets not only communicate via paracrine and hormonal pathways, but are richly innervated via parasympathetic, sympathetic and sensory nerves [Molina 2006].

   1b. Insulin release. The β-cell secretes insulin in response to nutrients, hormones and neurotransmitters [Molina 2006]. Several nutrients can act as insulin secretagogues including glucose, leucine, glutamine in the presence of leucine, and non-esterified fatty acids [MacDonald 2005]. Glucose is rapidly transported into the β-cell by facilitated
diffusion via a cell surface glucose transporter (GLUT) protein 2. Glucose then enters
the glycolytic pathway via the glucose-sensor, glucokinase, followed by the Krebs cycle
with the net production of ATP [Jitrapakdee 2010]. The formation of ATP increases the
cytoplasmic ATP:ADP ratio which closes ATP-dependent potassium (K\textsubscript{ATP}) channels
[Ashcroft 1984]. K\textsubscript{ATP} channel closure triggers plasma membrane depolarization followed
by the opening of voltage-gated calcium channels. The increase in intracellular calcium
leads to insulin granule exocytosis and release to the systemic circulation [Molina 2006].
Glucose not only releases preformed insulin granules but can also increase proinsulin
gene transcription, enhance mRNA stabilization, and modify mRNA translation. This
results in a biphasic release of insulin, with an initial rapid release of preformed insulin
followed by a second more sustained release of newly synthesized insulin [Straub 2004].
Although still requiring an increase in intracellular calcium levels, amino acids and fatty
acids stimulate insulin secretion primarily by K\textsubscript{ATP} independent pathway [Jitrapakdee
2010]. Evidence for this pathway was supported by studies of mice that could still
secrete insulin despite targeted disruption of the sulfonylurea receptor 1 (one of the two
subunits composing the K\textsubscript{ATP} channel) [Seghers 2000].

1c. Insulin Action. Insulin’s overall action is anabolic and promotes the synthesis
of carbohydrates, fat, and protein mediated through binding to the insulin receptor. The
insulin receptor protein is a heterodimer, consisting of two extracellular α-subunits and
two transmembrane β-subunits [Belfiore 2009]. The α-subunit contains the extracellular
ligand-binding domain. Insulin binding to the insulin receptor α -subunit regulates
intracellular tyrosine kinase activity of the β-subunit [Lin 2010]. The activated receptor
phophorylates several insulin receptor substrates (IRS) including IRS1, IRS2, IRS3, and
IRS4 [Molina 2006]. These IRS proteins facilitate the interaction of the insulin receptor with intracellular substrates by serving as a scaffold for the recruitment of proteins involved in signal transduction to downstream pathways. The classic insulin-signaling cascade involves phosphatidylinositol-3-kinase (PI-3) and Akt. Activated Akt phosphorylates many proteins involved in glucose transport, glycogen synthesis, and the regulation of protein synthesis [Taguchi 2008]. A second major pathway of insulin signaling is the mitogen-activated protein kinase (MAPK) cascade. The MAPK pathway is mainly responsible for the proliferative and differentiation effects of insulin [Molina 2006]. The number of available insulin receptors is modulated by exercise, diet, insulin, and other hormones.

The transport and utilization of glucose by skeletal muscle is a critical step in whole body glucose homeostasis and thus a key regulatory action of insulin [Ren 1993]. Insulin mediates about 40% of glucose disposal by the body, 80-90% of which occurs in skeletal muscle [Molina 2006]. Insulin-stimulated glucose transport into skeletal muscle occurs by facilitated diffusion through GLUT4. Upon insulin binding to the insulin receptor, PI-3 kinase activates Akt which facilitates the translocation of GLUT-4 to the sarcolemma and subsequent entry of glucose into the cell [Bjornholm 2005, DeFronzo 2009]. Insulin also promotes skeletal muscle protein synthesis via interaction with the mammalian target of rapamycin [Hay 2004].

In adipocytes, insulin decreases cAMP levels ultimately leading to hormone-sensitive lipase and lipolysis inhibition [Lonroth 1986]. Insulin regulates the expression of many enzymes involved in lipid synthesis by modulating the activity of sterol response element-binding proteins (SREBP) [Le Lay 2002]. SREBPs mediate the action of insulin
by activating the expression of over 30 genes involved in fatty acid, triglyceride, cholesterol and phospholipid uptake and synthesis [Horton 2003]. Insulin regulates the expression and activation of the peroxisome proliferator-activated nuclear receptor γ, which plays a critical role in adipocyte function by promoting fatty acid storage, increasing GLUT4 expression, and modulating adipokine secretion [Laviola 2006]. Insulin also inhibits the utilization of fat as an energy substrate by suppressing fatty acid oxidation [Randle 1998].

In the liver, insulin regulates fasting glucose concentrations by inhibiting hepatic glucose production (both glycogenolysis and gluconeogenesis) and stimulating glycogen synthesis [Weickert 2006]. Insulin suppresses the activity of phosphoenolpyruvate carboxykinase and glucose-6-phosphotase, both important enzymes in the gluconeogenesis pathway. However, hepatocytes can uptake glucose via GLUT-2, independent of insulin activation, and acute hyperglycemia can directly suppress phosphoenolpyruvate carboxykinase gene expression [Pilkis 1992, Nordlie 1999].

**1d. Glucagon.** Glucagon is a 29 amino acid polypeptide hormone secreted by the α-cells of the pancreas which plays an important role in the regulation of nutrient homeostasis by producing antagonistic effects on insulin action. Glucagon is secreted normally under fasting conditions when there is a decrease in plasma glucose levels. Glucagon primarily targets the liver and adipose tissue where it induces glycogen breakdown, promotes gluconeogenesis, and stimulates fatty acid mobilization [Lin 2010].

**2. Incretin Hormones**

The gastrointestinal tract is now a recognized organ critical to post-prandial glucose tolerance and perturbed in the diabetic state [DeFronzo 2009]. Two secreted
gastrointestinal hormones, GLP-1 and GIP, are incretins responsible for 50-70% of postprandial insulin secretion [Lynn 2001, Drucker 2003]. Incretins stimulate insulin secretion in response to enteral nutrients and work in an additive manner. The incretin effect describes the phenomenon that an oral glucose load results in a larger secretion of insulin compared to the intravenous administration of glucose at the same systemic concentration [Perley 1967]. GIP and GLP-1 can account for the great majority of the incretin effect [Drucker 2006].

2a. Glucagon-like Peptide-1. GLP-1 is derived from the proglucagon gene which is expressed in α-cells of the pancreas, L-cells of the intestine and the central nervous system (CNS) [Baggio 2007]. The proglucagon gene generates a single mRNA that undergoes tissue specific posttranslational processing to yield specific peptide profiles dependent on the originating tissue [Mojsov 1986]. In the intestine, posttranslational processing of proglucagon creates glicentin, oxyntomodulin, GLP-1, intervening peptide-2, and GLP-2. Enteroendocrine L-cells are primarily located in the distal small intestine and colon of the intestinal tract [Baggio 2007].

Biologically active GLP-1(7-37) and GLP-1(7-36)NH₂, are secreted from the basolateral surface of intestinal L cells in response primarily to apical carbohydrates and fats. L cell secretion is also regulated and response to neural and hormonal stimulants [Drucker 2003]. Previous studies of GLP-1 secretion in humans have found that a threshold rate of gastric emptying of glucose must be exceeded to result in GLP-1 secretion [Schirra 1996]. An increase in GLP-1 secretion with a larger meal is likely due to enhanced duodenal nutrient delivery that exceeds the absorptive capacity of the
proximal small bowel with significant nutrient spill over to the distal small bowel and L cells [Vilsboll 2003, Schirra 1996].

GLP-1 secretion in humans is biphasic. The early phase was initially thought to represent proximal neurohormonal stimulation, although recent evidence suggests that proximal small bowel L cells could account for the early rise in secretion [Theodorakis 2006]. The late phase is likely due to direct nutrient stimulation [Rocca 1999, Anini 2002] but the mechanisms whereby nutrients stimulate GLP-1 secretion remain under investigation. The sodium/glucose co-transporter, intracellular glucose metabolism, and K\textsubscript{ATP} channels may be involved in glucose-induced GLP-1 secretion [Gribble 2003, Reimann 2008]. Studies of GLP-1 release by protein and fat ingestion suggest a role of specific amino acid transporters and G-protein coupled receptor activation [Reimann 2004, Hirasawa 2005]. Nutrients also regulate proglucagon gene expression within the intestine [Hoyt 1996].

Once secreted, GLP-1 acts on the pancreatic β-cells to increase insulin secretion [D’Alessio 1989]. In addition to augmenting insulin secretion, GLP-1 promotes insulin biosynthesis [Baggio 2007]. GLP-1 also has proliferative and anti-apoptotic effects on β-cells [Farilla 2002]. GLP-1 inhibits glucagon secretion. In type 2 diabetic patients, GLP-1 can improve fasting hyperglycemia caused by hyperglucagonemia and inappropriate hepatic glucose production [Creutzfeldt 1996]. GLP-1 has also been found to have insulin-independent effects on enhancing glucose effectiveness. D’Alessio et al. studied the effects of a supraphysiologic dose of GLP-1 on intravenous glucose tolerance and found that GLP-1 enhanced glucose disappearance partly through its insulino­ptic effects by increasing the acute insulin response to glucose from 240 pM to 400 pM; in
addition, GLP-1 also increased glucose effectiveness at basal levels of insulin without a change in insulin sensitivity [D’Alessio 1994]. Subsequent studies have been unable to document an effect of GLP-1 on glucose turnover independent of the endocrine pancreas [Vella 2000]. GLP-1 also functions as one of the prominent ileal brake hormones. The presence of nutrients within the lumen of the ileum initiates GLP-1 release to inhibit gastric and pancreatic secretions [Layer 1995]. The intravenous infusion of GLP-1 during a meal retards gastric emptying, thus slowing nutrient absorption and the rise in glucose levels with a resultant decrease in insulin levels [Nauck 1997]. This suggests that the effect of GLP-1 on motility could even outweigh its insulinoactive activity.

The half-life of GLP-1 is less than two minutes due to rapid inactivation by dipeptidyl peptidase IV (DPPIV) [Deacon 1995]. DPPIV metabolizes GLP-1 to GLP-1 (9-37) or GLP-1 (9-36)NH₂ by cleaving a dipeptide from the amino terminus position [Deacon 1995]. Given this short half-life, it would be expected that the effect of GLP-1 can not only be limited to a direct hormonal effect but includes rapid signal relay by a neural pathway. Indeed, the administration of GLP-1 to the poral vein augments the insulin response by 81% and the effect of GLP-1 on insulin secretion is ablated with a ganglionic blocker [Balkan 2000]. Further, Vahl et al have found that nerve terminals that innervate the portal vein (the hepato-portal glucose sensor) contain the GLP-1 receptor (GLP-1R) and GLP-1R antagonism localized to the hepato-portal glucose sensor region impairs glucose tolerance [Vahl 2007].

GLP-1 decreases appetite and this effect is mediated by central signaling. It had been proposed that the anorectic effects of GLP-1R agonists are due to visceral illness rather than a primary effect on food intake. This was supported by the finding that the
illness effects of lithium chloride can be blocked by GLP-1R antagonism in rats [Seeley 2000]. It was later determined that the GLP-1R mediated visceral illness effect of lithium chloride is species dependent, and could not be replicated in mouse studies [Lachey 2005]. Alternatively, central administration of GLP-1R agonists in rodents reduced food and water intake, therefore decreasing body weight suggesting a direct effect on food intake [Tang-Christensen 1996]. The peripheral administration of GLP-1 also enhances satiety and reduces food intake in normal and type 2 diabetic patients possibly mediated by neural sensing within the lamina propria of the gastrointestinal tract as selective ablation of the nodose ganglion and vagus nerve in mice completely blocks the anorectic effects of peripheral exendin-4 [Flint 1998, Gutzwiller 1999, Talsania 2005].

The GLP-1R is a 7-transmembrane-spanning, heterotrimeric G-protein coupled receptor. The GLP-1R is located within α-, and β-cells of the pancreas, stomach, intestine, pituitary, nodose ganglion of the vagus nerve, heart, hypothalamus, brainstem, among other tissues [Baggio 2007]. GLP-1R stimulation in the pancreatic β-cell activates adenylate cyclase and increases intracellular cAMP eventually elevating intracellular calcium concentrations and enhancing insulin-granule exocytosis [Vilsboll 2009]. Exendin (9-39) (Ex9) is a specific antagonist of the GLP-1R derived from the lizard agonist of the GLP-1R, exendin-4 [Goke 1993]. Elimination of GLP-1 action by Ex9 reduces glucose-stimulated insulin secretion [Schirra 1998].

2b. Glucose-dependent Insulinotropic Polypeptide. GIP was the first incretin hormone to be identified and was initially isolated from extracts of the porcine small intestine. This hormone was given the name gastric inhibitory polypeptide based on its ability to inhibit gastric acid secretion [Brown 1975]. It was later named glucose-
dependent insulinotropic polypeptide to describe its physiologic function, as the effects on gastric acid secretion found only at supraphysiologic concentrations [Maxwell 1980]. GIP is a 42 amino acid polypeptide derived after posttranslational cleavage from a larger proGIP prohormone precursor [Ugleholdt 2006]. GIP is secreted from enteroendocrine K cells, primarily located in the duodenum and proximal jejunum, in response to glucose and fat ingestion. The half life of GIP is 5-7 minutes, and like GLP-1, is also inactivated by DPPIV. GIP is cleaved by DDPIV at the amide bond between Ala2 and Glu3, forming the inactive GIP (3-42) [Malde 2007]. GIP (3-42) represents approximately 60% of the circulating GIP and has been found to have antagonist activity of the GIP receptor (GIPR) at supraphysiologic doses [Gault 2002].

GIP secretion may be mediated by multiple mechanisms, including increases in intracellular calcium, activation of adenylyl cyclase, and potassium-mediated cellular depolarization [Baggio 2007]. Studies of GIP secretion, including a recent publication by our laboratory, demonstrate that GIP release appears dependent on the caloric-load of the nutrient and nutrient absorption, and not just the duodenal delivery rate or the luminal exposure to nutrients as indicated for GLP-1 [Schirra 1996, Yoder 2009]. Schirra et al reported that GIP secretion is governed by ongoing nutrient absorption rather than an effect of gastric emptying as demonstrated by the comparable total secretion of GIP after an iso-caloric oral load or continuous duodenal infusion [Schirra 1996]. Yoder et al has also demonstrated that lymphatic GIP concentrations increase dose-dependently with the lipid content of an intraduodenal lipid bolus suggesting a mechanism based on the caloric load [Yoder 2009]. Further research is needed to determine the regulation of K cell intracellular GIP production and secretion.
The GIPR is in the same family as the GLP-1R and is expressed in the pancreas, stomach, small intestine, adipose tissue, central nervous system, pituitary, heart, thyroid among a host of other tissues. GIPR activation increases intracellular calcium levels which enhances insulin exocytosis within the pancreatic β-cell. Table 1 displays a comparison between the routinely accepted, endogenous, physiologic actions of GLP-1 and GIP. Like GLP-1, GIP’s primary role is as an incretin hormone where it enhances glucose-dependent insulin secretion. The importance of GIP in oral glucose tolerance has been documented by studies of GIPR knockout mice. The GIPR knockout has hyperglycemia with an impaired initial insulin response after an oral glucose load compared to wild type [Miyawaki 1999]. GIP also increases insulin biosynthesis and proliferation and inhibits apoptosis within the β-cell [Wang 1996, Kim 2005]. GIP has a

Table 1. Physiologic actions of endogenous glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino-tropic polypeptide (GIP)

<table>
<thead>
<tr>
<th>Action</th>
<th>GLP-1</th>
<th>GIP</th>
</tr>
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<tbody>
<tr>
<td>Pancreatic β-cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-dependent stimulation of insulin secretion</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Glucose sensitivity</td>
<td>Increased</td>
<td>No effect or Increased</td>
</tr>
<tr>
<td>Insulin biosynthesis</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Differentiation of precursor cells</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Glucagon secretion</td>
<td>Decreased</td>
<td>No effect or Increased</td>
</tr>
<tr>
<td>Gastric emptying</td>
<td>Decreased</td>
<td>No effect</td>
</tr>
<tr>
<td>Postprandial hyperglycemia</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Appetite</td>
<td>Decreased</td>
<td>No effect</td>
</tr>
<tr>
<td>Body Weight</td>
<td>Decreased</td>
<td>No effect or Increased</td>
</tr>
<tr>
<td>Additional effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroprotection</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Cardioprotection</td>
<td>Increased</td>
<td>Unknown</td>
</tr>
<tr>
<td>Renal (diuresis, naturiesis)</td>
<td>Increased</td>
<td>Unknown</td>
</tr>
<tr>
<td>Bone formation</td>
<td>No effect</td>
<td>Increased</td>
</tr>
</tbody>
</table>

unique action compared to GLP-1 in stimulating bone formation and increasing bone
density via osteoblast action [Bollag 2001].

Unlike GLP-1, GIP has an important role in adipocytes by controlling lipid metabolism. In adipocytes, GIP stimulates fatty acid synthesis and re-esterification, triglyceride formation, and decreased lipolysis [Baggio 2007]. Surprisingly, many recent findings suggest an important role for GIP in the propagation and pathogenesis of obesity despite its dominant incretin action. Unlike the glucose intolerance of GIPR -/- mice on a chow diet, GIPR ablation in mice on a high fat diet prevents obesity due to increased energy expenditure and the use of fat as preferred energy substrate over triglyceride storage [Miyawaki 2002]. In further support of a link between GIPR signaling and obesity, McClean et al noted reversal of a diet-induced obesity phenotype and glucose intolerance with use of a GIPR antagonist [McClean 2007]. Treatment with a GIPR antagonist for 60 days in ob/ob mice significantly improved fasting glucose, hemoglobin-A1c, intraperitoneal glucose tolerance, meal tolerance and insulin sensitivity [Irwin 2007].

2c. Lymphatic Measurement of Incretin Hormones. Incretin secretion from the gastrointestinal tract is traditionally measured from the systemic circulation. However, as previously mentioned, plasma GIP and GLP-1 are quickly degraded by DPPIV to their corresponding inactive forms. Therefore, plasma may not be the ideal place to characterize incretin secretion in rodents due to the short half life, plasma dilution and assay detection sensitivity. The ability to sensitively and accurately measure incretin concentrations is critical to study in vivo meal-induced incretin secretion. We have previously documented that lymphatic concentrations of both GLP-1 and GIP in rats are higher than plasma concentrations; therefore, lymph can offer an alternative method of
measuring incretin hormone levels in rodents [D’Alessio 2007, Lu 2007]. This methodology involves the cannulation of the superior mesenteric lymphatic duct to allow continuous lymphatic sampling in conscious rats (Figure 1). Our lab traditionally has used this technique to study enteral lipid absorption and chylomicron secretion [Tso 1977].

Figure 1. Superior mesenteric lymphatic cannulation of a rodent.

Data from our lab and collaborators have shown that lymphatic incretin concentrations are significantly higher in lymph compared to not just peripheral but also portal plasma [D’Alessio 2007]. D’Alessio et al compared portal and lymphatic concentrations of GLP-1 and peptide YY (PYY) after a mixed meal. Lymphatic GLP-1 levels were two-fold higher in the fasting state than portal GLP-1 concentrations. Following the administration of a gastric Ensure bolus, lymphatic and portal GLP-1 concentrations both peaked at 30 minutes. Lymphatic GLP-1 concentrations were 20-fold greater compared to fasting lymph, whereas portal GLP-1 concentrations were only
six-fold higher compared to fasting plasma. In support of the use of lymph for the measurement of meal-induced incretin secretion *in vivo*, DPPIV levels were significantly lower in intestinal lymph than plasma offering one explanation for the enhanced GLP-1 concentrations in lymph. Interestingly, when comparing PYY between jugular plasma and lymph, PYY was only two fold higher in lymph compared to plasma after a nutrient bolus [D’Alessio 2007]. This suggests that GLP-1 may be specifically targeted for the lymphatic compartment but the mechanism and the biological role of this partitioning remains unclear. In addition, the lymphatic concentrations of incretins may more accurately reflect the local concentrations present within the lamina propria and exposed to enteric neurons.

3. Type 2 Diabetes Mellitus

Diabetes is the sixth leading cause of death in the US [Mokdad 2003]. In middle-aged adults, diabetes results in a five to ten year reduced life expectancy [Andreoli 2001]. Diabetes is the leading cause of renal failure, blindness, amputations, strokes and heart attacks [Pories 2001, Mokdad 2003]. The majority, 90-95%, of patients with diabetes have type 2 diabetes mellitus (DM). The prevalence of type 2 DM is increasing. The World Health Organization found that 171 million patients had diabetes in 2000, with 330 million cases expected by 2030 [Wild 2004]. In 2002, type 2 DM was responsible for over 130 billion dollars in health care expenses [ADA 2003]. Type 2 DM is strongly associated with obesity. Approximately 70-80% of individuals with type 2 DM are obese. The lifetime risk of acquiring type 2 DM in the morbidly obese is 50% [Mokdad 2003]. Unlike Type 1 DM which is the result of β-cell destruction, type 2 DM results from a loss of normal regulation of insulin secretion and action.
The pathogenesis of type 2 diabetes includes several core deficits, referred to in the 2009 Banting lecture by Ralph DeFronzo at the American Diabetes Association Meeting as the “ominous octet” [DeFronzo 2009]. This octet includes (1) decreased insulin secretion, (2) increased glucagon secretion, (3) increased hepatic glucose production, (4) decreased skeletal muscle glucose uptake, (5) central neurotransmitter dysfunction, (6) increased renal glucose reabsorption, (7) increased adipocyte lipolysis, and finally (8) a decreased incretin effect.

3a. Clinical Presentation and Diagnosis. The symptoms of diabetes can be vague and non-specific, but polyuria and polydipsia are common. Polyuria (increased urination) is due to increased urinary loss of glucose and subsequent water loss. Polydipsia (increased thirst) is due to dehydration and polyphagia (increased food consumption) due to the inability of the cells to utilize glucose as a substrate with a compensatory increase in free fatty acids and gluconeogenic amino acids. The diagnosis of diabetes differs based on the classifying organization but frequent common features include: (1) a fasting plasma glucose level greater than 126 mg/dL, (2) a random plasma glucose level higher than 200 mg/dL in association with symptoms of diabetes, or (3) a persistent elevation in plasma glucose following an oral glucose load (greater than 200 mg/dL two hours after glucose ingestion) [Molina 2006].

3b. Insulin Production and Secretion in Type 2 Diabetes Mellitus. As stated previously, glucose is tightly regulated in both the fasting and post-prandial states within a narrow range in healthy individuals. Type 2 DM is characterized by hyperglycemia, insulin resistance, β-cell dysfunction, and dysregulated hepatic gluconeogenesis [Andreoli 2001]. Initially, there is an early period of insulin resistance with
normoglycemia due to compensation of insulin secretion. Hyperglycemia develops when β-cells are no longer able to compensatory increase in insulin secretion to match the level of insulin resistance [DeFronzo 2004]. Figure 2 depicts the natural history of type 2 DM. In the setting of a genetic predisposition, there is a progression from normal glucose tolerance to impaired glucose tolerance with a 57% reduction in insulin sensitivity. There is a characteristic rise in insulin with increasing insulin resistance followed by a subsequent decline in insulin secretion and resultant hyperglycemia with the development of diabetes [DeFronzo 2009].

Figure 2. The natural history of type 2 diabetes. Top panel: the plasma insulin response (open circle); insulin-mediated glucose uptake (dark circle). From DeFronzo RA Diabetes (2009) 58: 773-795.

Not only are there secretory impairments of the β-cell, but there is also a loss of β-cell mass most likely due to increased apoptosis [Nauck 2009]. The etiology for increased apoptosis includes glucotoxicity, lipotoxicity, exposure to proinflammatory cytokines, hyperleptinemia, and amyloid deposition within the pancreas [Wajchenberg
2007]. Impaired β-cell function appears to be reversible in diabetes, particularly at early stages of the disease.

3c. Insulin Resistance in Type 2 Diabetes Mellitus. Insulin resistance plays a major role in the pathogenesis of type 2 DM and well precedes the onset of overt hyperglycemia and β-cell failure by several years [Lillioja 1988]. Insulin resistance is characterized by a decreased responsiveness of target tissues, including the liver, skeletal muscle and adipocytes, to insulin [DeFronzo 2009]. This includes a decrease in insulin receptor number as well as decreased receptor phosphorylation and subsequent decreased intracellular phosphorylation of IRS-1 and IRS-2 with decreased activation of PI-3 kinase [Caro 1986, Folli 1993].

Both the liver and skeletal muscle are severely resistant to insulin in type 2 DM [DeFronzo 2009]. Despite increased fasting insulin levels in type 2 diabetic patients, the liver is severely insulin resistant as documented by an overproduction of glucose via gluconeogenesis (2.5 mg/kg/min compared to 2 mg/kg/min in non-diabetic individuals) [DeFronzo 1989]. Contributing to inappropriate hepatic glucose production, type 2 diabetic patients have hyperglucagonemia and enhanced hepatic sensitivity to glucagon with an increased expression of gluconeogenic enzymes [Baron 1987, Gastaldelli 2000].

Muscle insulin resistance can account for over 85-90% of the impairment in total body glucose disposal in type 2 diabetic patients [Pendergrass 2007]. In the early stages of type 2 DM, impaired glycogen synthesis in muscle is the primary defect responsible for insulin resistance [DeFronzo 2009]. Gulli et al have studied lean, normo-glucose tolerant offspring of diabetic parents and found a 33-43% reduction in glucose uptake due to reduced glycogen synthesis with no abnormalities of hepatic glucose production [Gulli
While normoglycemia is maintained in an insulin resistant state with an increase in insulin secretion, the hyperinsulinemic state further propagates insulin resistance. Only 72 hours of physiologic hyperinsulinemia can significantly reduce insulin-stimulated muscle glycogen synthase activity, glycogen synthesis and total body glucose uptake [Iozzo 2001].

While the development of insulin resistance is impacted by age and underlying genetic predisposition, insulin resistance is strongly associated with obesity and lipid excess. The great majority of morbidly obese patients are insulin resistant. In support of this statement, weight loss and gain correlate with increasing and decreasing insulin sensitivity respectively [Salans 1974]. The effect of obesity on insulin resistance is multi-factorial. Increased systemic fat deposition not only within adipocytes, but also the liver and skeletal muscle, perturbs insulin signaling. Increased inflammatory mediator secretion from adipocytes, changes in adipokine secretion and signaling (most importantly adiponectin and leptin), defective substrate oxidation within myocytes, and increased plasma free fatty acids, all contribute to the development of insulin resistance within the obese individual [Machann 2004, Furler 2006, Moringo 2006].

The hyperinsulinemic euglycemic clamp has become the gold standard for quantifying insulin sensitivity in vivo [DeFronzo 2009]. Described in a publication by DeFronzo et al in 1979, this technique maintains glucose concentrations at a euglycemic level with an exogenous infusion of glucose (in addition to endogenous glucose production) in the setting of a fixed infusion of insulin to achieve a hyperinsulinemic state [DeFronzo 1979]. Insulin sensitivity measured with this technique primarily (80%) reflects skeletal muscle glucose uptake [Thiebaud 1982]. Given the invasive nature of a
hyperinsulinemic euglycemic clamp, other substitute methods of assessing insulin sensitivity have been developed. Insulin sensitivity measurements from an intravenous glucose tolerance test correlate well with the euglycemic clamp [Pacini 1982]. The homeostatic model assessment of insulin resistance correlates well with the euglycemic clamp but is a better measurement of hepatic insulin sensitivity rather than skeletal muscle as this calculation is derived from the fasting glucose and insulin levels [Matthews 1985].

3d. Reduced Incretin Effect in Type 2 Diabetes Mellitus. High-fat feeding in rodents has been shown to increase K cell hyperplasia, enhance GIP gene expression, and increase intestinal GIP content. This results in elevated fasting and feeding GIP concentrations [Bailey 1986, Flatt 1990]. In obese patients with impaired glucose tolerance, GIP secretion is significantly higher than healthy patients or type 2 diabetic patients [Theodorakis 2004]. With progression to type 2 DM in the obese, GIP secretion has been found to be normal or high [Vilsboll 2001]. More importantly, in type 2 DM, the incretin effect is reduced [Nauck 1986]. Defects in signaling pathways of GIP are considered among the most critical alterations underlying type 2 DM [Vilsboll 2003]. Attenuation of the GIP effect on insulin secretion with type 2 diabetes results in a deterioration of post-prandial glucose homeostasis [Zhou 2007, Xu 2007, Piteau 2007]. Lack of GIP amplification of the late-phase insulin response to glucose seems to characterize most forms of type 2 DM [Vilsboll 2003].

The effect of obesity on GLP-1 has been inconsistent in publications but favors that meal-stimulated GLP-1 secretion decreases with obesity [Naslund 1998]. Verdich et al found that GLP-1 levels were decreased in healthy obese subjects, and GLP-1
concentrations increased with weight loss [Verdich 2001]. Type 2 diabetic patients have a reduced secretion of meal-stimulated GLP-1 [Vilsboll 2001]. Unlike GIP, diabetic patients have a preserved insulinotropic response to GLP-1, although there are reports of a decreased potency of GLP-1 on β-cell responsiveness to glucose [Nauck 1993, Kjems 2003]. However, pharmacologic potentiation of the GLP-1 incretin response improves hyperglycemia [Nauck 1993]. The mechanism for the decrease of GLP-1 secretion with obesity and type 2 DM is unknown but insulin resistance has been a speculated causative factor suggesting that the reduced incretin secretion is a result of the diabetic state [Holst 2007].

Most studies that have examined the effect of diabetes on incretin secretion have examined obese, diabetic patients. Results from impaired glucose tolerant, non-obese patients have shown a decrease in GLP-1 secretion for the first thirty minutes compared to normal glucose tolerant patients [Rask 2004]. Relatively little information is known regarding GIP secretion in the lean, type 2 diabetic patient.

4. Obesity

4a. Impact of Obesity. Obesity is defined as the accumulation of excess body fat due to an excess consumption of calories in comparison to energy expenditure. Obesity is commonly measured by body mass index (BMI). An adult is categorized as overweight with a BMI between 25-30, obese with a BMI of 30-35, and morbidly obese when values exceed 35. Obesity affects over 30% of Americans with 10 million Americans classified as severely or morbidly obese [Clements 200, Bays 2007]. More than $50 billion is spent each year treating medical disease directly related to obesity and an additional $30 billion is spent per year on dietary programs and products [Patterson 2003]. The risk of mortality
for the morbidly obese is 5% per year [MacDonald 1997]. Obesity is associated with an increased risk of type 2 DM, coronary artery disease, cancer, metabolic syndrome, obstructive sleep apnea, gastroesophageal reflux disease, among a host of other diseases. The majority of these obesity-related morbidities can be reversed or prevented with significant or sustained weight loss [Buchwald 2004, Maggard 2005, Huerta 2007].

4b. Surgical Treatment of Obesity. Unfortunately, sustained weight loss using traditional medical management including specialized diets, behavior modification, and anorectic drugs fails greater than 90% of the time [Yanovski 2002]. Bariatric surgery is currently the most effective method for sustained weight loss [Huerta 2007]. Patients can expect to loss approximately 40% of their initial body weight [Sjostrom 2004]. In 1991, the National Institutes of Health endorsed bariatric surgery as an effective means of long-term weight control and developed specific criteria for surgeons to use to optimize patient selection [Gastrointestinal Surgery 1992]. The current guidelines issued by the American Society for Bariatric Surgery in 2004 identify suitable surgical patients as those with a BMI of >40 or have a BMI of 35-40 if an obesity-associated morbidity is present. These obese-associated morbidities include: type 2 DM, life-threatening cardiopulmonary problems, obesity-induced physical problems that interfere with a normal lifestyle, and body size problems precluding or severely interfering with employment, family function, and ambulation [Buchwald 2005].

A variety of surgical procedures have been developed to achieve weight loss, and they primarily involve either the mechanical restriction of caloric intake by creating a small gastric reservoir or the induction of malabsorption by bypassing variable lengths of small bowel. Historically, the jejunoileal bypass was a highly successful malabsorptive
operation for weight loss in which the proximal jejunum was anastomosed to the distal ileum, leaving approximately 45 cm of contiguous jejun-ileum. This surgery has been abandoned due to a wide variety of associated metabolic abnormalities including dehydration, electrolyte abnormalities, renal stones, bacterial overgrowth, D-lactic acidosis, and liver failure in up to 7% of patients [Griffin 1983].

Laparoscopic adjustable gastric banding and RYGB are the two most popular bariatric procedures performed in the United States. The laparoscopic adjustable gastric band received FDA approval in 2002 and has been in clinical use in the United States since that time. The procedure is purely restrictive and involves putting a silastic band around the proximal stomach creating a small gastric pouch with a narrow adjustable outlet. It is adjustable by changing the volume of saline in a surgically placed subcutaneous reservoir that is attached to the band, thereby increasing or decreasing the outlet diameter. Weight loss is less than with RYGB, approaching 45-50% of excess body weight; however, this surgery is associated with the lowest morbidity and mortality rate of currently available bariatric procedures [Sjostrom 2004, Buchwald 2005].

Gastric bypass is a combination of nutrient malabsorption and food restriction. It was developed as a combination operation due to difficulties in producing a surgically precise and effective restrictive operation for sustained long-term weight loss. Gastric bypass was originally performed in the 1960s by Mason and Ito with the creation of a small gastric pouch and a loop gastro-jejunostomy [Mason 1967]. Due to intolerable alkaline reflux and esophagitis, gastric bypass has been modified so that alkaline biliary contents mix with nutrients more distal in the small intestine by the creation of a Roux-
en-y anastamosis (named after the first physician to describe this procedure, Swiss surgeon Cesar Roux) rather than a gastro-jejunal anastamosis [Collins 2007].

RYGB, as shown in Figure 3, employs the creation of a new gastric pouch which is approximately 5% the size of the original stomach (generally 20-30 cc capacity). A limb of jejunum is divided 30-40 cm distal to the ligament of Treitz and a gastro-jejunostomy is created. The gastro-jejunostomy becomes the gastric outlet, instead of the pylorus. This, in addition to a loss of gastric reservoir, can create a rapid gastric emptying effect known as “dumping syndrome” and often results in patient aversion to sugary liquids. The Roux limb is 100-150 cm long and a jejuno-jejunostomy is created to allow mixture of food contents with bile and pancreatic secretions. Although various micronutrient and vitamin deficiencies occur after RYGB without routine supplementation, studies suggest that carbohydrate and fat absorption are not

significantly affected after RYGB (this contrasts to the macronutrient malabsorption seen after biliopancreatic diversion and jejunooileal bypass) [Rubino 2006, Malinowski 2006]. Patients generally lose 66% of their excess body weight within the first two years after RYGB and then level out to about 25% above their ideal body weight [Yale 1989, Pories 2001]. RYGB is the first therapy for morbid obesity to demonstrate a significant improvement in disease-related mortality due to a decrease in cardiovascular events, diabetes, and cancer [MacDonald 1997, Adams 2007]. This procedure has increased in popularity over the past 20 years, with over 140,000 procedures performed in the US in 2004 [Blackburn 2005].

Sleeve gastrectomy (SG) is another restrictive bariatric surgery attracting scientific and research attention for not only its ability to produce dramatic weight loss but also its surprising metabolic effects. SG involves the partitioning of the stomach into a long tube-like “sleeve” along the lesser curvature with removal of the remaining stomach. SG was first used for the removal of gastrointestinal stromal tumors and adapted to bariatric surgery given its success in producing weight loss. SG as a bariatric surgery was used initially in the severely, morbidly obese (BMI>60) as a “first stage” procedure because it is a quick surgery with short anesthesia time and less risk of surgical complications compared to RYGB in this high-risk patient group. Once patients had lost enough weight to endure the longer and more complicated RYGB, patients would have a second operation with conversion of the SG to a RYGB [Regan 2003]. SG is quickly becoming an accepted stand alone procedure for weight loss for patients with a BMI<60. The metabolic effects of SG include diabetes reversal, often paralleling the results produced by RYGB. Abbatini et al reported comparable three-year rates of resolution of
diabetes after SG and RYGB, 81.2% and 80.9% respectively [Abbatini 2009]. In addition, SG significantly increases insulin and GLP-1 levels to that achieved with RYGB, a finding not commonly reported after a restrictive procedure [Peterli 2009].

5. Metabolic Surgery

5a. Traditional Treatment of Type 2 Diabetes Mellitus. Diet modification, exercise and weight loss are all associated with an improvement in glycemic control. In the Diabetes Prevention Program, high-risk patients for the development of type 2 DM were placed on metformin, intensive lifestyle intervention or placebo [Knowler 2002]. Intensive lifestyle intervention included a goal to lose at least 7% of the starting body weight through a low-fat, low-calorie diet, moderate intensity physical activity for 150 minutes per week, and at least 16 weeks of individualized counseling. Intensive lifestyle modification reduced the incidence of type 2 DM by 58% compared to placebo and was more successful than metformin therapy. Unfortunately, long-term follow up of these patients revealed a similar incidence of diabetes among the three groups [DPPRG 2009]. The intensive lifestyle intervention group unfortunately partially regained the initial weight loss demonstrating how difficult long-term application of a lifestyle modification is as therapy for diabetes prevention or control.

The goal of any therapy is tight glycemic control, which has been shown to delay the development of complications associated with diabetes. This is supported by a study of metformin for intensive blood glucose control in overweight patients with type 2 DM which reduced mortality, stroke, and diabetes complications [UKPDS 1998]. The major pharmaceutical agents used for type 2 DM include: (1) insulin, (2) sulfonylureas, (3)
biguanides, (4) alpha-glucosidase inhibitors, (5) thiazolidinediones and (6) incretin mimetics [Molina 2006].

Because GIP action is reduced in type 2 diabetic patients, GLP-1 is the only incretin that has been developed for pharmacologic use. Intravenous administration of GLP-1 normalizes fasting glucose and significantly improves post-prandial glucose tolerance in type 2 diabetic patients [Toft-Nielsen 2001, Rachman 1997]. The pharmacologic administration of GLP-1 enhances glucose-stimulated insulin secretion relative to the plasma glucose concentration and decreases glucagon secretion [Zander 2002].

Exendin-4 was originally isolated from the venom of the *Heloderma suspectum* lizard in a search for biologically active peptides [Eng 1992]. Pharmaceutical GLP-1 mimetics have been designed to extend the naturally short half-life of endogenous GLP-1. This includes mimetics which are DDP-IV resistant, such as the GLP-1R agonist exenatide (synthetic exendin-4) and liraglutide which is GLP-1 conjugated to albumin. Not only do the GLP-1 analogs acutely stimulate glucose-dependent insulin release, but also sub-acutely increase insulin biosynthesis and the stimulation of insulin gene transcription [Wajchenberg 2007]. There is also a chronic enhancement of β-cell proliferation, islet neogenesis, and reduced β-cell apoptosis [Perfetti 2000]. DPPIV inhibitors (sitagliptin and vildagliptin), which enhance endogenous levels of the active forms of both GIP and GLP-1, have also been developed for oral administration [Deacon 2006].

5b. Effect of Bariatric Surgery on Type 2 Diabetes Mellitus. As early as the 1950s, there have been clinical reports of improved glucose homeostasis with surgeries
that involve gastric restriction and bypass of the duodenum for the gastrointestinal
reconstruction, such as a subtotal gastrectomy [Friedman 1955]. Pories et al published in
1995 results from 608 severely obese patients who underwent a standardized RYGB at a
single institution. They reported that 83% of the 146 diabetic patients experienced a
return to euglycemia post-operatively [Pories 1995]. A large meta-analysis by Buchwald
et al found an average remission rate of 84% for diabetic patients after RYGB [Buchwald
2004]. Bariatric surgery has also been shown to decrease the progression of insulin
resistance to clinical type 2 DM by 30 fold [Long 1994] and diabetes remission has been
maintained in prospective 14-year surveillance [Pories 2001]. Interestingly, the
improvement in glucose tolerance of diabetic patients occurs quickly after surgery despite
any significant weight loss. Schauer et al reported that 30% of diabetic patients
undergoing RYGB were able to leave the hospital off of all diabetes medications. The
average hospital stay in this study was 2.8 days [Schauer 2003]. A study of 10 morbidly
obese, diabetic patients found a significant improvement in \( \beta \)-cell sensitivity and
normalization of peripheral insulin sensitivity by one week after surgery [Guidone 2006].

The rapid improvement in fasting and post-prandial glucose concentrations
suggests weight-independent mechanisms mediated by RYGB surgery. Studies
comparing gastric banding and RYGB have shown enhanced diabetes remission with
RYGB compared to banding with equivalent amounts of weight loss [Korner 2007].
Common explanations for this response are based on changes in gastrointestinal hormone
release that occur due to alterations in gastrointestinal anatomy [Pories 2001, Rubino
2006, Morinigo 2006, Laferriere 2007, Pacheo 2007, Troy 2008, Bose 2009]. However,
there is as yet no direct evidence from clinical studies that the changes in gastrointestinal
hormone secretion after gastric bypass surgery cause the improvement in glucose tolerance.

RYGB enhances nutrient delivery to the distal small bowel. One potential mechanism for glucose homeostasis improvement after RYGB may originate from this enhanced nutrient delivery with augmentation of distal small bowel gastrointestinal hormones, such as GLP-1. Post-prandial GLP-1 levels are substantially increased after RYGB surgery [Morinigo 2006, Korner 2009, Kashyap 2009] consistent with the findings of an increased incretin effect after RYGB [Laferriere 2008].

RYGB also excludes nutrient delivery to the proximal small bowel, including all of the duodenum and proximal jejunum. Possible gastrointestinal mediators of this effect from the proximal small bowel include GIP. There have been a large number of conflicting reports regarding the changes noted in GIP secretion after RYGB, including increased, decreased or no change in secretion. In a published review of longitudinal studies of RYGB or biliopancreatic diversion and the effect on incretin secretion, results varied from a decrease in fasting and post-prandial GIP as early as one week after surgery, to no change at 12 months, to an increase in post-prandial levels one month after surgery [Schrumpf 1985, Guidone 2006, Laferriere 2007, Bose 2009]. Many of these discrepancies may be related to differences in surgical bypass limb length, time from surgery, fasting or post-prandial assessment, and the assay used for GIP measurement.

It is well accepted that weight loss and the subsequent reduction in visceral fat achieved with RYGB improve insulin sensitivity in type 2 diabetic patients [Williamson 2000, Thaler 2009]. RYGB improves insulin sensitivity by 4 to 5 fold, with an increase in plasma adiponectin. Skeletal muscle insulin receptor concentration increases and
muscle and liver lipid content decreases which are associated with enhanced insulin sensitivity [Thaler 2009]. However, there have been conflicting reports if RYGB improves insulin resistance by any independent mechanisms beyond weight loss. Ballantyne et al compared RYGB and gastric banding noting that percent excess weight loss predicted the postoperative insulin resistance during the first year [Ballantyne 2009]. In comparison, Wickremesekera reported significant changes in insulin resistance as early as 6 days after RYGB [Wickremesekera 2005]. The mechanism/s responsible beyond weight loss for an improvement in insulin resistance after RYGB could include signaling changes mediated from the gastrointestinal tract.

5c. Duodenal-jejunal Bypass as a Treatment for Type 2 Diabetes Mellitus.

Although the mechanism responsible for the resolution of diabetes after RYGB has yet to be elucidated, the surgical diversion of nutrients away from the duodenum appears to play an important role. DJB has been developed as an experimental surgical model to study the effect of duodenal exclusion, with a subsequent jejunal Roux-en-y reconstruction, on type 2 diabetes, insulin resistance, and obesity. This model also allows for the study of duodenal bypass independent of gastric exclusion and restricted food intake due to a decreased stomach capacity. In 2004, Rubino et al published the first study utilizing DJB (also referred to as duodenal-jejunal exclusion) as an experimental surgical model for RYGB in rodents [Rubino 2004]. In this study, lean, type 2 diabetic, GK rats were assigned to 4 groups: duodenal-jejunal exclusion, sham surgery, food restriction, or medical therapy. Rats undergoing DJB were reported to have an improvement in glucose tolerance by one week after surgery, achieving better glucose control than rats receiving rosiglitazone therapy. The DJB did not result in significant
changes in food intake or body weight compared to sham rats. There was a significant improvement in insulin sensitivity measured by an intraperitoneal insulin tolerance test.

The GK rat is a widely accepted inbred, lean model of type 2 DM derived from Wistar rats in the 1970s at Tohoku University [Kimura 1982]. Male rats develop type 2 DM by 12 weeks of age. Adults are characterized by fasting and post-prandial hyperglycemia, mild insulin resistance and decreased pancreatic insulin mass and secretory capacity with a polygenetic background [Giroix 1993, Ostenson 2007, Ghannat-Pour 2007]. Studies of the GK rat have focused on the defects in pancreatic β cell mass and insulin secretory capacity. In vivo and in vitro studies have shown a markedly decreased insulinotropic action of glucose on the pancreas, possibly mediated by impaired glucose oxidation [Giroix 1993]. The Paris GK colony shows impairment of several genes affecting insulin secretion as well as decreased β cell neogenesis [Ostenson 2007, Movasaat 2007]. Only a few authors have measured incretin secretion to a nutrient stimulus in GK rats; and of these cases, only one incretin was assessed or no comparison was made to non-diabetic animals [Patriti 2005, Patriti 2007, Pacheco 2007].

Although undoubtedly simplistic and under-appreciating of the complex interplay of mechanisms likely involved in improving glucose tolerance after RYGB, two theories have often been proposed to explain this effect. The “foregut hypothesis” supports the role of duodenal exclusion and the “hindgut hypothesis” promotes enhanced jejunal/ileal nutrient stimulation as the primary mediator. A subsequent study by Rubino et al in GK rats suggested that proximal small bowel exclusion may be the dominant mechanism for the improvement in glucose tolerance after DJB [Rubino 2006]. GK rats underwent DJB or a loop gastro-jejunostomy (LGJ). A LGJ also maintains gastric anatomy like DJB but
involves a loop anastamosis between the stomach and jejunum rather than a Roux-en-y reconstruction. This allows for enhanced nutrient stimulation of the distal small bowel with the maintenance of duodenal nutrient flow. They found superior glucose control in DJB rats compared to LGJ rats. When Rubino reversed the surgeries, he found that DJB rats had deterioration of glucose tolerance when converted to a LGJ, and gastro-jejunostomy rats had improvements in glucose tolerance when converted to a DJB. They also performed DJB in normal, Wistar rats with a slight deterioration of glucose tolerance in the DJB groups compared to pair-fed shams. The authors concluded that the proximal intestinal bypass of the duodenum is the key component for glucose control and that potentially undiscovered factors originating from the proximal small bowel might contribute to the pathophysiology of type 2 DM. In this study, DJB resulted in a significant reduction in food intake to less than 10 g/day, a finding not found in their previous study. Unfortunately, only two GK rats were able to survive the conversion of DJB to a LGJ, limiting the ability to interpret the results of this study. Pacheco et al has also performed DJB in GK rats, and compared this with control animals [Pacheco 2007]. They found that glucose tolerance had improved as early as one week after surgery without affecting glucagon, insulin, GLP-1 and GIP concentrations. Surgical rats had a significant reduction in leptin post-operatively compared to control rats.

DJB has been tested clinically in type 2 diabetic patients by Gleoneze et al [Geloneze 2009]. Twelve type 2 diabetic patients with a BMI of 25-30 underwent DJB and were compared at 24 weeks to BMI matched control patients who received standard medical therapy. The surgery produced no significant differences in body weight or fat mass compared to the control group. DJB patients had a greater reduction of fasting
glucose, hemoglobin A1C, and insulin requirements compared to control patients suggesting that DJB may have the potential to be a “surgical” treatment for type 2 DM.

5d. Ileal Interposition as a Treatment for Type 2 Diabetes Mellitus. IT was originally described in rats in 1982 by Koopmans and mimics the early delivery of nutrients to the distal small bowel produced in RYGB and DJB [Koopmans 1982]. In an IT, a distal segment of ileum is moved more proximally in the small bowel resulting in increased secretion of GLP-1 and PYY [Strader 2005]. This surgery has been shown to improve glucose homeostasis and insulin sensitivity in type 2 DM animal models. In high-fat fed Long Evans rats, IT resulted in decreased food intake, weight loss and improved insulin sensitivity compared to sham rats [Strader 2005]. IT can also attenuate the weight gain usually associated with consumption of a high-fat diet in rats [Boozer 1990]. It is often difficult to differentiate a cause-and-effect relationship between hormonal changes and insulin sensitivity in the context of superimposed weight loss. However, IT has been shown in GK rats to significantly improve glucose tolerance, insulin sensitivity and the acute insulin response compared to sham-operated controls despite similar food intake and weight gain post-operatively [Patriti 2005, Patriti 2007]. Further, IT increased the number of L-cells in the transposed segment, as well as increased GLP-1 production, compared to controls [Patriti 2007]. De Paula et al have performed an IT with a SG or diverted SG in diabetic patients who had a BMI less than 35 showing an early improvement in fasting hyperglycemia for both groups [De Paula 2006, De Paula 2008]. Whether the clinical effect is simply due to weight loss, or hormonal changes from gastric exclusion, duodenal bypass or IT, requires further investigation.
AIMS OF THE CURRENT INVESTIGATION

We hypothesized in this thesis that an important aspect of the resolution of diabetes after RYGB involves enhanced distal small bowel stimulation of GLP-1. Further, a decrease in GIP secretion due to duodenal bypass may have an additional role in improving glucose homeostasis with obesity or insulin resistance.

The following specific aims have been performed to test this hypothesis. Given the conflicting literature on the changes in GIP secretion after RYGB surgery, our first aim tested the hypothesis that DJB decreases gastrointestinal GIP secretion. Incretin assessment can be difficult because plasma incretins are subject to rapid dilution and degradation by the protease DPPIV. Previous results from our laboratory have shown that the concentration of incretins is several fold higher in the intestinal lymph compared to the portal or systemic circulation. We used lymphatic sampling as a novel approach to study nutrient-induced incretin secretion two weeks after DJB or Sham surgery in Wistar rats (paper II).

The second aim tested the hypothesis that duodenal bypass increases GLP-1 secretion and thus improves glucose tolerance in type 2 diabetes. We first needed to sufficiently characterize the incretin response to different nutrients in a commonly used, type 2 diabetic rodent model, the GK rat. Lymphatic incretins were measured in response to different macronutrient stimuli in comparison to Wistar controls (paper I). We then performed DJB or IT in GK rats to determine if duodenal bypass offers an additional benefit beyond enhanced distal small bowel nutrient stimulation. Systemic GLP-1R antagonism was used to determine what proportion GLP-1R signaling contributes to any improvement in glucose tolerance after DJB (paper III).
Finally, we tested the hypothesis that DJB improves insulin resistance independent of weight loss. To test this hypothesis we performed DJB or Sham surgery in DIO Wistar rats as well as DIO Long-Evans rats that had been fed a 45% high-fat diet for ten weeks prior to surgery. Both groups were assessed for improvements in glucose tolerance and DIO Wistar rats underwent formal tests of insulin sensitivity via a hyperinsulinemic-euglycemic clamp study (paper IV).
DISCUSSION

The results of this thesis have led to the following four general conclusions and will be discussed in detail below.

1. GK rats have a defect in carbohydrate-mediated incretin secretion.
2. Duodenal bypass does not suppress food intake or reduce body weight.
3. Except for the GK rat, duodenal bypass does not improve oral glucose tolerance.
4. Duodenal bypass does not improve insulin resistance.

GK rats have a defect in carbohydrate-mediated incretin secretion.

Studies of the GK rat have previously focused on the defects in pancreatic β cell mass and insulin secretory capacity. Over the past five years, the GK rat has been used and publicized as a type 2 diabetic rodent model that benefits from dramatic improvements in glucose tolerance after DJB and IT, by mechanisms which could include a change in meal-induced incretin secretion or an alteration of other gastrointestinal hormones that affect glucose homeostasis [Rubino 2004, Patriti 2005, Rubino 2006, Patriti 2007, Pacheco 2007]. However, relatively little is known regarding the pre-operative incretin secretion profile of these animals. In paper I, we examined the differences in nutrient-mediated lymphatic incretin secretion of GK rats compared to non-diabetic, Wistar rats.

Paper I, in combination with the results from paper III, highlight two important findings. First, we verified the sensitivity of lymph as a medium for the measurement of gastrointestinal incretins in both GK and Wistar rats. Second, when given a mixed meal, GK rats have a reduced ability to augment incretin secretion compared to Wistar rats, and
the loss of this augmentation with a mixed meal appears to be due to a dextrin-mediated incretin defect that applies to both GLP-1 and GIP.

We have previously published that lymph is a more sensitive medium than peripheral or portal plasma for the measurement of incretin concentrations in Sprague-Dawley rats [D’Alessio 2007, Lu 2008]. While the purpose of paper III was to determine the effect of DJB on GIP secretion, it secondarily verifies that lymphatic incretin profiles parallel that of plasma, but at a much greater concentration. We found a 3.3 fold average higher concentration of fasting lymphatic GIP (93.2 ± 11.41 pg/ml) compared to plasma GIP (28.6 ± 2.84 pg/ml) in Sham rats. We also demonstrated a 4.3-4.5 fold larger increase in peak GIP secretion of lymph compared to plasma with a gastric Ensure bolus. Lu et al found that a mixed meal results in a 3 fold higher secretion of lymphatic GIP (1,733 ± 257 pg/ml) compared to portal blood (603 ± 244 pg/ml), however this was with the intraduodenal administration of a 50:50 dextrin/Liposyn 20% mixture as the mixed meal (without the presence of protein).

This would argue that the benefit of measuring lymphatic GIP or GLP-1 over plasma is only in the enhanced sensitivity of lymph, and that lymphatic concentrations do not offer any additional information. However, D’Alessio et al observed the interesting finding that GLP-1 is disproportionately transported into the lymph compared to plasma (derived from comparisons to another gastrointestinal hormone, PYY) [D’Alessio 2007]. We do not know by what mechanism GLP-1 is targeted to the lymph and if this mechanism holds potentially valuable information regarding the physiologic function of GLP-1 or pathophysiologic dysfunction with diabetes. It would be useful to compare plasma and lymphatic GLP-1 levels in a diabetic or glucose intolerant rodent model to
see if the lymphatic secretion profile differs from that of plasma in a pathophysiologic state.

In Paper I, the delivery of a mixed meal in Wistar rats increased lymphatic GLP-1 and GIP concentrations to a greater extent than the delivery of either nutrient alone, despite a similar caloric content. This is in support of previous findings by this lab that a mixed meal augments the secretion of GIP and GLP-1 compared to the administration of a solitary isocaloric nutrient [Lu 2007, Lu 2008]. Contrastingly, the effect of a mixed meal on GIP and GLP-1 was diminished in GK rats. We believe the return of mixed-meal enhancement of incretin secretion creates an ideal end-point for future studies in GK rats which would test therapies aimed at enhancing native enteroendocrine incretin secretion.

The central finding of paper I is that there is a dextrin-mediated incretin defect in GK rats for both GLP-1 and GIP. While most publications have found that GLP-1 is decreased in type 2 DM as seen in this current study, most clinical reports have found that GIP secretion is increased or unchanged in type 2 diabetic patients compared to euglycemic patients [Vilsboll 2001, Rask 2004, Theodorakis 2004]. As lean, type 2 diabetic patients are a minority of the diabetic population and understudied, it could be that GIP secretion is reduced in the lean, diabetic state. It is also possible that the reduced secretion of GIP is a finding unique to the GK rat and can not be extrapolated to human disease. Because use of a GIPR antagonist improved diabetes and prevented obesity-associated morbidites in mice, we wondered whether suppression of GIP after DJB might account for the improvement in glucose tolerance [McClean 2007, Irwin 2007]. The reduced secretion of GIP in GK rats makes it unlikely that the mechanism
mediating the improvement in glucose tolerance with GK rats after DJB is further suppression of GIP. Further, the GK rat is probably not an ideal model to test the effect of any surgical therapy designed to endogenously reduce GIP secretion as these animals at baseline undersecrete GIP with glucose-containing meals and it therefore seems unlikely that they would benefit from further GIP suppression.

We doubt that the incretin difference between GK and Wistar rats is due to perturbed nutrient delivery, such as delayed gastric emptying, which should produce a solitary GLP-1 defect, or nutrient absorption, which should produce a solitary GIP defect. Although the mechanisms for nutrient-driven incretin secretion are relatively unknown, it appears that the reduced secretion of GLP-1 with diabetes is secondary to the diabetic state rather than a primary cause of diabetes [Rask 2004]. Studies such as the one by Martins et al which document an improvement in GLP-1 secretion with exercise-induced weight loss support that the incretin secretion defect of obesity/diabetes is a secondary response [Martins 2010]. We believe our study supports this finding as the nutrient defect applied only to glucose and similarly to both K and L cells. Lim et al have previously demonstrated that the L cell is insulin responsive and insulin resistance in vivo results in decreased meal-stimulated GLP-1 release [Lim 2009]. The GK rat does have mild insulin resistance compared to Wistar rats and therefore it is possible that the mechanism for reduced incretin secretion is because of enteroendocrine cell insulin resistance.

Because the defect was isolated to glucose or glucose-containing meals, we also wonder whether enteroendocrine cell secretion dysfunction is due to some aspect of intracellular glucose signaling or glucose regulation shared by K and L cells.
Glucotoxicity is another interesting potential mediator of the observed defect in Paper I. Exposure of pancreatic islets to intermittent high glucose levels impedes glucose-stimulated insulin secretion, activates apoptosis, causes alteration of mitochondrial morphology and increases intracellular nitrotyrosine content [Del Guerra 2007]. Glucotoxicity may also induce endoplasmic reticulum stress in susceptible pancreatic β-cells [Marchetti 2007]. Given that there are many similarities noted in signaling pathways between enteroendocrine cells and pancreatic β-cells, it is possible that glucotoxicity alters K and L cell signalling of enteral glucose in the GK rat. However, a recent study by Hojberg et al argues against a glucotoxicity mechanism. They found that four weeks of tight glucose control did not alter GIP and GLP-1 secretion but did improve meal-stimulated insulin secretion [Hojberg 2008]. Further studies are needed to determine the effect of chronic tight glycemic control on incretin secretion and responsiveness in type 2 diabetic patients.

**Duodenal bypass does not suppress food intake or reduce body weight.**

While the improvement in glucose tolerance after RYGB is undoubtedly a multi-factorial process, including the significant impact of post-operative weight loss and calorie restriction on glucose homeostasis, gastrointestinal specific mechanisms could include (1) exclusion of the majority of the endocrine stomach from nutrient flow, (2) proximal small bowel exclusion from nutrient stimulus, and (3) enhanced nutrient delivery to the distal small bowel. We used DJB as a model of RYGB for two reasons. First, while DJB is unable to address any hormone or neural factors that may be affected by gastric nutrient exclusion, it is an extremely useful experimental model of RYGB to study the affects of proximal small bowel exclusion and enhanced distal small bowel
stimulation on glucose homeostasis. And second, in theory, DJB should not alter food intake or body weight due to the lack of gastric restriction.

It is interesting to note that several previous publications have found dramatic reductions in food intake with duodenal bypass in rodents [Rubino 2005, Rubino 2006, Wang 2008, Inabnet 2010]. In a study of Zucker obese rats and lean Wistar rats undergoing DJB, only the Zucker obese rats had reduced food intake post-operatively leading the authors to conclude that the mechanism responsible for the reduction in food intake is only applicable in an obese phenotype [Rubino 2005]. In a subsequent study by Rubino et al comparing DJB to LGJ in lean GK rats, DJB dramatically reduced food intake to less than 10 g/day making an obesity-specific effect of DJB on food intake unlikely [Rubino 2006]. These studies would suggest that despite preserved gastric capacity and continuity there could be neural or hormonal changes induced by duodenal bypass that affect food intake.

In this thesis, we have performed DJB in several different rodent strains including diabetic GK rats (Paper II), lean Wistar rats (Paper III), and high-fat fed Long-Evans and Wistar rats (Paper IV). In GK rats, we found no effect of either DJB or IT on food intake compared to Sham rats by one month after surgery (Paper II). Supporting our previously published findings with DJB surgery in GK rats, we found in Paper III, only modest changes in early post-operative food intake between the two groups, with no difference in food intake noted after POD#9 and no affect of DJB on body weight compared to Sham surgery. Because GK rats are derived from Wistar rats, we wondered whether in a different background strain, we would see an effect of the surgery on food intake and
body weight. In high-fat fed Long Evans rats, we once again found that DJB did not affect food intake compared to Sham rats (Paper IV).

In contrast to our findings in GK, lean Wistar rats, and high-fat fed Long Evans rats, we found a significant increase in post-operative food intake of high-fat fed Wistar rats compared to Sham rats likely as a compensatory mechanism to balance mild fat malabsorption (Paper IV). Macronutrient deficiencies, including fat malabsorption, are uncommon after a standard limb-length RYGB. It is interesting to note that the percent of fat absorption for high-fat fed Wistar Sham rats was lower than previous publications from our laboratory and collaborators in non-operated rodents using a non-absorbable fat marker [Jandacek 2004, Lo 2010]. We have measured fecal fat absorption in a second, separate group of high-fat fed Wistar rats under the same diet and surgical conditions as the current experiment (unpublished findings). DJB rats (n=9) at six weeks post-op absorbed 77.6 ± 2.69% compared to 84.4 ± 1.4% in Sham rats (n=10) (p=0.05). This additional data suggests that our fat absorption findings from Paper IV are valid and reproducible, and may represent a strain difference in absorption for Wistar rats on this diet compared to our previously reported rates of absorption using this methodology. This may explain why we have only seen an increase in food intake in high-fat fed Wistar rats and not in any other tested strain. We did not measure fat absorption from any of the other DJB studies and therefore cannot comment on the general effect of DJB on fat malabsorption. However, we can soundly say that we have never documented a long-term suppressive effect of duodenal bypass on food intake.

We wonder what mechanisms account for the differences observed in food intake between these and the current study. The first possibility would be there is a subtle but
important difference in the way other laboratories have performed DJB compared to us that affects a neural or hormonal aspect of food intake regulation. There are clear differences between the use of a duodenal sleeve and DJB that may alter food intake signals, such as preserved mechano-perception in the duodenum despite the lack of nutrient uptake. The use of an endoluminal sleeve implanted into the duodenal bulb of diet-induced obese rodents resulted in a 27% reduction in kcal consumed per day as compared to sham-operated animals [Aguirre 2008]. Interestingly, the subsequent improvement in insulin sensitivity appeared related to the reduced food intake and body weight. Studies of duodenal sleeve involving a control group with a fenestrated sleeve would help answer these complex questions. The second possibility would be that there is a mismatch in the post-operative health and recovery of the DJB animals compared to Sham animals from previous publications. Without an appropriately matched surgical sham for comparisons, it is difficult to know if food intake changes are independent of the surgical stress. Regardless of the etiology of these differences, it is our conclusion that duodenal bypass as performed in this thesis is an insufficient procedure to reduce food intake and should not be considered as a stand-alone weight loss procedure without some form of gastric reduction or exclusion.

**Except for the GK rat, duodenal bypass does not improve oral glucose tolerance.**

We hypothesized in this thesis that an important aspect of the resolution of diabetes after RYGB involves enhanced distal small bowel stimulation of GLP-1. Further, a decrease in GIP secretion due to duodenal bypass may have an additional role in improving glucose homeostasis with obesity or insulin resistance. We were interested specifically in what effect DJB had on plasma and lymphatic GIP concentrations given
the hormone’s proximal small bowel site of secretion and the recent literature highlighting the beneficial effects noted with reduction of GIP signaling on obesity propagation and glucose intolerance [McClean 2007, Irwin 2007]. Additionally, Rubino et al, among others, have published animal and clinical data to support that a major mediator of the effect of gastric bypass might derive from a change in secretion of a probable hormone originating from the proximal small bowel that is a causative agent for insulin resistance and glucose intolerance, leading us to speculate if GIP could be mediating these effects [Pories 2001, Rubino 2006, Rubino 2008, Bikman 2008, Salinari 2009]. However, publications of GIP secretion after RYGB have been inconsistent, ranging from increased, decreased, to no change in concentrations from pre- to post-operatively [Schrumpf 1985, Guidone 2006, Laferre 2007, Bose 2009]. Given the conflicting clinical reports on the changes in GIP secretion after gastric bypass, in Paper III, we characterized the effects of DJB on GIP in lean, Wistar rats to determine if a reduction in GIP secretion could be a possible mechanism to explain any improvements noted in glucose tolerance after duodenal bypass. We found that DJB does not significantly alter systemic GIP concentrations by two weeks after DJB surgery compared to Sham rats despite bypassing the proximal small bowel, which is the major focus of GIP-secreting K cells.

It is important to note that while we found minimal effects of DJB on systemic GIP secretion, further studies are needed to determine if the sensitivity of the GIPR changes after DJB and RYGB surgery. As the profound reduction in GIP signaling is considered to be a critical dysfunction resulting in hyperglycemia and cannot be overcome with exogenous GIP administration [Vilsboll 2003, Knop 2007], it seems
reasonable that despite unchanged GIP concentrations, alterations in GIPR signaling induced by DJB could have dramatic effects on prandial glucose tolerance.

Our second aim tested the hypothesis that duodenal bypass improves glucose tolerance in type 2 diabetes by increasing GLP-1 secretion. Unexpectedly, in paper III we found no improvement in glucose tolerance in DJB rats by two weeks after surgery. We noted an insignificant trend towards increased insulin secretion to an oral glucose load in DJB rats, but this did not result in a physiologically relevant reduction in glucose concentrations. Given the early time point of evaluation in this study and the use of a glucose tolerant phenotype, it is unclear if a longer observation period would have resulted in a significant improvement in glucose tolerance. Rubino et al has published that DJB in lean Wistar rats actually impairs glucose tolerance compared to Sham controls [Rubino 2006]. In non-diabetic Wistar rats, DJB increased glucose excursions during an oral glucose tolerance test, as shown by a slightly higher glucose area under the curve compared with sham-operated, pair-fed Wistar controls (although not statistically significant). This led the authors to argue that the benefit of duodenal bypass is limited to diabetic models, and could possibly explain our lack of phenotypic findings with DJB in paper III.

As mentioned previously, GK rats have been the predominant rodent model used to document improvements in hyperglycemia with DJB [Rubino 2004, Rubino 2006, Pacheo 2007, Wang 2008, Inabnet 2010]. Ileal interposition mimics the early delivery of nutrients to the distal small bowel produced in RYGB and DJB and also has been used successfully in GK rats to document improvements in glucose tolerance [Patriti 2005, Patriti 2007]. For this reason, we compared DJB and IT to determine if one surgery
offered an advantage over the other regarding glucose tolerance in GK rats. Both surgeries increase distal small bowel exposure to nutrients but only DJB, like RYGB, bypasses the duodenum and proximal jejunum.

In Paper II, we found that both DJB and IT result in only a modest but statistically significant improvement in glucose tolerance by four weeks after surgery in GK rats. This finding supports that the responsible mechanism is mediated by enhanced nutrient delivery to the distal small bowel and not duodenal bypass. DJB increased post-prandial GLP-1 secretion and GLP-1 protein content within the jejunum and ileum. The small improvement in glucose tolerance noted after DJB in GK rats was mediated by GLP1R signaling as Ex9 ablated this effect on oral glucose tolerance. The increase in GLP1R signaling could be from the increase in GLP-1 secretion or due to increased sensitivity and enhanced incretin effect of GLP-1 on the GLP1R, regardless of the quantitative changes in GLP-1 secretion. This is supported by findings of Kjems et al that there is a decreased potency of GLP-1 on β-cell responsiveness to glucose [Kjems 2003]. To our knowledge, this study offers the first direct evidence documenting a causal relationship between a change in GLP-1 signaling induced by bypass surgery and the subsequent improvement in post-operative glucose tolerance. Clinical studies of RYGB patients using Ex9 could support this mechanism and offer needed insight regarding what extent GLP-1 augmentation contributes to the improvement in oral glucose tolerance post-op compared to weight loss.

Again in Paper IV, DJB did not improve glucose tolerance in high-fat fed Wistar rats by two months after surgery. Although this finding was concerning, the lack of a chow-fed control group made it difficult to determine if the animals had normal glucose
tolerance and if the lack of effect could be explained again because we used a normo-tolerant animal. However, the high-fat diet used in Paper IV produced significantly worse glucose tolerance in the Long-Evans rats compared to a chow diet and we still were unable to achieve an improvement in post-prandial hyperglycemia with DJB at one month post-op. To our knowledge, the study performed in Paper IV is the first to examine DJB in a rodent model of diet-induced obesity.

It is imperative to note that Paper II did not find the robust improvement in glucose tolerance as previously reported by some investigators after DJB in GK rats [Rubino 2004, Rubino 2006, Pacheco 2007]. Differences in surgical technique, post-operative care, observation duration, and GK colonies are all possible reasons for this difference. We are concerned however with the findings from Paper IV that regardless of what accounts for the differences between our GK study and other authors, it is a finding only producible in GK rats. The lack of a consistent improvement in hyperglycemia with DJB in glucose-intolerant rodents indicates a requisite need for the continued investigation of the mechanisms mediating an improvement in glucose tolerance with DJB and RYGB, and should caution the clinical use of duodenal bypass as a stand-alone procedure for type II diabetes mellitus as is currently being clinically undertaken [Geloneze 2009, Tarnoff 2009, Ramos 2009]. Further, our studies strongly suggest that there is not a factor released from the small bowel in response to duodenal nutrient stimulation that is responsible for type 2 diabetes mellitus and insulin resistance, as has been proposed in the literature as the “anti-incretin” theory, and removal of nutrient stimulation reverses this process [Rubino 2008].

Duodenal bypass does not improve insulin resistance.
Our final specific aim tested the hypothesis that DJB improves insulin resistance independent of weight loss. In Paper II and IV, we had found no changes in insulin sensitivity due to DJB as assessed by a subcutaneous insulin tolerance test in GK or high-fat fed Long-Evans rats, respectively. Insulin resistance was significantly worse for both DJB and Sham high-fat fed Long-Evans rats compared to chow-fed, Sham, Long-Evans rats. As previously mentioned, the hyperinsulinemic euglycemic clamp has become the gold standard for quantifying insulin sensitivity \textit{in vivo} and an insulin tolerance test can easily miss subtle changes in insulin resistance [DeFronzo 2009]. Therefore, in Paper IV, we performed a hyperinsulinemic euglycemic clamp at ten weeks post-operatively to test the effects of DJB on insulin resistance in high-fat fed Wistar rats. The hyperinsulinemic-euglycemic clamp study revealed that neither insulin sensitivity nor glucose metabolism was altered by duodenal bypass surgery in the setting of diet-induced obesity.

There is convincing data that RYGB may improve insulin resistance beyond that which can be explained by surgical weight loss [Kashyap 2009, Korner 2009]. Kashyap et al tested the effects of gastric bypass and gastric restrictive surgery on insulin sensitivity using a hyperglycemic clamp and C-peptide modeling kinetics. At four weeks post-operatively, insulin sensitivity had improved significantly with only RYGB despite similar weight loss between the two groups [Kashyap 2009]. There are also reports of significant changes in insulin resistance as early as three days after bilio-pancreatic diversion and six days after RYGB [Adami 2003, Wickremesekera 2005]. Yet when RYGB was rigorously matched to a diet restriction group, glucose uptake as measured by a hyperinsulinemic euglycemic clamp was similar between the two groups at two weeks [Campos 2010]. It is possible that the acute changes in insulin resistance after RYGB
may be predominately due to calorie restriction and the later improvements are due to weight loss as well as independent gastrointestinal mechanisms. The lack of an improvement in insulin resistance found with DJB surgery in paper IV by greater than two months after surgery however suggests that duodenal bypass alone is an insufficient mechanism to induce weight-independent changes in insulin sensitivity.

We have used DJB surgery in this study as a modified RYGB in order to eliminate the effect of a reduced gastric capacity on food intake and subsequently body weight and percent body fat, which directly relates to the level of insulin resistance. It is possible that gastric exclusion, as performed with RYGB and not DJB, is essential for the induction of any weight-independent improvements in insulin sensitivity. A recent study with SG, which parallels the gastric exclusion produced in RYGB without altering small bowel anatomy, found that insulin sensitivity had improved by five days after surgery [Rizello 2010]. This is in agreement with the rapid improvement in insulin sensitivity noted after gastric bypass. Another surprisingly similarity between RYGB and SG is that despite unaltered small bowel anatomy, SG can increase post-prandial GLP-1 and PYY secretion, a finding not commonly reported after gastric banding, and almost universally found after RYGB [Peterli 2009]. Further studies with SG are needed with appropriate weight-matched control groups to determine if gastric resection (or exclusion as performed in RYGB) is a required feature of a metabolic surgery to maximize insulin sensitivity changes beyond the reduction in gastric capacity and food intake.
GENERAL SUMMARY

In summary, the magnificent reversal of glucose intolerance and insulin sensitivity seen in type 2 diabetic patients after RYGB can not be solely explained by duodenal bypass suggesting that gastric exclusion of the neuro-endocrine stomach may be the most important contributing component beyond weight loss for a significant metabolic improvement after bariatric surgery. Our studies negate that there is a factor released from the small bowel in response to duodenal nutrient stimulation that is responsible for type 2 diabetes and removal of nutrient stimulation can reverse this process. Further animal and clinical studies are needed to determine how gastric exclusion or resection alters post-prandial glucose homeostasis with the goal of developing a safe and effective metabolic surgery for type 2 DM.
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Nutrient-driven incretin secretion into intestinal lymph is different between diabetic, Goto-Kakizaki rats and Wistar rats.

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ABSTRACT

Objective. The incretin hormones, GIP and GLP-1, augment post-prandial glucose-mediated insulin release from pancreatic β-cells. The Goto-Kakizaki (GK) rat is a widely-used, lean rodent model of type 2 diabetes; however, little is known regarding the incretin secretion profile to different nutrients in these rats. We have recently shown that lymph is a sensitive medium to measure incretin secretion in rodents and probably the preferred compartment for GLP-1 monitoring. To characterize the meal-induced incretin profile, we compared lymphatic incretin concentrations in the GK and Wistar rat after enteral macronutrient administration. Methodology. After cannulation of the major mesenteric lymphatic duct and duodenum, each animal received an intra-duodenal bolus of either a fat emulsion, dextrin, a mixed meal, or saline. Lymph was collected for 3 hours and analyzed for triglyceride, glucose, GLP-1 and GIP content. Results. There was no statistical difference in GIP or GLP-1 secretion after a lipid bolus between GK and Wistar rats. Dextrin and a mixed meal both increased incretin concentration AUC, however significantly less in GK rats compared to Wistar rats (dextrin GIP: 707 (pg/ml)hr ± 106 versus 1373 (pg/ml)hr ± 114 respectively, p<0.001; dextrin GLP-1: 82.7 (pM)hr ± 24.3 versus 208.3 (pM)hr ± 26.3 respectively, p=0.001). Conclusions. After administration of a carbohydrate-containing meal, GK rats were unable to mount as robust a response of both GIP and GLP-1 compared to Wistar rats, a phenomenon not seen after a lipid meal. We propose a similar, glucose-mediated incretin secretion pathway defect of both K and L cells in GK rats.

Key Words: Glucagon-like peptide-1 (GLP-1), Gastric inhibitory polypeptide (GIP), enteroendocrine cell, lymph, Goto-Kakizaki (GK) rat
INTRODUCTION

The incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) account for approximately 50-70% of insulin secretion from the pancreas after a meal (28,43). GIP is secreted from the enteroendocrine K cells, which are located throughout the entire small bowel but are primarily concentrated in the duodenum (49). GIP secretion from K cells is stimulated by both enteral glucose and lipid (7,21). GLP-1 is secreted from L cells in the distal small intestine and colon in response to enteral lipid, glucose and vagal stimulation (12,15). The specific intracellular mechanisms regulating incretin secretion from enteroendocrine cells after a meal have yet to be elucidated.

In obese patients with impaired glucose tolerance, GIP secretion is significantly higher than in healthy patients (43). With progression to type 2 diabetes, GIP secretion is normal to high and GLP-1 secretion is low compared to euglycemic individuals after a mixed meal (44,46). In addition, in both lean and obese diabetic patients, pancreatic GIP receptors show a markedly blunted stimulatory response to endogenous or exogenous GIP resulting in a lack of late-phase insulin secretion (29,47,48). There is also a decreased insulinotropic potency of GLP-1 in diabetic patients (19). Augmenting GLP-1 levels via dipeptidyl peptidase IV (DPPIV)-resistant analogues, DPPIV inhibitors, or surgical manipulation, have improved glucose homeostasis in type 2 diabetic patients (1,9,10).

The Goto-Kakizaki (GK) rat is an inbred, polygenetic, lean model of type 2 diabetes mellitus derived from Wistar rats in the 1970s (18). GK rats develop type 2 diabetes by 12 weeks of age. Adult GK rats are characterized by fasting hyperglycemia, hyperinsulinemia, mild insulin resistance and decreased pancreatic insulin mass and secretory capacity (13,30). GK rats have been utilized by investigators to study many different aspects of type 2 diabetes including the incretin changes after metabolic surgery and the effect of incretin modulation on insulin secretion and glucose homeostasis (33,37,40,42,45). However, baseline incretin secretion profiles in GK rats to various macronutrients have been largely unexplored.

Unfortunately, measuring incretins in systemic plasma has proven difficult due to assay detection sensitivity and the short plasma half-life of both GLP-1 and GIP (less than 2 minutes in rodents) (2). Our lab has shown that lymphatic measurement of incretins offers a sensitive medium for the detection of meal-induced hormone secretion (24,25). Furthermore, GLP-1 appears to be selectively targeted to the lymph compartment, in contrast to other gut hormones like peptide YY (8). In this study, we compared the incretin secretion profile of the GK and Wistar rat by characterizing lymphatic incretin secretion in response to different macronutrient administration. Appropriately characterizing incretin secretion in these animals will add an essential understanding of their native, physiologic secretion pattern which can then be applied to future, therapy-based, incretin studies in GK rats.

MATERIALS & METHODS
Animals. 14-16 week-old, male, Goto-Kakizaki rats (Taconic, Germantown, NY) and age-matched, Wistar rats (Charles River Laboratories, Wilmington, MA) were allowed to acclimate to their environment for two weeks prior to the beginning of the experiment. Rats had free access to standard, rodent chow and water except as noted for the experimental protocol. All procedures were approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Lymph and Duodenal Cannulation. Rats were fasted overnight but allowed free access to water. Under isoflurane anesthesia, the peritoneum was entered through a midline incision. The major mesenteric, lymphatic duct was identified and cannulated with tubing (medical grade; 0.50 mm ID and 0.80 mm OD; Tyco Electronics, Castle Hill, Australia). The lymphatic tube was secured in place with a drop of cyanoacrylate glue. A small enterotomy was made in the anterior wall of the stomach along the greater curvature, and a second silicone tube (0.04 in ID and 0.085 in OD; VWR International, West Chester, PA) was placed into the stomach and advanced past the pylorus into the duodenum. The duodenal tube was secured with a purse-string stitch. The abdominal wall was closed in two layers. Animals were kept in Bollman, restraint cages for the remainder of the study and were housed in temperature regulated isolates at 25ºC (4). Once the rat was fully awake after surgery, an infusion of 5% D-glucose in 0.9% NaCl solution was started through the duodenal tube at 3 ml/hr. That evening at 1700, the solution was switched to 0.9% NaCl without glucose at 3 ml/hr for the remainder of the experiment, except for administration of the nutrient bolus as described below.

Nutrient Administration and Sample Collection. The following morning after surgery, lymph was continuously collected for one hour on ice (fasting lymph). The rats were then given a single, intra-duodenal bolus of either 3 ml of saline or 3 ml of an iso-caloric meal (5-7 rats per group). The nutrient groups included (1) dextrin (Sigma Aldrich, St. Louis, MO) 1.1 g in 3 ml 0.9% NaCl, (2) 2.2 ml of Liposyn II 20% (Hospira, Lake Forest, IL) in 0.8 ml of 0.9% NaCl, or (3) a “mixed meal” of 0.55 g dextrin and 1.1 ml of Liposyn II 20% in 1.9 ml 0.9% NaCl. After 30 minutes, the rats were resumed on their intra-duodenal infusion of 0.9% NaCl for the remainder of the study. Lymph was continuously collected on ice for 30 minutes, 1 hour, 2 hours and 3 hours after the nutrient bolus. The collection tube was weighed before and after lymph collection to determine the lymphatic flow rate. A protective reagent was immediately added to the lymph samples to equal 10% total volume. The protective reagent consisted of 0.25 M EDTA (Sigma, St. Louis, MO), 80 U/ml heparin (American Pharmaceutical Partners, Schaumburg, IL), and 0.8 mg/ml aprotinin (Calbiochem, San Diego, CA). Lymph samples were stored at -20ºC until use.

Measurement of GIP, GLP-1, TG and Glucose. Intact GLP-1 concentrations were determined using a commercially available sandwich ELISA kit (LINCO Research, St. Charles, Missouri). The GLP-1 monoclonal antibody is specific to the N-terminal region of active GLP-1 forms including GLP-1 (7-36) and GLP-1 (7-37). The monoclonal antibody does not cross react with other forms of GLP-1 (1-36, 1-37, 9-36, 9-37), as well
as glucagon or GLP-2. As reported by LINCO, the intra-assay coefficient of variation (CV) is 7.4% and the inter-assay CV is 8%.

Total GIP concentrations were determined using a commercially available rat/mouse, sandwich ELISA kit (LINCO Research, St. Charles, Missouri). The GIP ELISA measures both active GIP (1-42) and inactive GIP (3-42) and does not cross react with glucagon, oxyntomodulin, GLP-1 or GLP-2. As reported by LINCO, the intra-assay CV is 2.6% and the inter-assay CV is 3.7%.

Triglyceride concentrations were determined using a triglyceride assay kit from Randox (Oceanside, CA). Glucose concentrations were determined using a modified glucose oxidase/peroxidase method (Diagnostic Chemicals Limited, Charlottetown, PE, Canada).

**Analysis and Statistics.** Total lymphatic flow was calculated for each nutrient as a sum of the hourly lymph flow not including the fasting values (i.e. cumulative lymph flow due to the experimental nutrient only). Area under the curve (AUC) was calculated using the trapezoidal rule. Comparisons of concentrations in lymph between different experimental groups were performed using a two-way analysis of variance assuming normality. All values are presented as the mean ± standard error (SE). Values were determined as statistically significant if p<0.05.

**RESULTS**

**Lymph Production.** Lymph flow rate was monitored continuously for 1 hour of fasting and 30 minutes, 1, 2 and 3 hours after the nutrient infusion. Total lymphatic production for the 3 hour study was compared for all 8 experimental groups (Figure 1). Lymph production increased with a lipid or mixed meal bolus in Wistar rats to 9.3 ml ± 1.1 and 11.1 ml ± 1.3 respectively, but this did not reach significance compared to the saline Wistar control at 7.0 ml ± 1.3. Dextrin did not increase total lymph production in either GK or Wistar rats compared to their respective saline, control animals. Total lymphatic production was significantly increased in GK rats after a lipid bolus to 14.9 ml ± 1.2 (p<0.05), and with a mixed meal bolus to 15.8 ml ± 1.3 (p<0.05) compared to GK saline controls at 5.2 ml ± 1.3. When comparing total lymphatic production between GK and Wistar rats, the diabetic GK rats had a greater total lymphatic production after both a lipid and mixed meal bolus (p=0.002 and p=0.019, respectively).

**Lymphatic Triglyceride Concentration.** As expected, a dextrin bolus did not significantly affect either triglyceride concentration at any time point or the triglyceride concentration AUC for both GK and Wistar rats. A lipid bolus significantly increased GK rat triglyceride concentrations above the GK saline control at 1, 2 and 3 hours. A lipid bolus in Wistar rats significantly increased the triglyceride concentration at all time points compared to the Wistar saline control. When comparing GK and Wistar rats, as shown in Figure 2, Wistar rats had a significantly higher triglyceride concentration AUC after both a lipid and mixed meal bolus compared to GK rats (lipid: 3438 (mg/dl)hr ± 204 versus...
1482 (mg/dl)hr ± 204 respectively, p<0.001; mixed meal: 3116 (mg/dl)hr ± 223 versus 1315 (mg/dl)hr ± 223 respectively, p<0.001).

Lymphatic Glucose Concentration. As expected for their diabetic state, GK control rats tended to have a higher lymphatic glucose concentration AUC, 290 (mg/dl)hr ± 42, compared to Wistar control rats, 216 (mg/dl)hr ± 42, p=0.23 (Figure 3). There was no difference in lymphatic glucose concentration AUC after a lipid bolus between GK and Wistar rats (p=0.92). When comparing lymphatic glucose concentration AUC, dextrin significantly increased the glucose AUC in GK rats greater than Wistar rats (602 (mg/dl)hr ± 36 versus 431 (mg/dl)hr ± 38 respectively, p= 0.002). A mixed meal bolus also increased GK rat glucose concentration AUC higher than Wistar rats, as expected for a type 2 diabetic rodent (593 (mg/dl)hr ± 42 compared to 513 (mg/dl)hr ± 42 respectively, p=0.19), although this did not reach statistical significance. The mixed meal bolus was a 50:50 combination of Liposyn and dextrin. It is possible that the lymphatic hyperglycemia noted in GK rats after a mixed meal bolus was not statistically higher than Wistar rats due to a similar glucose response in both groups to the Liposyn component.

Lymphatic GLP-1 Concentration. Figures 4A and 4B compare GLP-1 concentrations for both GK and Wistar rats after each nutrient. In GK rats, a carbohydrate-containing nutrient bolus (dextrin or a mixed meal) significantly increased GLP-1 concentrations at 30 minutes compared to a saline bolus (Fig. 4A). Wistar rats had a significantly elevated GLP-1 concentration after a carbohydrate-containing nutrient bolus at 30 minutes and 1 hour compared to a saline bolus. A lipid bolus significantly increased the lymphatic GLP-1 concentration of Wistar rats compared to a saline bolus at 1 hour (Fig. 4B).

As shown in Figure 6A, there was a trend, although not statistically significant, for a smaller increase in GLP-1 concentration AUC in GK rats after a lipid bolus compared to Wistar rats (116.1 (pM)hr ± 28.8 versus 172.1 (pM)hr ± 26.3 respectively, p=0.16). GK rats had a significantly smaller increase in GLP-1 concentration AUC after a dextrin bolus to 82.7 (pM)hr ± 24.3 compared to 208.3 (pM)hr ± 26.3 for Wistar rats, p=0.001. A similar smaller increase in GLP-1 concentration AUC in GK rats was seen after a mixed meal bolus compared to Wistar rats (149.2 (pM)hr ± 28.8 versus 416.1 (pM)hr ± 28.8 respectively, p<0.001). The impairment in GLP-1 secretion was so profound in GK rats that the GLP-1 concentration AUC was not significantly increased after any nutrient compared to the GK saline control (42.4 (pM)hr ± 28.8).

Lymphatic GIP Concentration. After all three nutrient boluses, GK rats responded with a significantly elevated GIP concentration at 30 minutes, 1 hour, and 2 hours compared to the GK saline control (42.4 (pM)hr ± 28.8).

When comparing the GIP concentration AUC between GK and Wistar rats (Figure 6B), GK control rats receiving a saline bolus had a trend, although not statistically significant, for a lower AUC compared to Wistar, control rats. A lipid bolus produced a similar GIP concentration AUC in GK rats (910 (pg/ml)hr ± 114) and Wistar rats (940 (pg/ml)hr ± 114, p= 0.86). Surprisingly following the GLP-1 pattern, a dextrin
bolus resulted in a significantly smaller increase in GK rat GIP concentration AUC compared to Wistar rats (707 (pg/ml)hr ± 106 versus 1373 (pg/ml)hr ± 114 respectively, p<0.001). The increase in GIP concentration AUC after a mixed meal bolus was also significantly smaller in GK rats compared to Wistar rats (927 (pg/ml)hr ± 125 versus 1750 (pg/ml)hr ± 125 respectively, p<0.001).

DISCUSSION

Studies of the lean, type-2 diabetic, GK rat have previously focused on the defects in pancreatic β-cell mass and insulin secretory capacity. However, GK rats have recently been used to study the response of various incretin-based therapies on glucose homeostasis (37,40,42,45). Furthermore, the GK rat has been utilized to study the improvement of diabetes after differing metabolic surgeries including duodenal-jejunal exclusion and ileal transposition, by mechanisms which augment meal-induced incretin secretion (31,32,33,36). However, only a few authors have examined the specific changes in incretin secretion to a specific nutrient in GK rats, and of these cases, only one incretin was assessed or no comparison was made to non-diabetic, Wistar animals, from which the GK rats were originally derived (31,32,33). In this study, we examined the differences in lymphatic incretin secretion of GK rats compared to non-diabetic, Wistar rats and how different nutrients affect incretin secretion.

Incretin secretion from the gastrointestinal tract is traditionally measured in the systemic circulation. However, plasma GIP (1-42) and GLP-1 (7-36) are quickly degraded by DPPIV to GIP (3-42) and GLP-1 (9-36) (2). Plasma may not be the ideal place to characterize incretin secretion in rodents due to the short half life, portal dilution, renal clearance, and assay sensitivity. Our laboratory has utilized lymph as a novel, alternative medium to measure incretin hormone levels in rodents. This technique involves the cannulation of the superior mesenteric lymphatic duct to allow for continuous lymphatic sampling of the gastrointestinal tract in conscious rats. Data from our laboratory and collaborators have shown that incretin concentrations are significantly higher in lymph than portal or systemic blood (8,24,25). Furthermore, GLP-1 appears to be specifically targeted to intestinal lymph, in comparison to other gut hormones (like peptide YY), making lymph an ideal medium to characterize meal-induced incretin secretion in GK rats (8).

This study supports the previous finding from our laboratory that in healthy animals, such as Sprague-Dawley rats, a mixed meal of carbohydrate and lipid increases incretin secretion greater than an iso-caloric challenge with either solitary nutrient (24,25). In this study we found that in Wistar rats, the delivery of a mixed meal increased lymphatic GLP-1 and GIP concentrations to a greater extent than the delivery of either nutrient alone, despite a similar caloric content. Contrastingly, this augmented incretin response to a meal of mixed nutrients was not as apparent in GK rats, suggesting that any potentiating effect of a mixed meal on incretin secretion is greatly diminished in this rodent model of diabetes. This observation creates an ideal study end-point for future incretin-centered therapies in GK rats, i.e. the return of mixed-meal augmentation of incretin secretion.
We were surprised to find that the GLP-1 secretion defect in GK rats did not affect all of the tested nutrients equally and was most prominent in dextrin-containing meals only. Clinical studies have shown that GLP-1 concentrations in type 2 diabetic patients are lower than in healthy subjects after a mixed meal (44,46). The responsible mechanisms that alter incretin secretion in diabetic patients are unknown although recent data suggest that these changes are a consequence, rather than a cause, of diabetes (20,26,48). When comparing type 2 diabetic patients to patients with diabetes secondary to chronic pancreatitis, both groups exhibit a similar loss of incretin effect (20). We therefore expected that GLP-1 in GK rats would also be decreased for all nutrients due to the diabetic state of these animals. To our knowledge, it is not known if the smaller increase in meal-induced GLP-1 secretion in type 2 diabetic patients applies to all types of nutrients equally, or to glucose-based nutrients only, as seen in this study.

In addition, this study found the surprising result that the dextrin-mediated incretin defect in GK rats applies similarly to both GLP-1 and GIP. Most clinical studies have found that GIP secretion is increased or unchanged in type 2 diabetic patients compared to euglycemic patients, although these findings may reflect the obese nature of the study population rather than diabetes (17,43). It is also possible that the decrease in GIP secretion is a finding unique to this rodent model, or in contrast, reflects a phenomenon of the lean diabetic state, as this is an understudied human population.

The exact mechanisms of nutrient based incretin secretion from enteroendocrine cells are unknown. In vitro studies of L and K cells have found that multiple intracellular signaling pathway are involved and may differ depending on the nutrient stimulus. Activation of cAMP pathways and increases in intracellular calcium stimulate GIP and GLP-1 release from both K and L cells (16,41). However, there also appear to be many differences in the ways that K and L cells sense enteral nutrients and signal incretin secretion. Several studies have identified an important role of vagal, cholinergic innervation in the secretion of GLP-1, while GIP secretion appears to be independent of vagal innervation (2,3,5,6). GLP-1 secretion from L cells has been linked to $K_{ATP}$ channels, while recent evidence suggests that the secretion of GIP may be $K_{ATP}$ independent (14,35,50). GLP-1 secretion may depend on the rate of delivery of nutrients to the intestine determined by gastric emptying (39), and GIP secretion may be dependent on, not just the presence of nutrients, but the rate of absorption and nutrient processing (11,15).

Because we found a relatively well-preserved secretion of both incretins in GK rats to lipid but with a diminished secretion to carbohydrate-containing meals, we speculate that the secretion defect is most likely of an incretin-signaling or regulatory mechanism shared by both K and L cells, rather than a defect of different mechanisms unique to each enteroendocrine cell population. There are data that support an effect of insulin resistance on mixed-meal induced incretin secretion. In a mixed-meal study by Rask et al on non-diabetic patients, a decreased secretion of both GLP-1 and GIP was found, which correlated with insulin resistance but not with obesity (34). Further, a recent article by Lim et al demonstrated that the L cell is insulin responsive, and insulin
resistance \textit{in vitro} and \textit{in vivo} results in decreased meal-stimulated GLP-1 release (23). Although rat enteroendocrine cells have not been found to express the insulin receptor (23), the impaired secretion of GIP and GLP-1 by enteroendocrine cells in the GK rat found in this study parallel the findings of Rask and suggest a defective mechanism that applies to both K and L cells during enteral-glucose stimulation, such as insulin resistance or hyperglycemia.

\textit{In vivo} and \textit{in vitro} studies of GK rats have shown a markedly decreased insulinotropic action of glucose on the pancreas (13). While some studies have shown impairment of several genes within the pancreas affecting insulin secretion as well as decreased β-cell neogenesis (27,30), this study offers the possibility that diminished incretin secretion to oral glucose may also contribute to \textit{in vivo} hyperglycemia in GK rats due to a reduced incretin effect and impaired insulin secretion from the pancreas.

This study also provides the interesting finding that GK rats have reduced lymphatic lipid absorption in the first three hours after a lipid-containing bolus compared to Wistar rats (as reflected by the lymphatic triglyceride concentration). This observation implies that in addition to the reduced incretin response to glucose, there are other intestinal defects present in GK rats of lipid processing or transport which might extend beyond the enteroendocrine cell to include the enterocytes. This conclusion counters recent evidence documenting overproduction of post-prandial lipids in the insulin-resistant state (22,38). Interestingly, despite this defect in lymphatic lipid absorption, the incretin response to the lipid meal was preserved in GK rats.

There is still a great deal to be learned regarding enteroendocrine signaling pathways and the similarities and differences between the mechanisms used by K and L cells to secrete incretins after a meal. Based on our current findings, we propose the possibility of a shared, enteral glucose-mediated, incretin pathway defect that applies to both K and L cells in GK rats. Determination of the exact mechanisms involved and the relevance to lean, type 2 diabetic patients is of significant, clinical interest, with the potential for the advancement of pharmaceutical agents aimed at enhancing native incretin secretion by a glucose-mediated pathway.

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Fig 1. Cumulative 3 hour lymphatic production for GK (black bar) and Wistar (white bar) rats after different experimental nutrient boluses. (a) represents significant differences of GK rats from Wistar rats given the same nutrient. (b) represents significant differences of the various nutrient boluses for GK rats compared to saline, GK controls. Statistical significance determined at p< 0.05. Data presented as mean ± SE.
Fig 2. Comparison of triglyceride concentration AUC after an isocaloric, intra-duodenal nutrient bolus for the 3-hour study period for GK (black bar) and Wistar (white bar) rats. (a) represents significant differences of GK rats from Wistar rats given the same nutrient. (b) represents significant differences of the various nutrient boluses for GK rats compared to saline, GK controls. (c) represents significant differences of the various nutrient boluses for Wistar rats compared to saline, Wistar controls. Statistical significance determined at p< 0.05. Data presented as mean ± SE.
Fig 3. Comparison of lymphatic glucose concentration AUC for the 3 hour study period between GK (black bar) and Wistar (white bar) rats. (a) represents significant differences of GK rats from Wistar rats given the same nutrient. (b) represents significant differences of the various nutrient boluses for GK rats compared to saline, GK controls. (c) represents significant differences of the various nutrient boluses for Wistar rats compared to saline, Wistar controls. Statistical significance determined at p< 0.05. Data presented as mean ± SE.
Fig 4. The changes in GLP-1 concentration with time after various nutrient boluses for GK rats compared to the saline, GK control (4A). The changes in GLP-1 concentration with time after various nutrient boluses for Wistar rats compared to the saline, Wistar control (4B). (*) represents significant differences between the experimental nutrient group compared to saline controls. Statistical significance determined at p< 0.05. Data presented as mean ± SE.
Fig 5. The changes in GIP concentration with time after various nutrient boluses for GK rats compared to the saline, GK control (5A). The changes in GIP concentration after various nutrient boluses for Wistar rats compared to the saline, Wistar control (5B). (*) represents significant differences between the experimental nutrient group compared to saline controls. Statistical significance determined at $p<0.05$. Data presented as mean ± SE.
Fig 6. Comparison of lymphatic GLP-1 concentration AUC for the 3 hour study period (6A). Comparison of lymphatic GIP concentration AUC for the 3 hour study period (6B). (a) represents significant differences of GK rats from Wistar rats given the same nutrient. (b) represents significant differences of the various nutrient boluses for GK rats compared to saline, GK controls. (c) represents significant differences of the various nutrient boluses for Wistar rats compared to saline, Wistar controls. Statistical significance determined at p< 0.05. Data presented as mean ± SE.
Duodenal-jejunal exclusion improves glucose tolerance in the diabetic, Goto-Kakizaki rat by a GLP-1 receptor mediated mechanism.

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Running Title: DJE effects on GLP-1 receptor

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Abbreviations: Roux-en-y gastric bypass (RYGB), Goto-Kakizaki (GK), duodenal-jejunal exclusion (DJE), ileal interposition (IT), oral glucose tolerance test (OGTT), glucagon-like peptide-1 (GLP-1), Ligament of Treitz (LOT), area under the curve (AUC)

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ABSTRACT

Background. Gastric bypass results in the rapid resolution of type 2 diabetes. No causal evidence exists to link specific gut hormone changes with improvements in glucose homeostasis post-operatively. We hypothesized that surgical augmentation of the glucoregulatory factor GLP-1 would improve glucose tolerance in diabetic GK rats. We compared 2 procedures that increase distal small bowel stimulation, ileal interposition (IT) and duodenal-jejunal exclusion (DJE). Methods. DJE, IT, DJE Sham, or IT Sham were performed in GK rats. Glucose tolerance was tested at 4 and 6 wks, the latter with and without Exendin-[9-39], a GLP-1 receptor antagonist. Small bowel segments were harvested for GLP-1 protein content 2 wks after DJE or Sham surgery. Results. Despite similar weight profiles, a significant improvement in the OGTT was noted at 4 and 6 wks after DJE and IT. Plasma GLP-1 levels were significantly elevated after DJE and IT. Intestinal GLP-1 was increased in the mid-jejunum and ileum after DJE. Exendin-[9-39] abolished the improvement in glucose tolerance after DJE. Conclusions. DJE increased GLP-1 secretion and improved glucose tolerance, an effect that was reversed by GLP-1 receptor antagonism. This study provides direct evidence that improvement of glucose tolerance following a gastric bypass-like surgery is mediated by enhanced GLP-1 action.

Key Words: gastric bypass, glucagon-like peptide-1, ileal interposition, incretin
INTRODUCTION

Roux-en-y gastric bypass (RYGB), the most commonly performed bariatric surgery in the United States, results in the rapid improvement of type 2 diabetes for morbidly obese patients [1]. The reported rate of resolution of diabetes after RYGB is approximately 80% [2-7]. Mechanisms beyond weight loss and calorie restriction are quite probable given the rapid and sustained improvement in type 2 diabetes found in post-RYGB patients. Common explanations for this response are based on changes in gastrointestinal hormone release that occur due to alterations in gastrointestinal anatomy [8-14]. However, there is as yet no direct evidence from animal or human studies that changes in gastrointestinal hormone secretion cause the improvement of glucose tolerance seen after gastric bypass surgery.

The distal jejunum and ileum contain the majority of enteroendocrine L cells, which secrete the incretin hormone, glucagon-like peptide-1 (GLP-1). The incretin hormones GLP-1 and gastric inhibitory polypeptide are responsible for up to 70% of post-prandial insulin secretion [15,16]. GLP-1 is a 30 amino acid peptide secreted by intestinal L cells in response to enteral carbohydrates and fats [16]. GLP-1 also decreases glucagon secretion, suppresses endogenous glucose production and enhances peripheral glucose uptake [17-19]. In addition, GLP-1 functions as an “ileal brake” by slowing gastric emptying, inhibiting food intake, and prolonging intestinal transit [20-22]. The administration of GLP1R agonists or DPP IV inhibitors, which retard the degradation of endogenous GLP-1, improve HgbA1c levels, and fasting and postprandial glucose concentrations of type 2 diabetic patients [23-25]. Post-prandial plasma GLP-1 levels are almost universally increased after RYGB, as early as 2 days after surgery, and this is likely due to increased delivery of nutrients to distal small bowel L cells [11, 26-28].

Duodenal-jejunal exclusion (DJE) is an experimental, metabolic surgery similar to RYGB, including duodenal and proximal jejunal exclusion to nutrients, a jejunal roux-en-y reconstruction, and early nutrient delivery to the distal small bowel. Several authors have shown dramatic, early improvements in glucose homeostasis in rodents following DJE surgery [9, 12, 29]. Ileal interposition (IT) is another experimental, metabolic, gastrointestinal surgery originally described in rats by Koopmans [30]. In an IT surgery, a distal segment of ileum is moved more proximally in the small bowel resulting in increased secretion of the ileal gut hormones, including GLP-1 and peptide YY [31-33]. Previously, a study comparing DJE and IT surgeries in lean, diabetic Goto-Kakizaki (GK) rats found that both surgeries resulted in the same improvement in glucose homeostasis, leading the authors to postulate that the distal small bowel was the responsible factor [34]. However, rats in this study had a significant weight loss after DJE and IT surgeries compared to sham controls, rendering definitive differentiation between the effect of weight loss and the surgical procedure itself difficult.

We hypothesized that DJE and IT surgeries would improve glucose tolerance in GK rats through early stimulation of the distal small bowel by nutrients resulting in increased secretion of GLP-1. We therefore directly compared the effects of DJE and IT on glucose tolerance and GLP-1 secretion in GK rats without a difference in post-surgical weight profiles. To further test if GLP-1 was the responsible hormone released from the distal small bowel, we acutely administered the GLP1R antagonist, Exendin-[9-39] (Ex-
9), during an oral glucose tolerance test (OGTT) performed 6 weeks after surgery in DJE and DJE Sham rats.

**METHODOLOGY**

***Animals and Experimental Design.*** At the time of study initiation, 12 to 14 week old, male, GK rats (Taconic, Germantown, NY), or age-matched Wistar rats (Charles River Laboratories, Wilmington, MA) were housed individually. GK rats are an inbred, lean model of type 2 diabetes derived from Wistar rats. Rats were allowed to acclimate to their environment for one week prior to the beginning of the study. All animal procedures and protocols were approved by the University of Cincinnati’s Internal Animal Care and Use Committee.

The first experiment involved rats in five different study groups (n = 9 per group). These groups included: (1) GK DJE, (2) GK DJE Sham, (3) GK IT, (4) GK IT Sham, and (5) Wistar IT Sham. A Wistar IT Sham group allowed for a comparison to non-diabetic animals. Food intake and body weight were followed for 30 days post-operatively. An OGTT was performed pre-operatively and at 2 and 4 weeks post-operatively. An insulin tolerance test (ITT) was performed at 3 weeks post-operatively. At 5 weeks post-operatively, a mixed meal test was performed following the insertion of a jugular cannula for the measurement of systemic incretin hormones.

The second experiment included GK rats in two different study groups, DJE (n = 7) and DJE Sham (n = 6 Sham). At two weeks after surgery, intestinal segments from the duodenum, mid-jejunum, and ileum were harvested for GLP-1 protein content. We chose this time point because we had seen from Experiment #1 an improvement in glucose tolerance in DJE rats compared to Sham rats during an OGTT as early as 2 weeks after surgery.

The third experiment again had two different groups of GK rats, DJE (n = 8) and DJE Sham (n = 6). Animals were followed for 6 weeks after surgery. After six weeks, the animals were acutely challenged with Exendin-9 during the administration of an OGTT to test the involvement of GLP1R signaling in the improvement in glucose homeostasis after duodenal-jejunal exclusion.

***Surgical Procedures.*** (1) *Duodenal-Jejunal Exclusion.* Animals were fasted for 18 hours pre-operatively. Under isoflurane anesthesia, the peritoneum was entered through a midline incision. Similar to the duodenal exclusion described by Rubino et al [29], the most proximal portion of the duodenum and the jejunum 10 cm distal to the Ligament of Treitz (LOT) were divided (Figure 1). The proximal segment of duodenum was anastomosed to the distal segment of divided jejunum in end-to-end fashion. The distal stump of duodenum was sewn closed. A partial enterotomy was made 15 cm distal to the duodeno-jejunosotomy and a jeju-jejunostomy was made with the proximal segment of divided jejunum in end-to-side fashion. The abdomen was irrigated and closed in two layers. Rats had free access to water for the first 24 hours post-operatively. Twenty-four hours after surgery, the rats were started on an *ad lib* liquid diet (Regular Ensure, Abbott Laboratories, Columbus, OH). After 24 hours of a liquid diet (post-operative day #2), the rats were transitioned back to their pre-operative standard chow diet (Harlan Teklad diet 7012).
(2) Ileal Interposition. An ileal interposition was performed similar to the procedure previously described by Strader et al [31]. Rats were also fasted for 18 hours pre-operatively. The abdomen was entered under isoflurane anesthesia. The cecum was identified, and the ileum was divided at 5 and 15 cm proximal to the cecum (Figure 1). After division of the jejunum 10 cm distal to the LOT, the isolated segment of ileum was interposed into the divided segment of proximal jejunum. The divided segment of proximal and distal ileum were then re-anastomosed in end-to-end fashion. The abdomen was irrigated and closed in 2 layers. The post-operative care was the same as that described for DJE above.

(3) Sham Surgeries. All Sham rats received the same pre- and post-operative care as the DJE and IT rats. For the DJE Sham surgery, a full enterotomy with division of the mesentery and re-anastomosis in end-to-end fashion was made at the proximal duodenum, 10 cm distal to the LOT, and 25 cm distal to the LOT. The IT Sham surgery included an enterotomy, mesenteric division, and re-anastomosis at 10 cm distal to the LOT, 5 cm proximal to the cecum, and 15 cm proximal to the cecum.

(4) Jugular Cannulation and Gastric Tube Insertion. Animals were fasted overnight. Under isoflurane anesthesia, the right internal jugular vein was identified and isolated. A catheter (0.014 ID/0.033 OD, Braintree Scientific, Braintree, MA) was inserted in the jugular vein and advanced to the level of the right atrium. The distal catheter was tunneled subcutaneously and exteriorized at the posterior aspect of the neck. Under the same anesthetic period, the abdomen was entered through the previous midline incision. The stomach was mobilized and a small enterotomy was made along the anterior aspect of the greater curvature. A catheter (0.04 ID/0.085 OD, VWR International, West Chester, PA) was inserted into the stomach and secured with a purse-string stitch. The gastric catheter was exteriorized through the right flank, and the abdomen was closed in two layers. Animals were kept in restraint cages post-operatively. The mixed meal study for experiment #1 was started after two hours of anesthetic recovery.

Insulin Tolerance Test. An ITT was performed at 3 weeks post-operatively in experiment #1. 0.5 U/kg of insulin was administered subcutaneously followed by blood sample collection from the tail vein at 15, 30, 45 and 60 minutes post-injection. Blood samples were immediately assayed in duplicate for glucose concentration using a handheld glucometer. Due to unacceptable hypoglycemia in Wistar rats, a 0.5 U/kg dose of insulin could not be used and subsequently the Wistar IT group was not used for comparison of insulin sensitivity.

Oral Glucose Tolerance Test. For experiment #1, a 2 g/kg D-glucose OGTT was performed pre-operatively and at 2 and 4 weeks post-operatively. Blood samples were collected from the tail vein at 0, 10, 30, 60, and 120 minutes after the glucose gavage and immediately assayed in duplicate for glucose concentration using a handheld glucometer. Blood samples from the 4 week OGTT were also collected in EDTA coated collecting tubes. Samples were spun at 4000 g for 10 minutes at 4°C, and the plasma was stored at -20°C until assayed for insulin concentration using a commercially available ELISA kit (Millipore, St Charles, MO). For experiment #2, an OGTT was performed on 2 separate days at 6 weeks post-operatively with the co-administration of either
subcutaneous saline or the GLP-1R antagonist Ex-9 as described below. To better characterize the glucose response, blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 minutes after the glucose challenge.

**Mixed Meal Test.** We have previously shown that GK rats have a more robust secretion of GLP-1 to a mixed meal bolus over a solitary nutrient, such as glucose [35]. We therefore used a mixed meal test in experiment #1 to maximize GLP-1 secretion and plasma measurement. A mixed meal of Regular Ensure (7.68 ml/kg) was given intragastric to all rats in Experiment #1 at 5 weeks post-operatively. Blood samples were collected from the jugular catheter at 0 and 30 minutes after the mixed meal bolus. Blood samples were collected into EDTA-coated collecting tubes with the addition of a 1% DPPIV inhibitor (Millipore, St Charles, MO) and spun at 4000 g for 10 minutes at 4ºC. Plasma was stored at -20ºC until assayed for GLP-1 concentration. GLP-1 samples were assayed using an active GLP-1 ELISA kit (Millipore, St Charles, MO).

**Small Bowel GLP-1 Protein Content.** For experiment #2, 2 cm intestinal segments were isolated from 3 separate sections of small intestine under anesthesia. These sections included (1) the second segment of the duodenum, (2) 25 cm distal to the LOT (Sham animals) or just distal to the jeju-jejunostomy (DJE animals), and (3) the distal ileum. Tissues were weighed and frozen at -20ºC. Frozen segments were homogenized in 2 M glacial acetic acid (5 ml/g tissue weight). Samples were incubated at 95ºC for 10 minutes followed by a 10 minute incubation on ice. After centrifugation at 4000 g for 10 minutes at 4ºC, the supernatant was removed, frozen, and lyophilized. Once lyophilized, the segments were resuspended in dH2O, diluted, and assayed the same day for total protein concentration and GLP-1 concentration using an active GLP-1 ELISA kit (Millipore, St Charles, MO).

**GLP1R blockade with Ex-9.** In experiment #3, GK rats at 6 weeks after surgery were given either a subcutaneous dose of 200 µl of saline or 25 nM of Ex-9 (Bachem, Torrence, CA). This was followed 10 minutes later by a 2 g/kg D-glucose OGTT as described above.

**Statistical Analysis.** Area under the curve (AUC) was calculated using the trapezoidal rule. Comparisons between surgical groups were made using a one-way analysis of variance (ANOVA) or a two-way ANOVA for the Exendin-9 study to account for separate treatments and surgeries. Comparisons between surgical groups over time were performed using a two-way repeated-measures ANOVA. A student’s t-test was used to compare GLP-1 content of the intestinal segments. All values are presented as the mean ± standard error. Values were determined as statistically significant if p<0.05.

**RESULTS**

**DJE and IT do not affect body weight or food intake in GK rats.**
In experiment #1, Wistar IT rats weighed significantly more than all of the GK surgical groups for every time point of the study (Figure 2A). There was no difference in body weight between any of the GK surgical groups for each day measured post-operatively.
As shown in Figure 2B, Wistar IT rats also ate significantly more food per day compared to all GK rat groups (excluding post-operative days 0-2 when rats were fasted or on a liquid diet). GK DJE rats ate the same amount of daily chow as GK DJE Sham rats except for post-operative day #28 (GK DJE 26.9 g ± 2.20 vs GK DJE Sham 22.1 ± 2.48, \( p < 0.05 \)). Similarly, GK IT rats ate the same amount of daily chow as GK IT Sham rats except for post-operative day #12 (GK IT 22.5 ± 0.98 versus GK IT Sham 26.8 g ± 2.25, \( p < 0.05 \)). In experiment #2 and 3, there was no difference in post-operative body weights for any day measured between GK DJE and GK DJE Sham rats (data not shown).

**DJE and IT significantly improves glucose tolerance by 4 weeks after surgery in GK rats without changing plasma insulin concentrations.**

An OGTT was performed at 0, 2, and 4 weeks post-operatively in experiment #1. There was no difference in pre-operative glucose tolerance AUC among the 4 GK groups (Figure 3), and pre-operative Wistar IT Sham rats had a significantly lower glucose concentration throughout the OGTT compared to the GK groups (data not shown). As shown in Figure 3, by 4 weeks after surgery, both GK DJE and GK IT rats had a significantly lower late-phase glucose AUC (60-120 minutes) compared to GK DJE Sham and GK IT Sham rats (GK DJE 13267 (mg/dl)min ± 457 vs. GK DJE Sham 15696 (mg/dl)min ± 663, \( p < 0.05 \); GK IT 13327 (mg/dl)min ± 936 vs. GK IT Sham 15769 (mg/dl)min ± 360, \( p < 0.05 \)).

At 2 weeks after surgery, both DJE and IT rats had a lower glucose concentration at 120 minutes compared to their respective sham groups during an OGTT (GK DJE 189.3 mg/dl ± 8.5 vs. GK DJE Sham 237.1 mg/dl ± 15.3, \( p < 0.05 \); GK IT 197.4 mg/dl ± 13.3 vs. GK IT Sham 238.0 (mg/dl)min ± 17.6, \( p = NS \); data not shown). As reflected in Figure 4A, the glucose concentration over time during an OGTT at 4 weeks was significantly lower in GK DJE compared to GK DJE Sham rats at 60 minutes (244 mg/dl ± 7.7 vs. 282 mg/dl ± 15.7 respectively, \( p < 0.05 \)) and 120 minutes (198 mg/dl ± 10.6 vs. 241 mg/dl ± 15.0 respectively, \( p < 0.05 \)). Similar to GK DJE rats, GK IT rats had a significantly lower glucose concentration compared to GK IT sham rats at 120 minutes (GK IT 192 mg/dl ± 17.4 vs. GK IT Sham 242 mg/dl ± 8.2, \( p < 0.05 \)). Surprisingly, there was no difference in insulin secretion profiles (Figure 4B) during the 4 week OGTT between the GK experimental and their respective GK sham group at any time point. GK rats lacked a rapid increase and peak in insulin secretion seen at 30 minutes in Wistar IT Sham rats (30 min. insulin concentration: 2.1 ng/ml ± 0.16, \( p < 0.05 \) compared to all GK groups).

**DJE and IT do not affect insulin sensitivity in GK rats.**

Plasma glucose concentrations were determined after the administration of 0.5 U/kg of insulin subcutaneously to all GK surgical groups (Figure 5) at 3 weeks post-operatively. There was no statistical difference in glucose concentrations at any time point between any of the GK surgical groups, suggesting that neither DJE nor IT surgery acutely affects insulin sensitivity in GK rats after surgery.

**DJE and IT increase post-prandial plasma GLP-1 concentrations.**

Plasma GLP-1 levels were measured from the jugular vein after administration of a mixed meal tolerance test at 5 weeks after surgery (Figure 6). There was a statistically
significant increase in fasting GLP-1 levels of GK DJE rats, 3.5 pM ± 0.20, compared to Wistar IT Sham rats, 2.3 pM ± 0.19, (p < 0.05). Both GK DJE and IT surgical groups had significantly higher plasma GLP-1 concentrations at 30 minutes post-prandial compared to their respective GK sham groups (GK DJE 4.5 pM ± 0.36 versus GK DJE Sham 2.7 pM ± 0.22, p < 0.05; GK IT 4.4 pM ± 0.52 versus GK IT Sham 3.1 pM ± 0.25, p < 0.05). Both GK DJE and IT groups also had a significantly higher GLP-1 concentration at 30 minutes compared to the Wistar IT Sham group, 2.5 pM ± 0.11.

**DJE increases distal small bowel GLP-1 protein content.**

By 2 weeks after surgery, there was a significant increase in the GLP-1 content of the distal small intestine (Figure 7). As expected, DJE did not significantly alter the duodenal GLP-1 concentration compared to sham animals (0.33x10^-6 % ± 0.067 vs. 0.263x10^-6 % ± 0.039 respectively, p = 0.43). DJE compared to Sham surgery significantly increased both mid-jejunal GLP-1 content (2.34x10^-6 % ± 0.29 vs. 1.44x10^-6 % ± 0.22 respectively, p = 0.03) and ileal GLP-1 content compared to sham rats (5.19x10^-6 % ± 0.42 vs. 2.88x10^-6 % ± 0.24 respectively, p < 0.001).

**Ex-9 administration ablates the significant improvement in glucose tolerance at 6 wks after DJE in GK rats.**

Similar to experiment #1 as seen 4 weeks after surgery, there was a statistically significant late-phase improvement in glucose concentrations in DJE rats compared to DJE Sham rats at both 60, 90 and 120 minutes after an oral glucose load performed at 6 weeks after surgery (Figure 8A). DJE rats at 60 minutes had an average glucose concentration of 285.0 mg/dl ± 5.9 compared to 316.9 mg/dl ± 4.1 for Sham rats, p = 0.007. At 120 minutes, the average glucose concentration for DJE rats was 211.1 mg/dl ± 10.3 compared to 255.7 mg/dl ± 13.5 for Sham rats, p < 0.001. As shown in Figure 8C, there was a significant improvement in glucose concentration AUC for DJE rats (28786 (mg/dl)min ± 571) compared to Sham rats (32113 (mg/dl)min ± 593, p = 0.035). The administration of Exendin (9-39) to DJE and DJE Sham rats resulted in similar glucose concentration curves, with the loss of the statistically significant late-phase improvement for the DJE group over time (Figure 8B). As shown in Figure 8C, there was no difference (p = 0.439) in OGTT AUC observed between the 2 groups after the administration of Ex-9.

**DISCUSSION**

In this study, we found that independent of weight loss, both DJE and IT in GK rats result in a statistically significant improvement in glucose tolerance by 4 weeks after surgery. Both metabolic surgeries did not acutely change plasma insulin concentrations or insulin sensitivity. Supporting a mechanism mediated by enhanced nutrient delivery to the distal small bowel, the common feature of DJE and IT, we found a similar magnitude of elevation of post-prandial plasma GLP-1. Furthermore, intestinal GLP-1 protein levels were significantly increased by 2 weeks after surgery in not only the ileum (the major focus of L cells in the non-operated gut) but also the mid-jejunum at the new post-surgical site of primary nutrient absorption. The administration of the GLP1R antagonist, Ex-9, ablated the significant improvement in glucose tolerance seen after DJE surgery at
6 weeks. Thus, the improvement in glucose tolerance noted after DJE in this model is mediated by GLP1R signaling.

RYGB results in the early and sustained improvement in glucose homeostasis for the majority of morbidly obese, type 2 diabetic patients. Multiple mechanisms stemming from the rearrangement of small bowel anatomy may be involved beyond weight loss and calorie restriction. For this reason, we compared two different experimental, metabolic surgeries, DJE and IT, to determine if one surgery offered an advantage over the other regarding glucose tolerance in a lean, rodent model of diabetes. Both surgeries increase distal small bowel exposure to nutrients but only DJE, like RYGB, bypasses the duodenum and proximal jejunum. It has been proposed that exclusion of the duodenum from nutrient stimulation is a predominant mechanism responsible for the improvement in glucose homeostasis after RYGB [8, 9]. Results of this study indicate that increased GLP-1 secretion and GLP1R stimulation, and not duodenal exclusion, is the predominant mechanism involved in the early improvement in glucose tolerance after DJE surgery in GK rats.

The GLP1R is a specific G-protein coupled receptor located on the lung, brain, kidney, pancreatic islets and gastrointestinal tract [36-38]. We were unable to specifically identify which action of GLP1R signaling was responsible for the improvement in glucose tolerance. Although we did not detect an absolute increase in plasma insulin levels following DJE, this does not exclude the possibility that the surgery enhances insulin secretion via increased GLP1R stimulation. Because both DJE and IT result in reduced glucose concentrations without a change in insulin sensitivity, it is possible that a relatively greater secretion of insulin for the given glucose concentration accounts for some of the effect of surgery. This relative increase in insulin secretion could be the dominate GLP1R mechanism in this model, as some clinical studies have found an increase in post-prandial insulin secretion after RYGB [39, 40].

Ayala et al have shown that GLP1R -/- mice have an impaired suppression of hepatic glucose production independent of insulin secretion [19]. Activation of GLP1R signaling suppresses glucagon secretion and could possibly mediate the suppression of hepatic glucose production. Le Roux et al administered octreotide as a non-specific blocker of GLP-1 and PYY to post-RYGB and gastric banding patients and found an increase in meal size and decrease in satiety unique to the RYGB group; however, the effect on glucose tolerance, insulin, and glucagon secretion was not assessed [28]. There is a lack of consensus regarding the changes in glucagon secretion after RYGB, including a decrease, no change or increase in glucagon secretion [41-44]. Because we did not measure plasma, or more specifically, portal vein glucagon concentrations, we cannot exclude the possibility that the effects of DJE surgery are mediated by the suppression of glucagon secretion via a GLP1R mechanism.

The administration of Ex-9 in vivo completely abolishes the stimulatory effect of endogenous GLP-1 on insulin secretion, with no effect on co-stimulators of insulin such as gastric inhibitory polypeptide and vasoactive intestinal polypeptide [45, 46]. While Ex-9 is specific for the GLP1R, there are cross-reactive hormones of the GLP1R besides GLP-1, including the intestinal proglucagon alternative splice product oxyntomodulin. Oxyntomodulin as yet does not have an identified separate receptor and has been found to mediate glucoregulatory actions including stimulation of insulin secretion through a functional GLP1R [47, 48]. We did not measure oxyntomodulin concentrations in this
study and are unaware of any published reports regarding the effect of RYGB on oxyntomodulin secretion. However, oxyntomodulin acts only partially via the GLP1R. Because we found a full reversal of the improvement in glucose tolerance after DJE with use of the GLP1R antagonist, we expect that the hormone involved is mediated only by GLP1R signaling, making GLP-1 the likely candidate. The increase in GLP1R signaling could be from a physiologically relevant increase in GLP-1 or due to increased sensitivity and enhanced incretin effect of GLP-1 on the GLP1R, regardless of the quantitative changes in GLP-1 secretion.

We found no change in insulin sensitivity assessed by a subcutaneous ITT. While the euglycemic-hyperinsulenic clamp offers greater precision compared to an ITT in assessing peripheral insulin sensitivity, we were not surprised to find that insulin sensitivity was not acutely affected by DJE or IT. Some studies have suggested unique changes in insulin sensitivity after RYGB [49]. However, when RYGB patients are compared to patients with similar degrees of weight loss (gastric banding patients), the improvement in insulin sensitivity correlates to the magnitude of post-surgical weight loss [50, 51], and thus would not be expected in our surgical model.

Our study does not find the robust improvement in glucose tolerance as previously reported by some investigators after DJE in GK rats [12, 29, 45]. Differences in surgical technique and post-operative care are possible reasons for this difference. Also, there are differences in phenotypic severity between different colonies of GK rats [52]. The GK rat is a lean, inbred model of type 2 diabetes derived from Wistar rats. These rats have reduced β-cell mass, decreased pancreatic insulin reserves, and a defective secretion of insulin to a glucose stimulus [53, 54]. With age, GK rat islets have a decreased number of β-cells, reduced islet insulin content, and exhibit abnormal islet morphology [55, 56]. It is possible that in a rat strain dominated by pancreatic insulin insufficiency, there is a point of “no return” in reversing pancreatic failure and a sub-maximal amount of recovery that can be obtained with DJE surgery. Recent data has shown that the rate of resolution of diabetes after RYGB is highest for patients who have had a short duration of disease (less than 4 or 5 years) or mild disease (diet-controlled) [4, 57]. The lack of a consentient improvement in glucose tolerance after DJE surgery points to the need for further research to determine what factors (duration of diabetes, type of diabetes, insulin requirements, beta cell reserve, etc.) enable or prevent a maximum surgical response.

While this study did not produce dramatic improvements in glucose tolerance by 4 to 6 weeks after DJE or IT, our results parallel the findings of recently published results with IT surgery. IT performed in streptozocin-induced diabetic rats had a similarly significant although small improvement in glucose homeostasis by 4 weeks after surgery without a change in insulin secretion [32]. By 11 weeks, IT surgery in these rats resulted in a more dramatic improvement in glucose concentrations after a glucose tolerance test. We suspect that with a longer observation period, improvements in glucose tolerance would have been more pronounced for both surgeries due to β-cell recovery as seen by other investigators after IT or with exogenous GLP-1 treatment [58, 59].

CONCLUSION
To our knowledge, this study offers the first direct evidence documenting a causal relationship between a change in GLP-1 signaling induced by bypass surgery and the subsequent improvement in post-operative glucose tolerance. It is possible that in other animal models, specifically in a diet-induced obesity model, DJE may cause other positive hormonal changes beyond GLP1R signaling that affect glucose tolerance. Clinically, it is yet unknown if the combination of effects that bypass surgery can achieve induced by weight loss, calorie restriction and augmented hormone signaling are superior to pharmacologic intervention in a population of type 2 diabetic patients with a BMI<35 (especially when considering cost effectiveness, morbidity and mortality). However, evidence, as shown in this study, that RYGB-like surgeries, independent of weight loss and calorie restriction, can benefit type 2 diabetes mellitus in animal models by enhancing incretin signaling, supports the further careful and cautious investigation of RYGB for the use as a treatment for type 2 diabetic patients without morbid obesity.
CITATIONS


FIG 1. Duodenal-jejunal exclusion (DJE) and ileal interposition (IT) are two experimental, metabolic surgeries used for the investigation and treatment of type 2 diabetes mellitus. As diagramed on the left, DJE bypasses the entire duodenum and 10 cm of proximal jejunum (dark grey color). IT (right panel) leaves anatomically normal nutrient flow to the proximal small bowel. Both surgeries offer early nutrient delivery to the distal small bowel (light grey color).
FIG 2. Body weight and food intake after gastrointestinal surgery in GK and Wistar rats. Body weights (Panel A) and food intake (Panel B) were assessed daily pre-operatively and for 30 days post-operatively. *Statistically different for all days when comparing Wistar IT sham to all GK surgical groups when p<0.05. Represents statistically significant comparisons between GK DJE and GK DJE Sham (a) and GK IT and GK IT Sham (b) when p < 0.05. Data are presented as mean ± SE.
FIG 3. Oral glucose tolerance test AUC was determined by measuring glucose concentrations before and after (10, 30, 60 and 120 minutes) the administration of an oral glucose load (2 g/kg D-glucose). AUC was determined using the trapezoidal rule. Glucose tolerance tests were performed pre-operatively and at 2 and 4 weeks post-operatively. *Statistically different AUC when comparing Wistar IT sham to all GK surgical groups when p<0.05 (intra-week comparisons only). Represents statistically significant comparisons between GK DJE and GK DJE Sham (a) and GK IT and GK IT Sham (b) when p < 0.05 (intra-week comparisons only). Data are presented as mean ± SE.
FIG 4. Plasma glucose (Panel A) and insulin (Panel B) concentrations were measured before and after (10, 30, 60 and 120 minutes) the administration of an oral glucose tolerance test (2 g/kg D-glucose) at 4 weeks post-operatively. *Statistically different for all time points (Panel A) or designated time points and groups (Panel B) when comparing Wistar IT sham to all GK surgical groups when p<0.05. Represents statistically significant comparisons between GK DJE and GK DJE Sham (a) and GK IT and GK IT Sham (b) when p < 0.05. Data are presented as mean ± SE.
FIG 5. An insulin tolerance test was performed at 3 weeks post-operatively. Plasma glucose concentrations were measured before and after (15, 30, 45 and 60 minutes) the administration of insulin (Humalin 0.5 U/kg sq for GK rats). Values are presented for each surgical group as a percent glucose concentration change compared to each groups respective fasting values. There were no differences in glucose concentrations between any of the GK surgical groups at any time point after insulin administration with significance determined as p < 0.05.
FIG 6. GLP-1 concentrations were measured from jugular plasma samples before and 30 minutes after a mixed meal bolus of Ensure (7.68 ml/kg) via an intragastric catheter. *Statistically different when compared to Wistar IT Sham rats when p < 0.05. Represents statistically significant comparisons between GK DJE and GK DJE Sham (a) and GK IT and GK IT Sham (b) when p<0.05. Data are presented as mean ± SE.
FIG 7. Percentage intestinal GLP-1 protein content was determined at 2 weeks after DJE (n=7) or DJE Sham (n=6) surgery in GK rats. Intestinal segments were taken from the second segment of the duodenum, mid-jejunum (distal to the jeju-jejunostomy in DJE rats or 25 cm distal to the ligament of Treitz in Sham rats), and distal ileum. *Statistically different for the tested segment of small bowel between DJE and Sham rats when p < 0.05. Data are presented as mean ± SE.
FIG 8. An OGTT was performed in male, GK rats 6 weeks after DJE (n=8) or DJE Sham (n=6) surgery. Plasma glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 minutes after a 2 g/kg D-glucose oral gavage with the co-administration of 200 µl of saline (Panel A) or 200 µl of 25 nM of the GLP1R antagonist Ex-9 (Panel B) subcutaneously. Panel C depicts glucose concentration AUC for the 6 week OGTT. *Statistically different for the designated time points (Panel A and B) or between groups (Panel C) when p < 0.05. Data are presented as mean ± SE.
The effect of duodenal-jejunal bypass on glucose-dependent insulinotropic polypeptide secretion in Wistar rats.

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Running Title: DJB and lymph GIP secretion.
Abstract

Background. Enteroendocrine K cells secrete the incretin hormone glucose-dependent insulino tropic peptide (GIP) and are predominately located in the duodenum. GIP levels should decrease after gastric bypass due to duodenal exclusion; however, studies have found conflicting data regarding the changes in GIP secretion after gastric bypass and duodenal-jejunal bypass (DJB).

Methods. We performed a DJB or Sham surgery on Wistar rats followed by an oral glucose tolerance test (OGTT) on post-op day #12 and superior mesenteric lymphatic cannulation on post-op day #14. We measured meal-stimulated GIP concentrations and small bowel GIP and GLP-1 protein content after DJB or Sham surgery.

Results. There was no difference in glucose tolerance by 10 days post-op. We found no difference in lymphatic GIP concentration AUC between DJB and Sham rats (15240 (pg/ml)*min ± 2651 vs. 17201 (pg/ml)*min ± 2763 respectively, p=0.62). GIP and GLP-1 protein content were both significantly increased only in the mid-jejunum in DJB rats compared to Sham rats, (p=0.009 and p=0.01 respectively).

Conclusions. Plasma and lymphatic GIP concentrations did not significantly change after DJB in Wistar rats. DJB increased GIP protein content in the mid-jejunum at the new site of nutrient absorption but this was surprisingly not countered by a decrease in GIP protein content in the bypassed duodenum. Further studies are need to determine the mechanisms that account for the discrepancy in GIP production and subsequent secretion after DJB as well as what role GIP plays in the effect of gastrointestinal surgery on glucose homeostasis.

Key Words: duodenal-jejunal bypass, glucose-dependent insulino tropic polypeptide, incretin, lymph, roux-en-y gastric bypass, K cell
Introduction

Roux-en-y gastric bypass (RYGB) results in the rapid improvement of type 2 diabetes in approximately 80% of morbidly obese patients [1-4]. Changes in secretion of one or both incretin hormones have been a commonly explored mechanism to explain the observed improvements in glucose homeostasis after RYGB. The changes in incretin secretion are likely yielded from the re-routing of the gastrointestinal tract after RYGB with exclusion of the majority of the stomach and entire duodenum and early delivery of nutrients to the distal small bowel.

The incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinoactive polypeptide (GIP), are responsible for 50-70% of post-prandial insulin secretion from the pancreas [5,6]. GIP is secreted from the enteroendocrine K cells in response to enteral nutrients. K cells are located throughout the entire gastrointestinal tract but are primarily concentrated in the duodenum [7]. Despite a primary role as an incretin hormone, recent evidence suggests that GIP over-secretion with a subsequent enhancement in receptor signaling may propagate the development of obesity, insulin resistance, and type 2 diabetes. This is supported by studies of GIP receptor -/- mice, which are resistant to diet-induced obesity and have preserved insulin sensitivity compared to high-fat fed controls [8]. Further, the antagonism of the GIP receptor prevents the development of diabetes in ob/ob mice and reverses obesity-associated metabolic disturbances in high-fat fed mice [9,10].

Given the proximal focus of GIP secreting K cells, bypassing the entire duodenum should theoretically result in a dramatic reduction in nutrient-mediated GIP secretion. However, there have been a large number of conflicting reports regarding the changes noted in GIP secretion after RYGB, including increased, decreased or no change in secretion. In a published review of longitudinal studies of RYGB or biliopancreatic diversion (BPD) and the effect on incretin secretion, results varied from a decrease in fasting and post-prandial GIP as early as one week after surgery, to no change at 12 months, to an increase in post-prandial levels one month after surgery [11-14]. Many of these discrepancies may be related to differences in surgical bypass limb length, time from surgery, fasting or post-prandial assessment, and the assay used for GIP measurement.

In this study, we utilized lymphatic incretin sampling as a more sensitive medium than plasma [15] to detect post-prandial changes in GIP secretion induced by duodenal-jejunal bypass (DJB, also known as duodenal-jejunal exclusion) surgery, an experimental surgery used initially in rodents to model RYGB [16-18]. DJB does not exclude the stomach as in a RYGB, but shares duodenal and proximal jejunal exclusion to nutrient flow, a jejunal roux-en-y reconstruction, and early nutrient delivery to the distal small bowel. We have previously published that in Goto-Kakizaki rats the primary mechanism for a modest improvement in glucose tolerance after DJB appears to be mediated by the surgical re-routing of the small bowel with a resultant increase in GLP-1 secretion and receptor signaling [19]. However, it is unclear if in other models of type 2 diabetes, if
hormonal mechanisms beyond the GLP-1 receptor, such as a reduction in GIP secretion or signaling, are involved.

We performed DJB or Sham surgery in chow fed Wistar rats, followed 2 weeks later by lymphatic cannulation for the assessment of gastrointestinal GIP secretion in response to a mixed meal. We hypothesized that bypassing the duodenum, the major site of enteroendocrine K cells, would result in a reduction in lymphatic GIP concentrations. To further characterize the changes in GIP secretion mediated by the gastrointestinal surgery, we compared GIP protein content within separate anatomic segments of small bowel of both surgical groups. In addition, GLP-1 protein content was analyzed within the small bowel of DJB and Sham rats as a comparison gastrointestinal hormone.

Methods

Animals. Eleven to twelve week-old, male, Wistar rats (Charles River Laboratories, Wilmington, MA) were allowed to acclimate to their environment for one week prior to the beginning of the experiment. Rats had free access to chow (Harlan Teklad standard rodent diet 7012, Indianapolis, IN) except as noted for the experimental protocols below. All procedures were approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Duodenal-jejunal bypass. DJB (n=10) and Sham surgeries (n=9) were performed as previously described under 18 hour fasting conditions [19]. In brief, the duodenum was divided just distal to the pylorus. The jejunum was divided 10 cm distal to the ligament of Treitz (LOT). A duodeno-jejunostomy was created by anastamosing the proximal segment of divided duodenum to the distal segment of divided jejunum. A jejun-jejunostomy was made 15 cm distal from the duodeno-jejunostomy in an end-to-side fashion. Postoperatively, rats were allowed access to water for the first 24 hours. On post-operative day (POD) #1, rats were started on a liquid diet of regular vanilla Ensure ad lib. On POD#2, all rats were returned to their standard chow diet. For Sham surgeries, the bowel was divided and re-anastamosed at the proximal duodenum, and 10 cm and 25 cm distal to the LOT. All DJB and Sham rats received the same pre and post-operative care. Body weight and food intake were measured daily for two weeks following surgery.

Oral Glucose Tolerance Test (OGTT). An OGTT was performed on all rats at approximately POD#12. D-glucose (2 g/kg) was administered by oral gavage to overnight fasted animals. Glucose was measured in duplicate from the tail vein using a hand-held glucometer at 0, 15, 30, 60, and 120 minutes after the gavage. Blood samples were simultaneously collected in EDTA-coated collecting tubes for the measurement of insulin concentration. Samples were spun at 4000 g for 10 min at 4º C and plasma was stored at -20º C for later analysis.

Lymph and Stomach Cannulation. Lymphatic cannulation was performed approximately two weeks post-operatively from the metabolic surgery (POD#14-16). Rats were fasted overnight but allowed free access to water. Lymphatic cannulation of the superior mesenteric lymphatic duct was performed as previously described [20]. A gastrotomy
was made along the greater curvature of the stomach, and a silicone tube (0.04 in ID and 0.085 in OD; VWR International, West Chester, PA) was placed into the stomach and secured with 4-0 silk suture. Both the lymphatic and gastric tubes were exteriorized thru the lateral abdominal wall. Animals were kept in Bollman, restraint cages for the remainder of the study and housed in temperature regulated isotes at 25º C. Successful lymphatic cannulation was performed in 8 of 10 DJB rats and 8 of 9 Sham rats. Once the rat was fully awake after lymphatic cannulation, an infusion of 0.9% NaCl solution was started through the gastric tube at 3 ml/hr to replenish the fluid and electrolyte loss from continuous lymphatic drainage. Nutrient administration and lymph collections were performed the same day, allowing the animals to recover from cannulation surgery for at least two hours.

**Nutrient Administration and Sample Collection.** Lymph was continuously collected on ice for ten minutes prior to the intragastric administration of a 7.68 ml/kg regular vanilla Ensure bolus. Lymph was subsequently collected in ten minute increments for one hour total. Blood samples were simultaneously collected from the tail vein corresponding to the lymph sample time points at fasting, 15, 35 and 55 minutes. Both lymph and blood samples were collected in EDTA coated tubes with the addition of a 1% DPPIV inhibitor (Millipore, St. Charles, Missouri). Blood samples were spun at 4º C at 4000 g for 10 minutes and the supernatant was stored at -20º C until later use.

**Hormone Assays.** Total GIP, active GLP-1 and insulin concentrations were determined using separate commercially available rat/mouse, sandwich ELISA kits (Millipore, St. Charles, Missouri). The GIP ELISA measures both active GIP (1-42) and inactive GIP (3-42) and does not cross react with glucagon, oxyntomodulin, GLP-1 and GLP-2. The GLP-1 ELISA measures the active forms of GLP-1, including GLP-1 (7-36) and GLP-1 (7-37), and does not cross react with inactive forms of GLP-1, glucagon, or GLP-2. The insulin ELISA is 100% specific for rat insulin and does not cross react with rat C-peptide.

**Intestinal GIP and GLP-1 Protein Content.** Four separate segments of small bowel were isolated for GIP and GLP-1 protein content. These segments included (1) the second segment of the duodenum, (2) the proximal jejunum 10 cm distal to the LOT (Sham rats) or just distal to the duodeno-jejunostomy (enteral limb of DJB rats), (3) the mid-jejunum 25 cm distal to the LOT (Sham rats) or just distal to the jeju-jejunostomy (common channel in DJB rats), and (4) the distal ileum. Intestinal isolation and processing was performed as previously described [19]. After homogenization in acetic acid and lyophilization, the dried segments were re-suspended in dH2O, diluted, and analyzed for total protein content, total GIP and active GLP-1 concentrations.

**Statistics.** Area under the curve (AUC) was calculated using the trapezoidal rule. Glucose, insulin and GIP AUC as well as incretin protein content for each intestinal segment were compared using a t-test. Comparisons of results between the two experimental groups over time (body weight, food intake, glucose, insulin and GIP concentrations) were performed using a two-way repeated-measures ANOVA. A Holm-Sidak test was used for post-hoc analysis. All values are presented as the mean ± SE. Values were determined statistically significant if p<0.05.
Results

Body Weight and Food Intake. We found no difference in daily post-operative body weights between the two surgical groups for the two week study duration. All animals lost weight until resumed on their chow diet and had returned to their pre-operative body weights by POD#5 (Figure 1a). At the completion of the study, DJB rats weighed 379.0 g ± 2.4 and Sham rats weighed 386.3 g ± 2.6 (p=0.44). As shown in Figure 1b, DJB rats ate significantly less chow than Sham rats from POD#3-8 (excluding POD#6). By POD#9, the DJB and Sham rats had no difference in daily food intake (30.04 g ± 0.96 vs. 30.51 g ± 1.02 respectively, p=0.77).

Glucose Homeostasis. A 2 g/kg D-glucose OGTT was performed approximately 12 days after DJB or Sham surgery. There was no difference in plasma glucose concentrations between the two groups for any time point measured (Figure 2a). Similarly as shown in Figure 2b, there was no difference in glucose concentration AUC between DJB and Sham rats (13851 (mg/dl)*min ± 442 vs. 13280 (mg/dl)*min ± 518 respectively, p= 0.41). As shown in Figure 2c, there was an insignificant trend for increased insulin secretion to an oral glucose load in DJB rats compared to Sham rats less than 2 weeks after surgery. The greatest difference was noted at 60 minutes after the administration of an oral glucose gavage (DJB: 1.87 ng/ml ± 0.19, Sham: 1.41 ng/ml ± 0.20; p=0.14). This trend was reflected in the insulin concentration AUC in Figure 2d. DJB rats had an insulin concentration AUC of 213.6 (ng/ml)*min ± 19.4 compared to Sham rats of 180.1 (ng/ml)*min ± 13.1 (p=0.18).

Plasma and Lymphatic GIP concentration. We performed superior mesenteric lymphatic cannulation with simultaneous placement of a gastric tube for nutrient delivery approximately 2 weeks after DJB or Sham surgery. Lymph and tail vein plasma samples were collected at fasting and for one hour after the administration of mixed meal gastric bolus. We found no difference in the lymphatic flow rates between DJB and Sham rats (data not shown). As shown in Figure 3a, we found no difference in plasma GIP concentrations at any time point measured between DJB and Sham rats. Supporting this finding, there was no difference in intestinal lymphatic GIP concentrations after a mixed meal bolus despite this being a more sensitive medium for incretin detection (Figure 3a). There was an insignificant trend towards a lower GIP peak concentration reached at 25 minutes after the nutrient bolus in DJB rats compared to Sham rats (353 pg/ml ± 63 vs. 466 pg/ml ± 110 respectively, p=0.18). There was also no difference between the two groups as shown in Figure 2b in plasma (DJB 3996 (pg/ml)*min ± 399, Sham 4772 (pg/ml)*min ± 559; p=0.27) or lymphatic GIP concentration AUC (DJB 15240 (pg/ml)*min ± 2651, Sham 17201 (pg/ml)*min ± 2763; p=0.62).

Small Bowel GIP and GLP-1 protein content. To determine how GIP protein content changed after DJB surgery, we examined four separate segments of the small bowel after duodenal bypass and the corresponding anatomical segments of small bowel in Sham rats. As shown in Figure 4a, at 2 weeks after surgery, there was a significant increase in midjejunal GIP protein content in DJB rats (the “common channel” for nutrient and
bile/pancreatic secretions) compared to Sham rats (% total GIP protein/total protein; 0.0037 % ± 0.0003 vs. 0.0026 ± 0.0002, p=0.009). Despite bypassing the duodenum with DJB surgery, there was a trend for increased GIP protein content in the duodenum of DJB rats compared to Sham rats (0.0029 % ± 0.0008 vs 0.0021 % ± 0.0003 respectively, p=0.07). We found no difference in proximal jejunal (the “enteral limb” of DJB rats) or ileal GIP protein content between the two surgical groups.

To see if a separate GI hormone followed the same pattern of production as GIP after duodenal bypass, we tested the effect of DJB on small bowel GLP-1 protein content, a potent incretin hormone previously implicated in improving glucose tolerance after metabolic surgery [7,13,14,19]. DJB, as shown in Figure 4b, again significantly increased GLP-1 protein content in the mid-jejunum compared to sham rats (0.81 x 10^{-6} % ± 0.048 vs 0.58 x 10^{-6} % ± 0.063 respectively, p=0.01). No difference in GLP-1 protein content was found among any of the other 3 intestinal segments analyzed (duodenum: p=0.65, jejunum: p=0.58, and ileum: p=0.84).

Discussion

Given the conflicting clinical reports on the changes in GIP secretion after gastric bypass, we studied the effects of DJB not only on plasma GIP secretion but also gastrointestinal lymphatic GIP, a newly-recognized, highly sensitive medium for the measurement of post-prandial incretins in rodents. We found that DJB does not significantly alter systemic GIP concentrations in plasma or lymph 2 weeks after DJB surgery compared to Sham rats despite bypassing the proximal small bowel, which is the major focus of GIP-secreting K cells. Intestinal GIP, as well as GLP-1, protein content was increased in the mid-jejunum at the common channel of DJB rats compared to Sham rats. Surprisingly, duodenal GIP protein content was not decreased by intestinal bypass of duodenum and can not account for the minimal differences noted in systemic GIP concentrations after DJB surgery.

In this study, we were interested specifically in what effect DJB has on plasma and lymphatic GIP concentrations given the hormone’s proximal small bowel site of secretion and the recent literature highlighting the beneficial effects noted with reduction of GIP signaling on obesity propagation and glucose intolerance [8-10]. There is an exaggerated secretion of GIP from K cells of obese type 2 diabetic patients and animals [21,22]. In support of a relationship between enhanced GIP signaling and the development of obesity and insulin resistance, Irwin et al has shown that antagonism of the GIP receptor in ob/ob mice prevents the development of obesity-associated diabetes with significant improvements in glucose concentrations and insulin sensitivity [10]. Rubino et al, among others, have published animal and clinical data to support that a major mediator of the effect of gastric bypass might derive from a change in secretion of a probable hormone originating from the proximal small bowel that affects insulin sensitivity and glucose homeostasis [17,23-26], leading us to speculate if GIP could be mediating these effects. While we found minimal effects of DJB on systemic GIP secretion, further studies are needed to determine if the sensitivity of the GIP receptor to stimulation changes after DJB and RYGB surgery. In addition, this study examined the
effects of DJB on lean, glucose-tolerant, Wistar rats. It may be that the effect of DJB on GIP secretion and signaling differs between lean, obese, and diabetic phenotypes.

The mechanism/s to the resolution of diabetes after gastric bypass has often been considered in terms of the proximal (“foregut”) and distal (“hindgut”) bowel hypothesis. While the improvement in glucose tolerance after RYGB is undoubtedly a multi-factorial process, including the significant impact of post-operative weight loss and calorie restriction on glucose homeostasis, gastrointestinal specific mechanisms could include (1) exclusion of the majority of the endocrine stomach from nutrient flow, (2) proximal small bowel exclusion from nutrient stimulus, and (3) enhanced nutrient delivery to the distal small bowel. While DJB is unable to address any hormone or neural factors that may be affected by gastric nutrient exclusion, it is an extremely useful experimental model of RYGB to study the affects of proximal small bowel exclusion and enhanced distal small bowel stimulation on glucose homeostasis independent of calorie restriction and weight loss. Supporting our previously published findings with DJB surgery in GK rats, we found in this study only modest changes in early post-operative food intake between the two groups, with no difference in food intake noted by POD#9 and no affect of DJB on body weight compared to Sham surgery [19]. While we are not aware of any published reports that document an affect of acute changes in food intake on subsequent post-prandial GIP secretion, it is possible that the early post-operative feeding differences between the two groups is masking an effect of the surgery on GIP secretion that would be revealed by examining a later time point.

We found no improvement in glucose tolerance in DJB rats by 2 weeks after surgery. We noted an insignificant trend towards increased insulin secretion to an oral glucose load in DJB rats, but this did not result in a physiologically relevant reduction in glucose concentrations. Rubino et al has published similar results in lean Wistar rats, showing no significant early effects of DJB on glucose concentrations over time [17]. Given the early time point of evaluation in our study and a glucose tolerant phenotype, it is unclear if a longer observation period would result in a significant improvement in glucose tolerance even in “normal”, lean rats as documented by other investigators with the use of ileal interposition [27].

Our laboratory and collaborators have previously published the enhanced sensitivity of lymphatic incretin sampling in rodents compared to plasma. Lu et al found that the duodenal administration of a mixed meal results in a 3 fold higher secretion of lymphatic GIP compared to portal blood [15]. D’Alessio et al have published that post-prandial GLP-1 levels are 5-6 fold higher in lymph compared to portal plasma [28]. In this study, we found that lymphatic GIP concentration curves parallel the pattern of secretion in plasma. However, there was a 3.3 fold average higher concentration of fasting lymphatic GIP compared to plasma GIP. We also demonstrated a 4.3 and 4.5 fold increase in peak GIP secretion of lymph compared to peak plasma GIP levels after a gastric administration of Ensure for DJB and Sham rats respectively, highlighting the increased sensitivity of post-prandial lymphatic sampling of GI hormones in rodents.
Our model of DJB bypasses approximately 30% of the small intestine (the entire duodenum and 25 cm total of jejunum before the jejun-jejunostomy). This is similar to the percent of bypassed intestine in a standard RYGB which bypasses the duodenum, first 30 cm of jejunum with a 100-150 cm enteral limb (approximately 25-30% of bypassed small bowel). Supporting an animal model that bypasses a similar amount of small bowel as RYGB, we found comparable lymphatic flow rates after a mixed meal. Lymph flow is predominately driven by enteral fat absorption and chylomicron secretion suggesting that DJB does not produce a significant amount of fat malabsorption. The conflicting reports on GIP secretion in the literature may well be related to the surgical length of bypass performed by the primary surgeon. Although GIP-secreting K cells are located throughout the gastrointestinal tract, as seen in this study, there is a marked reduction in GIP content of the ileum. Studies of BPD reporting changes in GIP secretion almost universally demonstrate a decrease in post-prandial GIP secretion possibly due to primary nutrient absorption occurring in the ileum of BPD patients rather than the jejunum of RYGB patients [11,26,29].

We used intestinal protein content as a surrogate marker of segmental K cell secretion after DJB surgery. While we anticipated that DJB would increase common channel GIP production due to increased nutrient absorption within the jejunum and decrease duodenal GIP production due to lack of nutrient stimulation, we found the surprising trend of increased GIP protein production in not only the jejunum but also the duodenum. This finding suggests that there is not a decrease in intracellular GIP protein production due to the lack of enteral nutrient stimulation of the duodenum as we had hypothesized to account for the minimal systemic changes found in GIP concentrations. Unfortunately, mesenteric lymphatic cannulation can not be used to segregate what percentage the different segments of small bowel contribute to the systemic concentration. Lymphatic concentrations instead represent total gastrointestinal secretion. Because we failed to find a difference between GIP concentrations of the two groups, we wonder to what extent the increased production of GIP in the duodenum translates into secretion changes. Studies of GIP secretion in response to fat, suggest a role for not just mucosal nutrient exposure, but also cellular uptake of the nutrient by the K cell (which would not occur in the excluded duodenal segment) [30]. Further research is needed to determine how the mechanisms for K cell intracellular GIP production and subsequent extracellular secretion are regulated, as well as how the duodenal K cell population contributes to systemic GIP secretion after gastric bypass surgery. In addition, we wonder what mechanisms govern the increased production of GIP and GLP-1 within the common channel including proximal stimulation affected by duodenal exclusion, early nutrient delivery to the distal small bowel, or the new concentration of bile and pancreatic secretions entering the common channel after DJB.

In conclusion, we found that 2 weeks after DJB surgery in chow-fed Wistar rats, there is a minimal change in systemic GIP concentrations despite bypassing the proximal small bowel. GIP protein content of the mid-jejunum was significantly increased after DJB with a surprising trend towards increased GIP production within the duodenum despite the intestinal bypass. Mechanisms responsible for the discrepancy in GIP protein content of the duodenum and subsequent nutrient-driven GIP secretion requires further
investigation. Additional studies are also needed to determine if GIP receptor sensitivity changes after RYGB even if no systemic changes in GIP concentration occur following surgery.

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Fig 1. Body weight and food intake after gastrointestinal surgery in Wistar rats. Body weights (a) and food intake (b) were assessed daily pre-operatively and for 2 weeks post-operatively. *Statistically different between the two surgical groups when p<0.05. Data are presented as mean ± SE.
Fig 2. An oral glucose tolerance test (2 g/kg D-glucose) was performed 12 days after DJB or Sham surgery. Plasma glucose concentrations (a) and insulin concentrations (c) were measured at fasting and 15, 30, 60 and 120 minutes after the glucose gavage. Glucose concentration AUC (b) and insulin concentration AUC (d) were determined using the trapezoidal rule. Data are presented as mean ± SE.
Fig 3. Total GIP concentrations were measured from plasma or gastrointestinal lymph (a) at fasting and for one hour following the administration of a mixed meal (7.68 ml/kg Ensure). Plasma and lymphatic GIP concentration AUC (b) was calculated using the trapezoidal rule. Data are presented as mean ± SE.
Fig 4. Percentage intestinal GIP (a) or GLP-1 protein content (b) was determined at 2 weeks after DJB or Sham surgery in Wistar rats. Intestinal segments were taken from the duodenum, proximal jejunum (distal to the duodeno-jejunostomy in DJB rats or 10 cm distal to the ligament of Treitz in Sham rats), mid-jejunum (distal to the jeju-jejunostomy in DJB rats or 25 cm distal to the ligament of Treitz in Sham rats), and distal ileum. *Statistically different for the tested segment of small bowel between DJB and Sham rats when p<0.05. Data are presented as mean ± SE.
Bypassing the duodenum does not improve insulin resistance associated with diet-induced obesity in rodents.

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Abstract

**Aims.** Roux-en-y gastric bypass (RYGB) surgery rapidly improves glucose tolerance and reverses insulin resistance in obese patients. It has been hypothesized that this effect is mediated by the diversion of nutrients from the proximal small intestine. We utilized duodenal-jejunal bypass (DJB) as a modification of gastric bypass to determine the effect of nutrient diversion from the foregut on insulin resistance in obese rats.

**Methods.** The effects of DJB or Sham surgery on glucose homeostasis were determined in high-fat fed Long-Evans and Wistar rats. Body weight and food intake were measured weekly post-op, and body composition was monitored before and after surgery. Glucose tolerance was tested before and as early as one month post-op; additionally, in Wistar rats, insulin sensitivity was determined by a hyperinsulinemic-euglycemic clamp (HIEC) study at 10-12 weeks post-op.

**Results.** DJB did not affect body weight, body composition, glucose tolerance or insulin secretion over the period of the study. The average glucose infusion rate during the HIEC was $6.2 \pm 1.16 \text{ mg kg}^{-1} \text{ min}^{-1}$ for Sham rats compared to $7.2 \pm 1.71 \text{ mg kg}^{-1} \text{ min}^{-1}$ for DJB rats ($p=0.62$), and neither endogenous glucose production ($p=0.81$) nor glucose utilization ($R_d$, $p=0.59$) differed between DJB and Sham rats.

**Conclusions.** DJB does not affect insulin resistance induced by a high fat diet in Long-Evans and Wistar rats. These data suggest that duodenal bypass alone is an insufficient mechanism to alter insulin sensitivity independent of weight loss and does not support that duodenal bypass mediates the metabolic improvements of RYGB.

**Key Words:** duodenal-jejunal bypass, insulin resistance, Roux-en-y gastric bypass, hyperinsulinemic-euglycemic clamp study, diet-induced obesity

**Abbreviations:** duodenal-jejunal bypass (DJB), endogenous glucose production rate (EGP), free fatty acid (FFA), glucagon-like peptide-1 (GLP-1), glucose infusion rate (GIR), Goto-Kakizaki rat (GK), HOMA for insulin resistance (HOMA-IR), hyperinsulinemic-euglycemic clamp (HIEC), insulin tolerance test (ITT), laparoscopic adjustable gastric banding (LAGB), rate of glucose appearance (Ra), rate of glucose disappearance (Rd), Roux-en-y gastric bypass (RYGB)
Introduction

The development of obesity invariably leads to a reduction of insulin sensitivity. As a result, obese, insulin-resistant individuals have decreased peripheral glucose uptake and poorly suppressed endogenous glucose production. While weight loss by dieting improves insulin resistance, sustained weight loss is difficult to achieve, and the majority of obese patients regain a significant amount of lost weight [1,2]. Roux-en-y gastric bypass (RYGB) is among the most successful therapies for sustained weight loss and results in the rapid improvement of Type 2 diabetes in approximately 80% of patients [3,4].

There are multiple studies demonstrating that RYGB improves both systemic and hepatic insulin sensitivity [5,6]. After RYGB-mediated weight loss, there is an increased expression of muscle insulin receptors and decreased hepatic and skeletal muscle lipid content [7-9], important correlates of enhanced insulin action. This is at least partially due to reduced body weight. When comparing RYGB and laparoscopic adjustable gastric banding (LAGB) by HOMA for insulin resistance (HOMA-IR), HOMA-IR was correlated with the percent of excess body weight lost after RYGB and LAGB [10,11]. However, there is increasing evidence that RYGB may improve insulin resistance independent of weight loss [12-14]. Studies which document a rapid reversal in insulin resistance post-operatively suggest weight-independent mechanisms that may be mediated from the gastrointestinal tract.

One proposed explanation for the improvement of glucose metabolism following RYGB is that exclusion of the duodenum and proximal jejunum from nutrient stimulation enhances insulin sensitivity [15-17]. In support of this theory, duodenal-jejunal bypass (DJB), a procedure that simulates the bypass portion of a RYGB, but without gastric reduction, not only decreased food intake and increased insulin secretion, but also improved hepatic insulin sensitivity due to an increase in intestinal gluconeogenesis in mice [18]. Implantation of a duodenal sleeve in high-fat fed rats resulted in a 55% improvement in peripheral insulin resistance compared to sham controls [19]. We recently reported that DJB modestly improved the glucose intolerance of Goto-Kakizaki (GK) rats, but did not alter insulin sensitivity [20]. However, in our previous study, we used insulin tolerance tests as the measure of insulin sensitivity, a method that is not the most sensitive measure of this parameter.

In the current study, we tested the hypothesis that RYGB improves insulin resistance by a weight-independent mechanism, mediated by bypass of the proximal small bowel. To test this hypothesis, we performed DJB in a diet-induced obesity model of insulin resistance. We first examined the effect of DJB on oral glucose tolerance and insulin resistance in high-fat fed Long-Evans rats. To confirm these results in a separate rat strain, we repeated the measures in high-fat fed Wistar rats after DJB and Sham surgery, and rigorously assessed insulin sensitivity with a hyperinsulinemic-euglycemic glucose clamp (HIEC).

Methods
Study Design. We examined the effect of DJB and Sham surgery on two separate strains of high-fat fed rats, Wistar and Long-Evans (Charles River Laboratories, Wilmington, MA). The first study included: (1) a high-fat fed, DJB, Long-Evans group (DJB-HFLE), (2) a high-fat fed, Sham, Long-Evans group (Sham-HFLE), and (3) a chow-fed, Sham, Long-Evans group (Sham-CLE). Eight-week old, male rats were fed 10 weeks of Open Source Diet D12451 (Research Diets, New Brunswick, NJ), which contains 45% kcal from fat. All three groups underwent pre-operative body composition and an OGTT. Rats were examined at four weeks post-operatively for body composition changes, oral glucose tolerance and insulin sensitivity via an insulin tolerance test (ITT).

The second study included two groups of Wistar rats (DJB-W and Sham-W) of the same age and high-fat diet protocol as the first study in Long-Evans rats. Wistar rats underwent pre-operative body composition and an OGTT. Post-operatively, body composition was performed at four, nine, and eleven weeks (the morning of the HIEC study). Dietary fat absorption was performed at six weeks and an OGTT was performed at seven weeks. Formal tests of insulin sensitivity by HIEC were performed at 10-12 weeks (as described below).

Animals. Individually housed rats were maintained on the same diet for the duration of the study, either Open Source Diet D12451 or chow (Harlan Teklad standard rodent diet 7012, Indianapolis, IN) as indicated by the study protocol, except for the first 48 hours post-operatively when rats were on a liquid diet. All procedures were approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Surgical Procedures. DJB or Sham surgeries and post-operative care were performed as previously described [20]. In brief, the duodenum was divided just distal to the pylorus and the jejunum was divided 10 cm distal to the ligament of Treitz. A duodeno-jejunostomy was created by anastamosing the proximal segment of divided duodenum to the distal segment of divided jejunum, and a jejuno-jejunostomy was made 15 cm distal from the duodeno-jejunostomy.

OGTT. A fixed dose of 2 g of D-glucose was administered by oral gavage to five-hour fasted animals. Glucose was measured using a hand-held glucometer at 0, 15, 30, 60, and 120 minutes after the gavage. Blood samples were simultaneously collected in EDTA-coated collecting tubes for the measurement of plasma insulin.

Body Composition. Total body fat was measured by an EchoMRI whole-body composition analyzer (Houston, TX). Fat mass was calculated as a percentage of total body mass.

ITT. Insulin, 0.5 U/kg, was administered subcutaneously to five-hour fasted rats. Glucose was measured immediately in duplicate using a hand-held glucometer from tail vein blood samples at 0, 15, 30, 45, and 60 minutes post-injection.

Dietary Fat Absorption. Fecal fat absorption, as a marker for intestinal malabsorption, was assessed as previously described [21]. In brief, rats were fed a measured amount of a
33% kcal from fat diet for three days containing a non-absorbable fat marker (sucrose polybehenate). The primary fat source of this pelleted diet was butter fat. Intestinal pellets were collected on day four and quantitative fat absorption was calculated by comparing the ratio of fecal and dietary fatty acids to behenic acid.

**Hormone Analysis.** Insulin concentrations were determined using a commercially available rat, sandwich ELISA kit (Millipore, St. Charles, MO). The insulin ELISA is 100% specific for rat insulin. The inter-assay precision is 6.0–17.9% and the intra-assay precision is 0.9–8.4% as reported by the manufacturer. Leptin was measured in Wistar rats from fasting plasma taken prior to the seven-week OGTT (Rat Leptin ELISA, Millipore, St. Charles, MO). The inter-assay precision is 3.0–3.9% and the intra-assay precision is 1.9–2.5% as reported by the manufacturer. Non-esterified free-fatty acid (NEFA) concentrations were determined using a colorimetric kit (Wako Chemicals USA, Richmond, VA).

**HIEC.** HIEC studies were performed in awake, unrestrained, chronically catheterized rats for the determination of insulin sensitivity. Wistar rats underwent surgical catheterization of the left jugular vein and right carotid artery at 10-12 weeks post-op, as previously described [22]. The jugular catheter served for infusion and the carotid catheter for blood sampling during the HIEC. Rats were allowed at least a week of recovery prior to beginning the clamp study. The HIEC consisted of a 180-minute protocol, initiated with 30 minutes of animal acclimatization to their HIEC environment. At 30 minutes, a prime-continuous infusion (bolus 20.8 µCi, then 0.52 µCi/min) of [3H-3]-glucose (Perkin-Elmer NEN, Boston, MA) was started and maintained throughout the duration of the study. After 30 minutes of tracer equilibration, blood samples for glucose and plasma radioactivity were taken at 10-minute intervals for the next 30 minutes to establish a basal period (non-hyperinsulinemic condition). Plasma glucose concentrations were measured real-time during the HIEC using a GM7 Micro-stat Analyzer (ANALOX instruments, London, UK). At 90 minutes, a prime-continuous infusion (bolus 44.3 mU/Kg for 1 minute, then 2.5 mU Kg\(^{-1}\) min\(^{-1}\)) of regular human insulin (Novolin, Novo Nordisk, Princeton, NJ) and a variable infusion of 25% glucose solution (IVX Animal Health, St. Joseph, MO) were started. The rate of glucose infusion was adjusted as needed in order to clamp the plasma glucose concentration at 130-140 mg/dL. Blood samples for glucose and plasma radioactivity were taken every 10 minutes during the 60 minute hyperinsulinemic period. Extra blood samples at 60, 90, 120, 150 and 180 minutes were taken and stored for the subsequent assay of plasma levels of insulin and NEFA. Plasma radioactivity from [3H-3]-glucose was determined after deproteinization with Ba(OH)\(_2\) and ZnSO\(_4\) and subsequent evaporation to remove tritiated water as described elsewhere [23].

**Clamp parameter calculations.** We assumed that under steady-state conditions for plasma glucose concentration, the rate of glucose disappearance (Rd) equals the rate of glucose appearance (Ra). Therefore, Ra was measured by dividing the infusion rate of [3H-3]-glucose (dpm) by the specific activity of plasma [3H-3]-glucose (dpm/mg of glucose). The mean values of Ra were calculated during the basal (60-90 min) and clamp (150-180 min) periods. In steady-state clamp conditions, the endogenous glucose production rate
(EGP) was calculated as the difference between Rd (calculated the same as Ra) and the glucose infusion rate (GIR).

Statistics. AUC was calculated using the trapezoidal rule. Fecal fat, leptin, glucose and insulin AUC, Ra, GIR, Rd, and EGP were compared using a student’s t-test. Comparisons of results between the experimental groups over time were performed using a two-way repeated-measures ANOVA. A Tukey test was used for post-hoc analysis. All values are presented as the mean ± SE. Values were determined statistically significant if p<0.05.

Results

Long-Evans. DJB-HFLE (n=8) and Sham-HFLE rats (n=8) did not differ in postoperative body weights or food intake (Figures 1A and 1B). Sham-CLE rats (n=11) weighed significantly less and ate significantly more food at every time point compared to both high-fat fed surgical groups. As shown in Figure 1C, both high-fat surgical groups had significantly more body fat both pre- and post-op compared to Sham rats. There was no difference in percent body fat of DJB-HFLE compared to Sham-HFLE at four weeks post-op (23.3 ± 1.08% vs. 25.3 ± 1.64% respectively, p= 0.22). While there was no statistical difference in the change in percent body fat for Sham-CLE from the pre- and post-op measurements, both DJB-HFLE and Sham-HFLE had lost a significant amount of body fat by four weeks post-op (p<0.001 and p=0.001, respectively).

A 2 g D-glucose OGTT was performed at one month post-op. There was no statistical difference at any time point between DJB-HFLE rats and Sham-HFLE rats (Figure 2A). Sham-HFLE rats had significantly worse glucose tolerance compared to chow-fed controls at 30 minutes (189 ± 11.1 mg/dL vs. 154 ± 9.4 mg/dL respectively, p=0.02), 60 minutes (174 ± 9.5 mg/dL vs. 126 ± 5.1 mg/dL respectively, p<0.001), and 120 minutes (153 ± 8.3 mg/dL vs. 115 ± 4.0 mg/dL respectively, p<0.008). DJB-HFLE rats also had statistically significant worse oral glucose tolerance compared to chow-fed controls at 60 minutes (164 ± 12.7 mg/dL, p= 0.008) and 120 minutes (145 ± 5.8 mg/dL, p=0.05). Sham-CLE rats had a significantly smaller post-prandial glucose concentration AUC (5045 ± 552 mg dL\(^{-1}\) min) compared to Sham-HFLE rats (8063 ± 738 mg dL\(^{-1}\) min, p=0.04) and DJB-HFLE rats (7708 ± 1164 mg dL\(^{-1}\) min, p=0.03). There was no difference in post-prandial glucose concentration AUC between the two high-fat fed groups (p=0.77).

An ITT was performed at one month post-op as shown in Figure 2B. Both DJB-HFLE and Sham-HFLE surgical groups had significantly worse insulin sensitivity at 45 and 60 minutes compared to Sham-CLE rats. The Sham-CLE group had a reduction in glucose concentrations at 45 minutes after the insulin injection to 70 ± 3.7% of fasting values and 66 ± 3.7% at 60 minutes. This is compared to 89 ± 5.5% (p=0.007) at 45 minutes and 90 ± 5.6% (p<0.001) at 60 minutes for the DJB-HFLE group, and 85 ± 4.9% (p=0.04) at 45 minutes and 88 ± 5.3% (p=0.002) at 60 minutes for the Sham-HFLE group.
There was no significant effect of duodenal bypass on insulin resistance at any time point compared to Sham-HFLE rats.

**Wistar rats.** To verify the lack of effect of DJB on obesity, oral glucose tolerance, and insulin resistance in a diet-induced obesity model, DJB (n=9) and Sham (n=10) surgeries were performed in high-fat fed Wistar rats. As shown in Figure 3A, by two months after surgery, there was also no effect of DJB on body weight when compared to obese, Sham rats. At 9 weeks after surgery, DJB-W rats weighed 621 ± 18.9 g compared to 624 ± 15.4 g in Sham-W rats (p=0.88). Moreover, DJB in this diet-induced obesity model did not decrease food intake. In fact, starting one month after surgery, there was a trend towards increased food intake (Figure 3B) for DJB-W rats who ate significantly more food than Sham-W rats on post-op day 35, 52 and 53 (p=0.04, 0.05, and 0.05, respectively).

Unlike the Long-Evans surgical groups, DJB and Sham surgery in high-fat fed Wistar rats did not significantly alter body fat percentage by four weeks after surgery as shown in Figure 3C. At nine weeks after surgery, both DJB-W and Sham-W groups had significantly increased their percent body fat compared to their respective pre-operative values. DJB-W rats had a pre-operative percent body fat of 18.1 ± 1.70% compared to 21.4 ± 1.28% (p=0.02) at week nine. Sham-W rats had a pre-operative percent body fat of 19.3 ± 1.19% compared to 22.1 ± 1.15% (p=0.002) at week nine. There was no difference in percent body fat at any time point measured between the DJB-W and Sham-W groups. Given the similar percent body fat and body weights, it is not surprising that there was no difference in fasting leptin levels between DJB-W and Sham-W groups at seven weeks after surgery (22.1 ± 2.67 ng/mL vs. 24.3 ± 2.68 ng/mL, respectively, p=0.56).

As expected for a mildly malabsorptive surgery, there was a trend towards decreased fat absorption in the DJB-W group compared to the Sham-W group (73.4 ± 4.5% vs. 81.5 ± 1.4% respectively, p=0.08) as shown in Figure 3D. The different diet used for the fecal fat experiment, with a lower fat content (33% vs. 45%) and, therefore, fewer calories per gram of food, likely explains the increased food consumption during this time period.

We found DJB did not improve glucose tolerance by seven weeks after surgery as assessed by an OGTT. As shown in Figure 4A, DJB-W rats had a fasting glucose concentration of 91 ± 1.4 mg/dL, and Sham-W rats had a fasting glucose concentration of 95 ± 2.3 mg/dL, p=0.90. Both groups peaked at 15 minutes after the gavage, but continued to have significantly elevated glucose concentrations above fasting levels at two hours after the oral gavage. Post-prandial glucose concentration AUC was 3706 ± 581 mg dL⁻¹ min for Sham-W rats compared to 3989 ± 553 mg dL⁻¹ min for DJB-W rats, p=0.73. Paralleling the lack of difference in glucose concentrations, we found no difference at any time point in insulin concentrations for the OGTT (Figure 4B). Both groups had a peak insulin concentration at 15 minutes, 14.5 ± 2.48 ng/mL for DJB-W rats compared to 16.7 ± 1.69 ng/mL for Sham-W rats, p=0.18. We also found similar post-prandial insulin concentration AUC for the two groups (DJB-W: 594 ± 92.3 ng mL⁻¹ min, Sham-W: 592 ± 30.6 ng mL⁻¹ min, p=0.98).
Hyperinsulinemic-Euglycemic Clamp Study in Wistar rats. Approximately 10-12 weeks after DJB or Sham surgery, animals were tested unrestrained, under hyperinsulinemic-euglycemic clamp conditions, to measure insulin sensitivity. On the day of the clamp study, Sham-W rats weighed 634 ± 16.5 g and DJB-W rats weighed 628 ± 17.8 g (p=0.80). Body fat composition was performed on the morning of the clamp procedures with no difference noted in percent fat mass between the groups (DJB-W 22.7 ± 1.59% vs. Sham-W 23.3 ± 0.92%, p=0.78). The percent fat mass at the time of the clamps was not significantly different from the values obtained at the 9 week post-op assessment (DJB-W p=0.53, Sham-W p=0.46), reflecting a similar post-catheter recovery for both groups.

Plasma glucose concentrations were measured every ten minutes starting at 30 minutes after the initiation of the HIEC and continued for the duration of the study. There was no difference in average basal (60-90 minutes) or clamp (150-180 minutes) glucose concentrations as shown in Figure 5A. DJB-W rats had an average basal glucose concentration of 142 ± 3.7 mg/dL compared to 150 ± 3.0 mg/dL for Sham-W rats, p=0.19. Both groups were effectively clamped between the goal glucose concentrations of 130-140 mg/dL during the clamp period. DJB-W rats had an average clamp glucose concentration of 136 ± 1.6 mg/dL compared to 133 ± 2.2 mg/dL for Sham-W rats, p=0.33. As demonstrated in Figure 5B, there was also no difference in average basal or clamp insulin concentrations between the two groups (p=0.79 and p=0.74, respectively). We found no difference in NEFA at any time point assessed during the HIEC (data not shown).

The Sham-W group had an average Ra of 7.6 ± 0.09 mg kg⁻¹ min⁻¹ during the basal period compared to 8.1 ± 0.49 mg kg⁻¹ min⁻¹ for the DJB-W surgical group, p=0.28 (Figure 6A). We found no difference in GIR, EGP or Rd between the two groups during the clamp period (Figure 6B-D) suggesting that duodenal bypass does not alter peripheral insulin sensitivity or the rates of glucose production and utilization. The average GIR during the clamp period for Sham-W rats was 6.2 ± 1.16 mg kg⁻¹ min⁻¹ and 7.2 ± 1.71 mg kg⁻¹ min⁻¹ for DJB-W rats, p=0.62. The average EGP for Sham-W and DJB-W rats was 4.7 ± 0.62 mg kg⁻¹ min⁻¹ and 4.4 ± 1.15 mg kg⁻¹ min⁻¹ respectively, p=0.81. The average Rd for Sham-W and DJB-W rats was 10.9 ± 0.6 mg kg⁻¹ min⁻¹ and 11.6 ± 1.21 mg kg⁻¹ min⁻¹ respectively, p=0.59.

Discussion

In this study, we tested the effect of duodenal bypass on insulin resistance in a rodent model of diet-induced obesity. Feeding of a 45% high-fat diet for ten weeks prior to surgery resulted in an obese, insulin resistant, glucose intolerant phenotype compared to chow fed rats. DJB compared to Sham surgery did not affect body weight, percent body fat or oral glucose tolerance in either Long-Evans or Wistar rats. High-fat fed, Long-Evans rats were significantly insulin resistant during an insulin tolerance test compared to chow fed, Long-Evans rats, but this was not affected by DJB. In high-fat fed Wistar rats, DJB did not affect insulin sensitivity as studied using HIEC. These results
indicate that exclusion of nutrients from the upper gut has a minimal effect on glucose tolerance and insulin action. Further, these data do not support the hypothesis that inhibiting nutrient interactions within the duodenum can account for any metabolic improvements noted with RYGB.

There are compelling data that RYGB may improve insulin resistance beyond that which can be explained by surgical weight loss [12,14]. Kashyap et al calculated the effects of gastric bypass and gastric restrictive surgery on insulin sensitivity using C-peptide modeling kinetics derived from a hyperglycemic clamp. At four weeks post-operatively, insulin sensitivity had improved significantly with only RYGB, despite similar weight loss between the two groups [12]. Yet when RYGB has been rigorously matched to a diet restriction group, glucose uptake, as measured by a HIEC, was similar between the two groups at two weeks [24]. It is possible that the acute changes in insulin resistance after RYGB may be predominantly due to calorie restriction and the later improvements are due to weight loss as well as independent gastrointestinal mechanisms. However, our results with DJB suggest that if RYGB does have a weight-independent affect on insulin sensitivity, this is not mediated by duodenal bypass alone.

We have used DJB in this study as a modification of RYGB in order to eliminate the effect of a reduced gastric capacity on food intake and subsequently body weight and percent body fat, which directly relates to the level of insulin resistance. It is possible that gastric exclusion, as performed with RYGB and not DJB, is essential for the induction of any weight-independent improvements in insulin sensitivity. A recent study with sleeve gastrectomy, which parallels the gastric exclusion produced in RYGB without altering small bowel anatomy, found that insulin sensitivity had improved within five days after surgery [25]. This finding is in agreement with the rapid improvement in insulin sensitivity noted after gastric bypass [13,26]. Another surprising similarity between RYGB and sleeve gastrectomy is that despite unaltered small bowel anatomy, sleeve gastrectomy can significantly increase post-prandial GLP-1 and peptide YY secretion, a finding not commonly reported after gastric banding, and almost universally found after RYGB [27]. Further studies with sleeve gastrectomy are needed with appropriate weight-matched control groups to determine if gastric resection (or exclusion as performed in RYGB) is a required feature of a metabolic surgery to maximize insulin sensitivity changes.

In previous studies of GK and lean Wistar rats, we have not found a suppressive effect on long-term food intake [20,28]. In contrast, in this study we found a significant post-operative increase in food intake of high-fat fed, Wistar rats, likely as a compensatory mechanism to balance mild fat malabsorption. It is interesting to note that several previous publications have found dramatic reductions in food intake with duodenal bypass in rodents. The use of a duodenal sleeve implanted in diet-induced obese rodents resulted in a 27% reduction in kcal consumed per day compared to sham-operated animals [19]. While there are clear differences between the use of a duodenal sleeve and DJB (such as preserved mechanoreception in the duodenum of sleeve animals), previous studies in DJB have also resulted in a reduction in food intake after surgery [29-32]. In a study of Zucker obese rats and lean Wistar rats undergoing DJB,
only the Zucker obese rats had reduced food intake post-operatively leading the authors to conclude that the mechanism responsible for the reduction in food intake is only applicable in an obese phenotype [29]. In a subsequent study by Rubino et al comparing DJB to gastro-jejunostomy in lean GK rats, DJB dramatically reduced food intake to less than 10 g/day making an obesity-specific effect of DJB on food intake unlikely [30]. It is not clear what mechanisms account for the differences observed in food intake between these and the current study, and if the responsible factor could have an independent effect on insulin resistance.

We were surprised to find a lack of effect of DJB on oral glucose tolerance in both rat strains utilized for this study. We have previously published a modest effect of DJB on glucose tolerance in GK rats mediated by GLP-1 receptor signaling [20]. GK rats have been the predominant rodent model used to document improvements in hyperglycemia with DJB [30-34]. In a previous study, we found that DJB did not affect glucose tolerance of lean, Wistar rats by two weeks after surgery despite an increase in jejunal GLP-1 protein content compared to Sham controls [28]. We wondered whether the lack of effect was due to the fact that the Wistar rats were euglycemic and therefore would receive minimal additional metabolic benefit from DJB. To our knowledge, the current study is the first to examine DJB in a rodent model of diet-induced obesity. The high-fat diet used in this study produced significantly worse glucose tolerance in the Long-Evans rats compared to a chow diet and we still were unable to achieve an improvement in post-prandial hyperglycemia with DJB. The lack of a consistent improvement in hyperglycemia with DJB in glucose-intolerant rodents indicates a requisite need for the continued investigation of the mechanisms mediating an improvement in glucose tolerance with DJB and RYGB, and should caution the clinical use of duodenal bypass as a stand-alone procedure for Type 2 diabetes mellitus.

In conclusion, this study in a diet-induced obesity model of insulin resistance in rats did not find that duodenal bypass significantly improves insulin sensitivity as measured by a HIEC study. This suggests that duodenal bypass alone is an insufficient mechanism to alter insulin sensitivity independent of weight loss. Further studies are needed to investigate the role of gastric exclusion as in RYGB or gastric resection as in a sleeve gastrectomy in improving insulin sensitivity beyond the reduction in gastric capacity and food intake.
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Duality of Interest

R.J.S. has received research support and/or served as an advisor or consultant to Amylin Pharmaceuticals, Eli Lilly, Johnson & Johnson, Novo Nordisk, Zafgen Inc. (Equity Stake <1%), Merck, Roche, and Mannkind. D.A.D. and S.O. have received research support from Ethicon.
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Figure Legends

**Fig 1.** The effect of duodenal-jejunal bypass (DJB) or Sham surgery in Long-Evans rats fed a high-fat diet (DJB-HFLE, —▼—, and Sham-HFLE, ···●···) or chow diet (Sham-CLE, --○--). Body weight (A) and daily food intake (B) were followed for four weeks post-operatively. Percent body fat (C) was measured pre- and four weeks post-operatively by EchoMRI. Statistically different compared to Sham-CLE for the given time point (*) or compared to pre-operative values within the same group (#) when p<0.05. Statistical data are presented as mean ± SE.

**Fig 2.** Blood glucose concentrations (A) during a 2 g D-glucose OGTT test or (B) during a 0.5 U/kg sc insulin tolerance test (ITT) performed at 4 weeks post-operatively in high-fat fed duodenal-jejunal bypass (DJB-HFLE, —▼—), high-fat fed Sham (Sham-HFLE, ···●···) and chow fed Sham Long-Evans rats (Sham-CLE, --○--). Glucose concentrations from the ITT are displayed as the percentage of the fasting glucose concentrations. Statistically different compared to Sham-CLE for given time point (*) when p<0.05. Statistical data are presented as mean ± SE.

**Fig 3.** The effect of duodenal-jejunal bypass (DJB, —●—) or Sham surgery (···○···) in high-fat fed Wistar rats on body weight (A), and food intake (B). The percent body fat was measured by EchoMRI pre-operatively, and at four and nine weeks post-operatively (C). Fecal fat absorption was quantified with the use of a non-digestible fat marker, sucrose polybehenate, added to a 33% kcal from fat diet fed to all groups for three days (D). Statistically different between the two groups for the given time point (*) or compared to pre-operative values within the same group (#) when p<0.05. Statistical data are presented as mean ± SE.

**Fig 4.** Blood glucose (A) and plasma insulin (B) concentrations during a 2 g D-glucose OGTT at seven weeks post-operatively in duodenal-jejunal bypass (DJB, —●—) or Sham (···○···) high-fat fed Wistar rats. Data are presented as mean ± SE.

**Fig 5.** The average plasma basal (60-90 min) and clamp period (150-180 min) glucose (A) and insulin (B) concentrations during a hyperinsulinemic-euglycemic clamp study performed approximately 11 weeks after duodenal-jejunal bypass (DJB) or Sham surgery in high-fat fed, Wistar rats. Statistical data are presented as mean ± SE.

**Fig 6.** The average rate of glucose appearance (Ra) in the basal period (A), glucose infusion rate (GIR) during the clamp period (B), rate of endogenous glucose production (EGP) during the clamp period (C), and rate of glucose disappearance (Rd) during the clamp period (D) from a hyperinsulinemic-euglycemic clamp study performed approximately 11 weeks after duodenal-jejunal bypass (DJB) or Sham surgery in high-fat fed, Wistar rats. Statistical data are presented as mean ± SE.
Figure 1

A

Body Weight (g)

Time (weeks)

B

Daily Food Intake (g)

Time (days)

C

% Body Fat

Pre-op

Post-op

Sham CLE

Sham HFLE

DJB HFLE

Sham CLE

Sham HFLE

DJB HFLE

* * # * #
Figure 2

A

Glucose Concentration (mg/dL)

0 20 40 60 80 100 120

Time (minutes)

B

% of Fasting Glucose Conc.

0 10 20 30 40 50 60

Time (minutes)
Figure 3
Figure 5
Figure 6

A

B

C

D