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Effects of Acute Nutrient Stimulation and Chronic High-Fat Feeding on GIP and GLP-1 Secretion in the Lymph Fistula Rat

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Effects of Acute Nutrient Stimulation and Chronic High-Fat Feeding on GIP and GLP-1 Secretion in the Lymph Fistula Rat

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by

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ABSTRACT

Obesity is an expanding global health problem. It is estimated worldwide that 1.6 billion adults are overweight (body mass index [BMI] > 25 kg/m²) and 400 million additional adults are obese (BMI > 30 kg/m²). Obesity is associated with several co-morbidities; many of these conditions are mediated through insulin resistance and glucose intolerance, which can be partially attributed to an altered incretin response. The two incretin hormones GIP and GLP-1 enhance postprandial insulin secretion. Ingestion of nutrients strongly promote the release of both GIP and GLP-1 from the enteroendocrine cells K and L cells, respectively; however, the mechanisms underlying these processes are largely unestablished. Additionally, the relationship between diet-induced obesity and the postprandial release of GIP and GLP-1 has not been clearly demonstrated. It is unclear if the alterations are caused by obesity or the consumption of a high-energy diet that often induces obesity. Therefore, the goal of this dissertation was to investigate how incretin secretion is regulated by various macronutrients and how this process is altered following the chronic feeding of a high-fat diet using the lymph fistula rat model. Previously, we demonstrated the benefits of using the lymph fistula rat model to study the secretion of the incretin hormones. The concentrations of both hormones are significantly higher in intestinal lymph than in plasma, and the model also allows for the continuous collection of fluid from conscious animals. Finally, the lymphatic incretin concentrations more accurately represent the concentrations within the intestinal mucosa where the hormones may interact with other cells or neurons.

The dose-response relationships between the amount and type of ingested macronutrient and the secretion of incretins are not well-defined. Previous reports investigating nutrient-stimulated incretin secretion have examined either individual macronutrients or mixed meals over a limited range of doses. Using the lymph fistula rat model, we determined that both GIP and GLP-1 respond dose dependently to increasing amounts of either acute carbohydrate or lipid in chow-fed Sprague-Dawley rats. However, there is
minimal incretin response to dietary protein. We further demonstrate that lipid and carbohydrate are equally effective at stimulating GLP-1 release, while carbohydrate is the more potent GIP secretagogue.

In the second portion of this dissertation, we investigated the effects of chronic high-fat feeding on incretin secretion. Rats were provided a high-fat (HF) or low-fat (LF) diet ad libitum for 3 or 13 weeks; a high-fat pair-fed (HF-PF) group was included as a control during the 3-week feeding trial. At the culmination of the feeding period, all animals were challenged with a duodenal mixed meal to measure lymphatic incretin secretion. Despite demonstrating no signs of obesity beyond hyperphagia, both the 3-week and 13-week HF-fed animals had elevated lymphatic GIP and GLP-1 concentrations compared to animals fed a LF diet following an identical mixed meal challenge with Ensure. Using our HF-PF group, we further demonstrate that, following chronic consumption of a HF diet, the elevated GIP concentration is driven by the greater percentage of fat intake, whereas the increased GLP-1 secretion is caused by the excess caloric intake.

Together, the findings from this dissertation have added important information to the field of incretin biology. These results may have important bearings on the nutritional and pharmacological regulation of GIP and GLP-1 secretion. Currently, GLP-1 mimetics are used for the treatment of type 2 diabetes. An additional potential therapeutic avenue is stimulating the release of the incretin hormones at the intestinal level; this would allow individuals to produce and secrete their own hormones, ideally replicating the physiological secretion time course and response to various nutrients. To accomplish this task, a greater understanding of the physiological and cellular mechanisms underlying nutrient-induced incretion secretion is needed. The results from the first portion of this dissertation provide steps in that direction. Furthermore, in order to provide incretin-based treatment to obese and type 2 diabetic patients, it is important to first understand the physiological defects in incretin secretion for both of these conditions. In regards to obesity, our findings suggest that mere consumption of a high-fat diet (for as little as 3 weeks) can result in significant changes in incretin secretion following an acute mixed nutrient challenge.
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This dissertation is based on the following papers, referred to in the text by their Roman numeral.

I. Stimulation of incretin secretion by dietary lipid: is it dose-dependent?

**SM Yoder, Q Yang, TL Kindel, P Tso.**


II. Differential responses of the incretin hormones GIP and GLP-1 to increasing doses of dietary carbohydrate but not dietary protein in lean rats.

**SM Yoder, Q Yang, TL Kindel, P Tso.**


III. Chronic high-fat feeding increases both GIP and GLP-1 secretion without altering body weight.

**SM Yoder, Q Yang, TL Kindel, P Tso.**

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LIST OF ABBREVIATIONS

GABA      /g534-amino butyric acid
ANOVA     analysis of variance
AUC       area under the curve
BMI       body mass index
carb      carbohydrate
CCK       cholecystokinin
CV        coefficient of variance
cAMP      cyclic adenosine monophosphate
K\textsubscript{ATP} channel
          ATP-sensitive potassium channel
DPP-IV    dipeptidyl peptidase-IV
ELISA     enzyme-linked immunosorbent assay
EDTA      ethylenediaminetetraacetic acid
ERK1/2    extracellular signal-related kinase 1/2
FRIC      fetal rat intestinal cell
GPCR      G-protein coupled receptor
GLP-1     glucagon-like peptide-1
GLP-1R    glucagon-like peptide-1 receptor
GLP-2     glucagon-like peptide-2
GIP       glucose-dependent insulinotropic polypeptide
GIPR      glucose-dependent insulinotropic polypeptide receptor
GLUT2     glucose transporter 2
HC        high-carbohydrate
HF        high-fat
HF-PF     high-fat pair-fed
LF        low-fat
MAPK      mitogen-activated protein kinase
NPY       neuropeptide Y
PP        pancreatic polypeptide
PYY       peptide tyrosine-tyrosine
PC        prohormone convertase
SE        standard error
SGLT1     sodium-coupled glucose transporter 1
TG        triglyceride
wk        week(s)
BRIEF EXPLANATION OF THE DISSERTATION FORMAT

The following dissertation is divided into three main sections. The first section is the Introduction; this section is primarily a literature review. I included my perspective on current interpretations in the literature; I also highlighted various questions that remain in the field. The second section is the Experimental Findings and Discussion. Here, I review the main findings from the dissertation. I also discuss the implications of those results and how they integrate into the current body of literature highlighted in the Introduction. Throughout this dissertation project, additional research questions arose; I address a few of those interesting questions following the Experimental Findings and Discussion section. The research papers (the third section) on which this dissertation is based are located after the Future Directions. Please consult the Table of Contents for the page numbers that correspond with each of these sections.
INTRODUCTION

1 - The Incretin Hormones

1.1 - Discovery

Inspired by Bayliss and Starling’s discovery of an intestinal factor (termed secretin) that stimulates the exocrine secretion of the pancreas (10), Moore et al. (112) aimed to uncover a ‘chemical excitant’ that stimulates the internal secretion of the pancreas. They further speculated that this chemical excitant may be important in glucose homeostasis. Prior to the discovery of insulin, it was known that the internal secretion of the pancreas is necessary for the regulation of carbohydrate metabolism (176). Moore et al. thus hypothesized that an intestine-derived chemical excitant stimulates the internal secretion of the pancreas, which ultimately maintains proper glucose metabolism. They suggested that functional derangements at any step in this pathway may lead to the appearance of a diabetic condition. Using a method similar to that described by Bayliss and Starling, Moore et al. isolated and extracted mucosal contents from the proximal small intestine of pigs. The resulting preparation was provided by mouth to three hospitalized diabetics. Following 3 months of duodenal extract treatment, the first patient’s diabetes had resolved (assessed by the absence of glycosuria). Moore et al. saw similar results with the second and third patients. Upon hospital admission, both patients were placed on a strict diabetic diet and treated with the duodenal extract; after 6 weeks (second patient) and 3.5 weeks (third patient) of treatment, sugar levels in the urine were undetectable and remained negative during the follow-up period. The authors stated that although no generalized conclusions can be drawn because of the small number of cases studied, their results did suggest that the duodenum secretes a pancreatic-stimulating factor that is involved in carbohydrate metabolism and that defects in the secretion of this factor may lead to glycosuria (i.e. diabetes).
Bainbridge and Beddard (8) countered Moore et al.’s findings by arguing that in all three cases the patients were not only treated with the duodenal extract but also with a diabetic diet; in each case, the resolution of diabetes may be due to the diet alone (and not an intestinally-derived chemical excitant). Bainbridge and Beddard treated three severe diabetics with duodenal extracts and a strict diabetic diet and were unable to reproduce the findings of Moore et al. (112). Furthermore, Moore et al. (113), in a follow-up study, were only able to significantly reduce glycosuria when the duodenal extracts were prepared “as soon as possible after removal from the animals [pigs]”. In retrospect, as these treatments were provided orally to patients, it is possible that the insulinotropic gut hormones were degraded in the stomach, as well as within the intestinal lumen, prior to reaching the circulation.

After the discovery of insulin in 1921, a rigorous search for gut hormones influencing carbohydrate metabolism began. Several contradictory accounts were published examining glucose levels following the provision of duodenal extracts (via intravenous or subcutaneous injection or via an oral route); no change, hyperglycemia followed by mild hypoglycemia, and sustained, significant hypoglycemia were all reported. Zunz and La Barre (186) determined that the conflicting results may be due to the different quality and preparation of the duodenal extracts. Accordingly, La Barre and Still (87) described a method to prepare the duodenal extracts into two fractions, one of which (secretin) stimulates secretion of pancreatic juice with no effect on blood sugar and the other (Fraction B) only produces hypoglycemia. They further demonstrated using sophisticated cross-circulation experiments that Fraction B lowered blood glucose levels by stimulating the secretion of insulin from the endocrine pancreas. In these studies, the pancreatic vein from a depancreatized dog (Dog A) was connected to the jugular vein of a second dog (Dog B) with an intact pancreas. Injection of Fraction B into Dog A only moderately reduced glucose levels but substantially reduced those in Dog B, demonstrating that Fraction B is capable of stimulating insulin secretion from the pancreas. This substance (Fraction B) was termed ‘incrétine’ (incretin) by La Barre in 1932 (86). La Barre and others (69, 86) suggested that incretin may have potential applications in the treatment of human diabetes. Despite this exceptional work, following an influential series of
papers by Loew et al. (96, 97, 98) that demonstrated no effect of duodenal extracts on the blood glucose level of fasted dogs, research on incretin was halted. Closer study of these manuscripts, however, reveals an inherent flaw in the experimental design. The intestinal extracts were tested on fasted dogs; the authors disregarded (perhaps accidentally, since the definition of incretin had yet to be fully developed) the primary characteristic of an incretin hormone: their ability to stimulate insulin secretion only occurs at elevated blood glucose levels. Unfortunately, this is an example of how flawed research by an influential investigator can hinder promising research. Additionally, the start of World War II aligned with the publication of these three papers, and during the war, all biomedical research not related to wartime medical problems was restricted, further stifling incretin research.

The field of incretin biology flourished once again in the 1960s. Around this time, several investigators observed that the plasma insulin response of individuals to intravenous glucose was significantly lower than that seen after an oral glucose load (47, 106). This suggested that an alimentary mechanism, in addition to elevated blood glucose levels, regulated insulin release. Perley and Kipnis (125) estimated that up to half of the insulin secreted following a meal could be attributed to incretin(s). This postprandial enhancement of insulin secretion by incretin(s) was termed the incretin effect.

As the search for incretin(s) began, the following criteria were proposed (31): 1) the factor must be released from gastrointestinal cells following nutrient ingestion, 2) the factor, at physiological concentrations, must stimulate insulin secretion, and 3) the factor must stimulate insulin release only in the presence of elevated blood glucose levels. Over the next 25 years, gastric inhibitory polypeptide (GIP), later named glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1 (GLP-1) were discovered to be the incretin hormones involved (GIP – 18) (GLP-1 – 85, 111, 151). Currently, GIP and GLP-1 are the only known incretin hormones, and together they account for most, if not all, of the incretin effect in humans (118, 173).
1.2 - Production and Secretion

GIP is synthesized and released from enteroendocrine K cells (23), which are primarily located in the duodenum and proximal jejunum. It is a 42-amino acid polypeptide derived from the posttranslational cleavage of a larger 153-amino acid proGIP precursor; prohormone convertase (PC) 1/3 is the enzyme responsible for this posttranslational cleavage (167). GIP is secreted in response to nutrient absorption (6). The basal circulating levels range from 60 to 100 pM in humans and increase to between 200 and 500 pM after a meal (172).

GLP-1 is secreted from enteroendocrine L cells located mainly in the distal jejunum, ileum, and proximal colon; however, it has been reported that a small subset of duodenal endocrine cells produce both GIP and GLP-1 (115, 160). GLP-1 results from a tissue-specific posttranslational proteolytic cleavage of the proglucagon gene (110). In pancreatic α-cells, proglucagon is cleaved by PC 2 into glucagon, glicentin-related polypeptide, intervening peptide-1, and the major proglucagon fragment. In intestinal L cells, on the other hand, proglucagon is processed by PC 1/3 into GLP-1, glicentin, oxyntomodulin, intervening peptide-2, and glucagon-like peptide-2 (GLP-2). The tissue-specific expression of the PC enzymes regulates the posttranslational cleavage of the proglucagon gene (149). GLP-1 secretion is stimulated by a variety of nutrient, neural, and endocrine factors (39). Fasting plasma levels range between 5 and 10 pM and increase approximately 2 to 3 fold after a meal (172).

As previously mentioned, the primary stimulus for incretin secretion is the ingestion of nutrients. Carbohydrate, protein, and lipid alone, as well as mixed meals, have all been documented to induce the release of GIP and GLP-1 from enteroendocrine cells. Based on observations from patients with intestinal malabsorption (12) and studies using pharmacological agents that impede nutrient uptake (54, 56), it is thought that GIP secretion is dependent on nutrient absorption rather than the mere presence of
nutrients in the intestinal lumen. Whether or not absorption is essential for GLP-1 secretion appears to be nutrient-specific. The mechanisms underlying these processes remain largely unestablished.

1.2.1 - Carbohydrate

Because both K and L cells express glucokinase (a tissue-specific isoform of hexokinase – the first enzyme in the glycolytic pathway) (78), it was hypothesized that GIP and GLP-1 secretion utilized machinery similar to that employed in pancreatic β-cells. Once transported into the β-cell via glucose transporter 2 (GLUT2), glucose is metabolized via glycolysis and the citric acid cycle, resulting in an elevated cytosolic ATP/ADP ratio. This, then, closes the ATP-sensitive potassium ($K_{\text{ATP}}$) channels, which leads to β-cell membrane depolarization and opening of the voltage-gated calcium ($Ca^{2+}$) channels. The rise in intracellular $Ca^{2+}$ levels triggers fusion of insulin granules to the plasma membrane and subsequent secretion of insulin into the circulation (139). Studies aimed at deciphering the mechanism underlying carbohydrate-induced GLP-1 secretion found that glucose and other metabolizable sugars acted through glucose metabolism, closure of $K_{\text{ATP}}$ channels, and action potential production (133), indeed analogous to the process in the pancreatic β-cell. More recently, Parker et al. (124) and Reimann et al. (134) generated transgenic mice with fluorescently labeled GIP-secreting K cells and GLP-1-secreting L cells, respectively; studies from these transgenic mice have also implicated $K_{\text{ATP}}$ channels in glucose-stimulated incretin secretion.

Along similar lines, Ritzel et al. (142), using an in vivo perfused rat ileum model, demonstrated that various carbohydrates (metabolizable and non-metabolizable) successfully stimulated GLP-1 release; however, only those carbohydrates which were substrates for glucose or fructose transporters promoted secretion, suggesting that uptake, even in the absence of metabolism, may be sufficient to induce secretion of GLP-1. Whereas metabolizable sugars, such as glucose, depolarize the cell via closure of $K_{\text{ATP}}$ channels, non-metabolizable sugars, on the other hand, generate small inward currents and
subsequent depolarization when transported via sodium-coupled glucose transporter 1 (SGLT1) (64, 114).

Although apparent that both pathways (cell depolarization due to metabolism-induced K$_{ATP}$ channel
closure or due to sodium (Na$^+$) influx via SGLT1) are involved in carbohydrate-induced GLP-1 secretion,
it is unknown which pathway, if any, is the primary system.

Both Cheung et al. (27) and Ramshur et al. (130) created K cell lines that secrete GIP and insulin; in these
cell lines, insulin secretion is controlled under the GIP promoter. Release of insulin from the cell line
generated by Cheung et al. was glucose-dependent. On the other hand, glucose was unable to stimulate
insulin secretion in the GIP/Ins cell line from the lab of Ramshur et al.; however, insulin was released in
response to the intracellular metabolites glyceraldehyde and methyl pyruvate. Additional studies revealed
that insulin secretion from the GIP/Ins cells is K$_{ATP}$ channel independent (130, 177). Furthermore, Wang
et al. (177) failed to identify in vivo a single GLP-1-positive L cell that was also positive for the
potassium channel subunit Kir 6.2, in stark contrast to the previously described work by Reimann and
Gribble (133) in the GLUTag cell line (a murine cell line derived from a colonic tumor) and by Parker et
al. (124) and Reimann et al. (134) in primary cells isolated from transgenic mice.

Recently, it has been suggested that sweet taste receptor pathways are actively involved in glucose-
sensing by the K and L cells (83). The sweet taste receptor found in lingual taste buds is a G-protein
coupled receptor (GPCR); it is a heteromer, comprised of two subunits, Tas1R2 and Tas1R3, coupled to
heterotrimeric gustducin (α-gustducin, Gβ3 and Gγ13) (84). The 3 gustducin subunits, as well as the
receptor, have been reported to be expressed by human and murine incretin-secreting cells (76, 104).
GLP-1 secretion from NCI-H716 cells (a human cell line derived from cecal adenocarcinoma) was
stimulated by glucose, sucrose, and the non-caloric sweetener, sucralse; additionally, Tas1R3 and α-
gustducin knockout mice displayed deficiencies in their glucose-stimulated GIP and GLP-1 secretion.
Margolskee et al. (104) further suggested that the intestinal sweet taste receptor functions as a luminal
sugar sensor which increases the expression of SGLT1. However, expression of Tas1R2, Tas1R3, and α-
gustducin was undetectable in primary murine K and L cells and sucralose failed to stimulate GIP and GLP-1 secretion from mixed intestinal epithelial cultures (124, 134). In spite of this data, the role of the sweet taste receptor pathway is still far from clear, and whether or not it is a potentially promising therapeutic avenue remains to be determined.

1.2.2 - Protein

According to Gunnarsson et al. (66), glucose-induced incretin release is differently modulated by the ingestion of fat and protein. They observed that the addition of whey protein increased the levels of biologically active GIP and GLP-1, markedly augmented the plasma insulin response, and enhanced glucose disposal in mice. Similarly, supplementing typical breakfast and lunch meals with whey protein was found to increase GIP and insulin secretion and diminish postprandial blood glucose levels in humans (52). Karamanlis et al. (80) also noted a comparable response in humans given a glucose, protein (gelatin), or glucose plus protein drink.

The underlying mechanism behind protein-stimulated GLP-1 secretion is still under investigation. It has been reported, though, that glutamine triggers membrane depolarization, amplifies intracellular calcium concentrations, and induces GLP-1 release (135). Membrane depolarization was attributed to small inward currents generated during the uptake of glutamine via a Na⁺-coupled transporter (similar to the membrane depolarization following glucose uptake via SGLT1). Asparagine, another amino acid that utilizes a Na⁺-coupled transporter for uptake, generated similar inward currents but was a less potent GLP-1 secretagogue than glutamine (135), suggesting that glutamine may have additional stimulatory effects downstream of membrane depolarization. Alanine and glycine are also capable of inducing GLP-1 release; however, unlike glutamine and asparagine, the mechanism is Na⁺-independent. Gameiro et al. (57) discovered that these amino acids act through glycine and γ-amino butyric acid (GABA) receptors.
The glycine and GABA receptors are ligand-gated chloride (Cl⁻) channels that open when activated, allowing the inward flux of Ca²⁺ ions and subsequent GLP-1 release.

Downstream of membrane depolarization, Reimer (136) revealed the involvement of extracellular signal-related kinase 1/2 (ERK 1/2) and p38 mitogen-activated protein kinases (MAPKs) after stimulation with meat hydrolysate and essential amino acids. As suggested by the previous finding, protein hydrolysates and peptones are documented secretagogues of GLP-1 secretion. Incubating STC-1 (a cell line derived from a murine duodenal tumor) and GLUTag cells with both albumin egg and meat hydrolysates increased proglucagon gene transcription and proglucagon promoter activity (29). Uptake of oligopeptides produces membrane depolarization, suggesting that the electrogenic co-transport of peptides and protons may be inducing this response. PEPT1, a proton/oligopeptide transporter, has been implicated as the effector of this membrane depolarization. Matsumura et al. (105) demonstrated that STC-1 cells depolarized and secreted GLP-1 when stimulated with dipeptides. However, STC-1 cells do not endogenously express PEPT1 and were only capable of producing this effect when transfected with PEPT1; thus, it remains to be established if PEPT1 is a physiological regulator of GLP-1 secretion in vivo. It is not clear if the transfected protein is regulated or functioning properly or if primary enteroendocrine cells even express the transporter.

In regards to GIP secretion, much less is known. Wolfe et al. (180) observed that peptones increased the circulating levels of GIP in the rat. However, they argued that the increase in secretion was not necessarily due to direct interaction of protein with the K cell; rather, the acid-stimulatory properties of protein mediated the release of GIP, since protein-induced GIP secretion no longer occurred after treatment with a proton pump inhibitor.
Lipid-induced incretin secretion has also been well-documented; however, the mechanisms underlying this phenomenon have yet to be elucidated. Investigations looking at the potential of individual fatty acids have found that monounsaturated fatty acids are more potent stimulators of incretin secretion than both saturated and polyunsaturated fatty acids. Thomsen et al. (162) observed increased GIP and GLP-1 secretion in humans following a carbohydrate meal supplemented with olive oil (rich in monounsaturated fatty acids) compared to one supplemented with butter (saturated fatty acids). Similarly, when fat was provided as an individual macronutrient to humans, Beysen et al. (13) documented a greater GLP-1 response after the ingestion of olive oil compared to that following the ingestion of safflower oil (polyunsaturated fatty acids) and palm stearin (saturated fatty acids). Rocca et al. (146) further demonstrated that chronic exposure to oleic acid over palmitic acid enhanced GLP-1 secretion in GLUTag cells. Additionally, only those fatty acids with a chain length greater than 14 carbons were capable of stimulating GLP-1 secretion from fetal rat intestinal cells (FRICs) (144). In contrast to the described studies, Relling and Reynolds (138) found in lactating dairy cows that monounsaturated and polyunsaturated fatty acids were both capable of inducing GLP-1 secretion and that saturated, monounsaturated, and polyunsaturated fatty acids were equally potent stimuli of GIP secretion. Here, the different animal and cell models may be accountable for the lack of agreement.

The hydrolysis of triglycerides appears to be a crucial step in incretin secretion. Ellrichman et al. (46), Enç et al. (48), and Pilichiewicz et al. (126) all reported significant reductions in GIP secretion and modest to significant decreases in GLP-1 secretion following treatment with the pancreatic lipase inhibitor orlistat. On the other hand, Damci et al. (37) reported increases in both GIP and GLP-1 secretion following orlistat treatment in type 2 diabetic patients; the authors contend that enhanced delivery of lipid to the distal ileum, the primary site of the GLP-1-secreting L cells led to the amplified GLP-1 levels; however, this does not suffice to explain the elevated postprandial GIP levels. In spite of
these results, the majority of studies support the need for triglyceride hydrolysis to obtain proper incretin secretion. Beglinger et al. (11) further suggest that hydrolysed long-chain fatty acids stimulate cholecystokinin (CCK) release, which in turn stimulates GLP-1 secretion via the CCK\textsubscript{1} receptor (referred to as the CCK\textsubscript{A} receptor in older literature); CCK is a hormone released from the duodenum that signals the release of bile from the gallbladder and digestive enzymes from the pancreas. The authors tested this hypothesis with three experiments. The first two experiments recapitulate previous findings: 1) treatment with orlistat significantly attenuates olive oil-induced CCK and GLP-1 secretion and 2) long chain, but not short chain, fatty acids simulate CCK and GLP-1 secretion. In the third experiment, subjects were provided with duodenal infusions of sodium oleate and intravenous dexloxiglumide (a CCK\textsubscript{1} receptor antagonist). Dexloxiglumide treatment abolished the lipid-induced GLP-1 increase but amplified the increase in CCK. The authors propose that following adequate fat hydrolysis, the products of fat digestion stimulate CCK release, which stimulate GLP-1 secretion via CCK\textsubscript{1} receptors. However, the authors do not acknowledge the possibility that blocking CCK action at the pancreas severely reduces secretion of digestive enzymes necessary for fat hydrolysis and that the absence of GLP-1 secretion is not due to inhibition of CCK signaling at the L cell but rather to the significant reduction in fat hydrolysis products to directly stimulate GLP-1 release. Thus, more work is needed to elucidate the role of CCK in lipid-induced GLP-1 secretion.

Although triglyceride hydrolysis is essential, fatty acid oxidation does not appear to be involved in GLP-1 secretion. FRICs treated with methyl palmoxirate (a fatty acid oxidation inhibitor) did not affect the release of GLP-1 (144). However, a role for fatty acid reesterification is not as clear. In the same study, GLP-1 secretion was prevented when FRICs were incubated with oleic acid that had a methyl ester blockage of the free carboxyl group. Despite these results, the authors argue that if reesterification was indeed important, then GLP-1 secretion should have been observed when the cells were incubated with saturated or polyunsaturated fatty acids during which reesterification was not inhibited (see discussion
above; 144). Although the latter is correct, it is not clear if additional upstream processes in addition to reesterification are involved in the release of GLP-1.

Recently, Hirasawa et al. (73) have implicated the G-protein coupled receptor, GPR120, in mediating lipid-induced GLP-1 secretion. GPR120 is specific for unsaturated long-chain fatty acids, as neither medium-chain nor saturated long-chain fatty acids produced a substantial GLP-1 response in STC-1 cells. In accordance with these findings, expression of GPR40, GPR119, and GPR120 (all documented fatty acid GPCRs) has been detected in primary K (124) and L cells (134). Further investigations have looked at potential downstream targets of GPR120. Iakoubov et al. (75) examined the role of an atypical protein kinase C isoform, aPKCζ, in FRICs and GLUTag cells and found that oleic acid-induced GLP-1 secretion was highly dependent on activation of aPKCζ, as GLP-1 release was abrogated by an aPKCζ inhibitor and aPKCζ siRNA transfection. Whether or not these same signaling pathways are used for all fatty acids is of great interest.

1.2.4 - Non-Nutrient Secretagogues

In addition to direct nutrient stimuli, GLP-1 secretion is induced by a variety of neural and endocrine factors (39). Rocca and Brubaker (145) demonstrated the importance of the vagus nerve in stimulating GLP-1 secretion. Bilateral subdiaphragmatic vagotomy completely abolished fat-induced GLP-1 secretion, and direct stimulation of the celiac branch of the vagus nerve resulted in significant GLP-1 release. Similarly, Hira et al. (72) also demonstrated indirect stimulation of GLP-1 release via the vagus nerve following a protein hydrolysate duodenal challenge in rats. GIP has been implicated in regulating GLP-1 secretion via the vagus nerve (143, 145).

Additionally, murine, human, and fetal rat L cells express the leptin receptor, and when exposed to leptin, all three cell types secreted GLP-1 (5). More recently, Katsuma et al. (81) and Thomas et al. (161)
described a role for the bile acid GPCR, TGR5, in GLP-1 secretion and glucose homeostasis. Along the same lines, Takahashi et al. (159) reported that chronic bile diversion in dogs abolished fat-induced GIP secretion and that it is restored upon oral supplementation of the collected bile with the fat meal. Although suggestive that aspects of bile are involved in GIP secretion, it is unclear whether it is the direct effect of bile on GIP-secreting K cells or the restoration of proper fat digestion and absorption that causes the subsequent release of GIP; further work is needed to clarify this issue. Interestingly, both hyperglycemia (175) and hyperinsulinemia (95) appear to negatively affect GLP-1 secretion, which may have important implications in the pathophysiology of type 2 diabetes and obesity.

1.3 - Action
1.3.1 - GIP

GIP effects its action through the GIP receptor (GIPR). The GIPR is a member of the 7-transmembrane-spanning, heterotrimeric G-protein coupled receptor superfamily (168). The GIPR gene is expressed in a variety of tissues, including small intestine, pancreas, and adipose tissue. Signaling through the GIPR increases cytoplasmic cyclic adenosine monophosphate (cAMP) and Ca²⁺ levels and activates several different intracellular kinases (6).

The main function of GIP is to enhance insulin secretion from the pancreatic β-cell in response to elevated blood glucose concentrations. GIP binding to the β-cell stimulates increases in intracellular cAMP and Ca²⁺ levels, closure of K_{ATP} channels, and exocytosis of insulin granules (50). GIP also up-regulates β-cell insulin gene transcription and biosynthesis, stimulates β-cell proliferation, and reduces β-cell apoptosis (82, 178). Additionally, GIP enhances lipogenesis by stimulating lipoprotein lipase activity, enhancing fatty acid synthesis and incorporation into triglycerides, and down-regulating glucagon-stimulated lipolysis, all of which promote fat deposition rather than mobilization (58). In fact, mice lacking the receptor for GIP are resistant to diet-induced obesity (108, 184).
1.3.2 - GLP-1

The GLP-1 receptor (GLP-1R) also belongs to the family of 7-transmembrane-spanning, heterotrimeric G-protein coupled receptors. Like the GIPR, the GLP-1R is also expressed in a wide range of tissues, including pancreas, stomach and intestine, nodose ganglion of the vagus nerve, and regions of the central nervous system, such as hypothalamus and brainstem (6).

Similar to GIP, GLP-1 enhances glucose-dependent insulin secretion (85, 111), stimulates β-cell proliferation, and decreases β-cell apoptosis (74), and in the presence of high glucose concentrations, GLP-1 has been shown to reduce glucagon secretion (70). GLP-1 additionally improves glycemic control by decreasing gastric emptying via the ileal brake reflex, thereby reducing the delivery of absorbed nutrients to the circulation over time (90, 102, 120, 156). GLP-1 provides another level of glycemic control by regulating food intake (67, 88, 117, 166).

1.4 - Degradation

Once secreted from the intestine into the circulation, both GIP and GLP-1 are rapidly degraded by dipeptidyl peptidase-IV (DPP-IV) (107). DPP-IV cleaves dipeptides from the amino terminus of proteins that contain a penultimate alanine or proline; the GIP and GLP-1 metabolites produced by DPP-IV are inactive. DPP-IV is ubiquitously expressed and found in multiple tissues and cell types. Most notably, DPP-IV is located on the surface of endothelial cells, particularly the cells lining the blood vessels of the intestinal mucosa. Hansen et al. (68) estimated that greater than 50% of GLP-1 released from the L cells is degraded in the capillaries draining the intestinal mucosa. Furthermore, after a single pass through the liver, approximately 40% of the remaining bioactive GLP-1 is degraded (41). Consequently, the half-life for both incretin hormones is quite short, lasting only 2-3 minutes.
2 - Measurement of Incretin Secretion

2.1 - In Vitro Models

Studying incretin secretion at the cellular level has been hindered by the lack of appropriate cell models. Almost all in vitro studies have made use of the murine and human cell lines that secrete GIP, GLP-1, or both incretin hormones. The GIP/Ins, GLUTag, and STC-1 cells are the commonly used murine lines, while the NCI-H716 is the predominantly used human cell line. The NCI-H716 cell line was derived from the ascites fluid of a male with poorly differentiated carcinoma of the colon. These cells have dense core granules in the cytoplasm, suggesting possible endocrine function (123). Indeed, further evaluation demonstrated nutrient- and non-nutrient-induced GLP-1 secretion from these cells (137). Drucker et al. (44) created the GLUTag cell line by generating transgenic mice that express a glucagon – simian virus-40 large T-antigen fusion protein specifically in the distal ileum and proximal colon. These mice develop endocrine carcinoma of the large bowel (91). GLUTag cells secrete GLP-1 in response to a variety of stimuli and display morphology characteristic of enteroendocrine cells (43). Similar to the GLUTag cell line, the STC-1 line was derived from intestinal tumors of transgenic mice - in this case, however, harboring two oncogenic fusion genes, rat insulin promoter – simian virus-40 large T-antigen (RIP1Tag2) and rat insulin promoter – polyoma small T antigen (RIP2PyST1) (141). The STC-1 cell line is plurihormonal, secreting CCK, GIP, GLP-1, and secretin. The GIP/Ins line, as discussed previously, was derived from the STC-1 line. Two groups (27, 130) selectively transfected GIP-specific STC-1 cells with a construct containing the human insulin gene under control of the rat GIP promoter. Although these cells were originally engineered for insulin gene therapy, they still secrete GIP alongside insulin.

In spite of having several incretin-secreting cell lines, the lines each respond differently to a variety of nutrient secretagogues. For example, GLUTag (75) and NCI-H716 (137) cells respond to fatty acids, while STC-1 cells may not; Hirasawa et al. (73) were able to demonstrate fatty acid-induced GLP-1
secretion from STC-1 cells, while Brubaker et al. (20) were unsuccessful. Furthermore, Cheung et al. (27) reported glucose-dependent insulin (i.e. GIP) release in their engineered GIP/Ins cells, whereas Ramshur et al. (130) was unable to stimulate insulin (i.e. GIP) secretion with glucose. However, investigators must rely on these lines because of the lack of appropriate primary cell models. Until recently, FRICs were the only described primary cell model. As the name implies, these cells are derived from the intestines of fetal rats (21); however, these cells contain a mixture of cell types, including other enteroendocrine cells and the absorptive enterocytes. Due to the heterogeneous cell population, it is difficult to ascertain if the incretin-secreting machinery are located within the K and L cells themselves or if the machinery requires communication with other intestinal cell types. The latter point is particularly relevant, as we have recently found that chylous lymph collected from animals actively absorbing fat stimulates the release of incretins in both the GIP/Ins and STC-1 cell line. Regardless, this cell model (FRIC) is commonly used in conjunction with the cell lines described above. Parker et al. (124) and Reimann et al. (134) have recently developed a technique to allow isolation of primary K and L cells, respectively. In both reports, transgenic mice expressing a yellow fluorescent protein under the control of the GIP promoter for K cells or the proglucagon promoter for L cells were generated; the fluorescently labeled cells were separated by flow cytometry and used for subsequent characterization studies. Although the authors use the pure primary cells to determine the expression level of nutrient-sensing machinery, the fluorescence-sorted cells are unable to survive in primary culture, thus all incretin secretion studies were performed in primary intestinal cultures, which run the same problems as the FRICs. Until techniques that allow successful culture of primary K and L cells are developed, investigators will still be at a disadvantage in unraveling the intracellular pathways utilized in GIP and GLP-1 secretion.
2.2 - In Vivo Models

Just as the in vitro systems are limited by the available cell lines, in vivo study of GIP and GLP-1 is also fraught with difficulties. Traditionally, in vivo study of the incretin hormones requires measurement of the circulating levels in the peripheral blood and occasionally the portal blood. Due to the low concentration of the incretin hormones in plasma, investigators studying GIP and GLP-1 secretion in small animal models are limited by the amount of blood that can be removed from an animal during the course of a study. Because of these limitations, continuous monitoring of GIP and GLP-1 secretion becomes difficult. A more effective means of studying incretin secretion in small animal models is therefore desirable. Since lymph collects fluid drained from the intestinal lamina propria, it is conceivable that the concentrations of incretin hormones would be higher in the intestinal lymph than in plasma. Additionally, since lymph has a lower flow rate than blood (lymph flow rate = 2-3 ml/h; portal blood flow rate = 8-20 ml/min) (154, 165), the hormones would be less diluted, thereby raising the concentration of GIP and GLP-1.

Indeed, the presence of incretin hormones in lymph was reported in the early 1980s. Although no measurements of plasma levels were reported, Adrian et al. (2) successfully demonstrated the presence of GIP in lymph sampled from the intestinal lymphatic duct of conscious calves. Using a conscious pig model, Manolas et al. (103) compared GIP concentrations following consumption of a standard meal (13.01 kJ/kg: 50% carbohydrate, 14% protein, 6% fat, 30% non-nutrient residual material) in plasma and lymph collected from the cisterna chyli. The test meal stimulated the release of GIP, which was observable in both plasma and lymph; however, both the integrated and total incremental responses (postprandial peptide response above basal levels and total postprandial peptide response, respectively) were greater in plasma than in lymph. Regardless, the lymph:plasma ratio was higher for GIP compared to the pancreatic hormones measured in the study. The authors suggested that the close proximity of the intestinal secretory cells to the lymphatic system may account for this difference.
3 - Anatomy and Physiology of the Gastrointestinal and Lymphatic Systems

To facilitate a better understanding of the rationale behind using lymph to measure incretin secretion, a brief overview of the anatomy and physiology of the gastrointestinal and lymphatic systems will be presented.

3.1 - The Gastrointestinal System

The gastrointestinal system consists of the gastrointestinal tract and the gastrointestinal glands. The gastrointestinal tract is essentially a tube divided into several segments: the oral cavity, esophagus, stomach, small and large intestine, rectum, and anus. The small intestine is further divided into three parts: the duodenum, jejunum, and ileum. The small intestine is the site of terminal food digestion, nutrient absorption, and endocrine secretion. The lining of the small intestine consists of a series of permanent spiral or circular folds, termed the plicae circulares, which amplify the organ’s surface area, promoting efficient nutrient absorption. The mucosal surface area is increased further by fingerlike projections and depressions, called villi and crypts, respectively. The villi and crypts are covered by a continuous sheet of epithelial cells. The predominant epithelial cell type is the absorptive cell, the enterocyte; however, a variety of other cell types, such as enteroendocrine cells, can be found on the intestinal villus (79). The enteroendocrine cells are specialized cells of the gastrointestinal system that produce and secrete hormones.

The enterocytes lining the villi are the cells responsible for nutrient absorption. Once absorbed, nutrients are either transported to the circulation via the portal blood or, in the case of lipid digestive products, to the lymph via chylomicrons (triglyceride-rich lipid particles). The structure of the villi (Figure 1) facilitates this divergent nutrient transport. Directly below the epithelial lining of the villi lies the
intestinal lamina propria. The lamina propria is composed of loose connective tissue with nerve fibers and smooth muscle cells. Additionally, located within each villus is a specialized lymphatic capillary, called a lacteal, which is surrounded by a blood capillary network (62).

3.2 - The Lymphatic System

The lymphatic system is comprised of a series of lymphatic vessels and lymph nodes. Lymph is formed when fluid and proteins from the interstitial space that are not reabsorbed by the blood capillaries enter the lymphatic capillaries. Following formation, lymph drains from the lymphatic capillaries into afferent (prenodal) lymphatic ducts, which transport lymph to regional lymph nodes. Efferent (postnodal) lymphatic ducts transport the lymph through a series of successive lymph nodes and progressively larger lymphatic vessels. Lymph drained from the upper right half of the body culminates in the right lymphatic duct, whereas lymph drained from the remainder of the body terminates in the thoracic duct, before first collecting in the cisterna chyli. Both the right lymphatic duct and the thoracic duct drain into the circulatory system at the right and left subclavian vein, respectively (158). Unlike the circulatory system, the lymphatic system does not have a muscular pump to move fluid through its vessels. Instead the lymphatic ducts make use of pressure differences, skeletal muscle contraction, and one-way valves to propel the lymph (139).

Beyond draining excess fluid and proteins, the lymphatics of the gastrointestinal tract are also responsible for the transport of lipoproteins and lipophilic compounds to the circulatory system. After being absorbed by the lacteals in the intestinal villi, these particles are transported via first the intestinal (mesenteric) lymphatic duct and then the thoracic duct before being drained into the circulation through the subclavian vein (32).
Structure of the Intestinal Villus. The mucosal surface of the small intestine is comprised of fingerlike projections and depressions, called villi and crypts. The villi and crypts are covered by a continuous sheet of epithelial cells. The primary epithelial cell is the absorptive enterocyte; however, a variety of other cells, such as enteroendocrine cells, can also be found on the villus. The enteroendocrine cells are sparsely distributed throughout the intestinal villus; in general, there is 1 enteroendocrine cell for every 1000 enterocytes. The GIP-secreting K cells and GLP-1-secreting L cells are two different types of enteroendocrine cell. Directly below the epithelial lining of the villus lies the intestinal lamina propria. The lamina propria is composed of loose connective tissue with nerve fibers and smooth muscle cells. Within each villus is a specialized lymphatic capillary, called a lacteal, which is surrounded by a blood capillary network. Lipid and lipophilic compounds are transported from the enterocyte into the lacteal. The lymph in the lacteal also contains material drained from the lamina propria, such as hormones secreted from the enteroendocrine cells. (© 2010 Marcia Hartsock, MA, CMI.)
Molecules transported by the lymph after being absorbed by the gastrointestinal system can be studied using lymph cannulation in a variety of animal models. Two general lymph cannulation techniques have been described in the literature: cannulation of the intestinal lymphatic duct or cannulation of the thoracic duct. Intestinal lymphatic cannulation allows for the collection of lymph from the stomach, intestine, pancreas, spleen, and portions of the liver, without contamination of lymph from other organs. Cannulation of the thoracic duct, on the other hand, allows for the collection of lymph not only from the intestinal lymphatic duct, but also from the remainder of the body, excluding the lymph drained from the upper right quadrant of the body. In some animals, such as the pig, the intestinal lymphatics are highly branched. In these animals, complete collection of the intestinal lymph is extremely difficult; cannulating the thoracic duct would ensure complete collection of intestinal lymph. Numerous lymph fistula animal models have been developed over the past few decades. The most widely used animal is the rat; however, several larger animal models are also used to study the transport of lipids and lipophilic compounds. A few of these models will be discussed.

4.1 - The Lymph Fistula Rat Model

The lymph fistula rat model has been described and utilized by several investigators. Variations in methodology involve differences in cannulation sites, use of anesthesia, and pre- and post-operative care. Bollman et al. (16) provided one of the first reports of thoracic and intestinal lymphatic duct cannulation in the anesthetized rat. In this model, the rats are initially anesthetized with ether and then injected with 0.1 ml of a 0.5% solution of Evans blue dye to facilitate visualization of the lymphatic vessel. To assist locating the intestinal lymphatics, Bollman et al. suggest providing the rats a lipid-based meal prior to surgery; the intestinal lymphatic duct will appear a milky-white color rather than clear. After the duct (thoracic or intestinal) has been located and incised, polyvinyl chloride tubing is inserted into the duct and
ligated into place (Figure 2). The tubing is then exteriorized and placed into a graduated centrifuge tube below the plane of the animal to allow continuous lymph collection by gravity. Following the operation, the rats are positioned in restraint cages for the duration of the lymph collection period to prevent the animals from removing the lymphatic cannula. Although they prevent the animals from turning around, the cages do allow some forward and backward movements (15). One of the limitations of the Bollman model is the absence of rehydration. Subsequent lymph fistula rat models describe the addition of either a duodenal infusion tube or jugular vein cannula for saline rehydration.

Porter and Charman (128) describe an updated anesthetized lymph fistula rat model. In this model, the investigators use a triple-cannulation method, in which the intestinal lymphatic duct, jugular vein, and duodenum are cannulated. Prior to the operation and every 2 h thereafter the animals were kept anesthetized with sodium pentobarbitone. Following the procedure the animals remained anesthetized during the entirety of the study, while lymph is continuously collected from the intestinal lymphatic duct cannula, plasma is sampled via the jugular vein cannula, and nutrients and saline (for rehydration) are provided through the duodenal cannula.

While the use of anesthetized models has many advantages, specifically elimination of any problems associated with animal movement during lymph collection, the lymph flow rate is affected by the use of anesthesia. In conscious animals, the fasting lymph flow rate ranges from 2-3 ml/h; however, in anesthetized rats, the fasting lymph flow rate drops dramatically to 0.1-0.6 ml/h (34, 45, 127). This reduction in lymph flow may be attributed to changes in gastrointestinal motility, altered capillary permeability, reduced interstitial fluid formation, and decreased blood flow. Additionally, the continuous use of anesthesia poses several economical and logistical problems. Not only does the cost of the study increase, but the animals require constant observation to monitor the effects of anesthesia on respiration and heart rate.
**Lymph Fistula Procedure.** After opening the abdominal cavity of the rat, the stomach, small intestine, and colon are retracted and gently pushed under the abdominal muscle walls. The surgeon’s fingers can be used to keep the liver out of the surgical plane. The inferior vena cava and left renal vein will now be exposed and the intestinal lymphatic duct can be seen as a clear vessel running parallel to and lying directly above the superior mesenteric artery. With proper anatomical references, locating the intestinal lymphatic becomes less challenging. The inset shows the site of the cannula placement. The incision should be made proximal to the vena cava, allowing insertion of the cannula and advancement approximately 3-5 mm. Once secured by a drop of cyanoacrylate glue, the cannula is positioned beneath the liver and exteriorized through the right flank of the animal. (© 2010 Marcia Hartsock, MA, CMI.)
Due to alterations in lymph flow rate from anesthesia, the use of conscious rat lymph fistula models to study the transport of lipids and lipophilic compounds is increasing. Tso and Simmonds (164) provide an excellent detailed description of the lymph fistula procedure and collection in unanesthetized animals. Similar to the procedure described by Bollman et al. (16), once the duct has been cannulated, the cannula is ligated into place at two locations; however, unlike Bollman et al., the duct is further secured using a drop of methyl cyanoacrylate glue. Following surgery the animals are allowed to recover from anesthesia and are then placed in restraint cages. Rehydration occurs via a duodenal cannula; however, a jugular vein cannula can be used instead. Beyond presenting a meticulous description of the procedure, Tso and Simmonds (164) additionally provide several hints for the successful completion of the procedure and discuss the advantages and disadvantages of cannulating the intestinal lymphatic duct over the thoracic lymphatic duct. The procedure described by Tso and Simmonds is economical and has a high success rate (approximately 90%, when performed by a trained surgical technician). This model has been extensively used in lipid absorption and transport studies.

4.2 - The Lymph Fistula Model for Large Animals

In addition to the described rat models, the lymph fistula procedure has been adapted for use in larger animal models, such as the dog and pig. Collection of lymph from the dog typically occurs through the thoracic duct. For short-term lymph collection, an external thoracic duct fistula, which continually drains the lymphatic fluid, is suitable. In these procedures, once the duct has been cannulated, the tube is exteriorized at the neck and attached to a collecting flask (65). The dogs used by Grindlay et al. were trained to remain still for extended periods of time, thus not requiring the use of a restraining device. Several years later, Rampone (129) designed a leather harness that could be used to hold the collecting flask in place and still permit movement in the animals. For long-term lymph studies, complete drainage and collection have been argued to alter the physiological state of the animal by altering the dynamics of the thoracic duct circulation and depleting the animal of fluid, salts, and proteins, despite the use of
rehydrating fluids. To circumvent this concern, thoracic duct lymph can be sampled from a side-fistula, in which the cannula is still exteriorized but occluded with a plastic clamp and secured around the neck of the dog (59). As an alternative to the side-fistula, a thoracic duct-to-duct (59) or thoracic-duct venous shunt (42) can be employed to sample lymph during long-term studies. In both of these shunt procedures, lymph is diverted from the thoracic duct into the cannula and returned to the circulation (via the thoracic duct for the thoracic duct-to-duct shunt or the jugular vein for the thoracic duct-venous shunt).

Intestinal lymphatic duct and thoracic duct cannulations can also be performed in anesthetized or conscious pigs. To collect lymph from the gastrointestinal system, either the cisterna chyli (103) or a branch of the intestinal lymphatics (179) can be cannulated. The intestinal lymphatics of the pig are highly branched; thus, cannulation of a duct that allows complete collection of the intestinal lymph is challenging. Because of this, only qualitative measurements of the collected lymph can be obtained. Collection of lymph from the thoracic duct, however, allows for quantitative analyses since drainage of the entire intestinal lymph pool is possible. As with the dog, long-term lymph drainage has been argued to affect the physiological state of the animal; a thoracic duct-venous shunt model can be used for long-term lymph sampling to avoid these potential issues (77). In this model, both the left external jugular vein and thoracic duct are cannulated; the catheters are then connected by two three-way valves connected in series. Although lymph cannot be continuously collected, the shunts allow the animals to move freely and allow lymph sampling for several days post-operatively (shunt patency averaged 5.5 d).

Descriptions of intestinal lymphatic cannulations in other large animal models, such as the cat (157), cow (148), and sheep (89), can be found elsewhere.
5 - Using the Lymph Fistula Rat Model to Study Incretin Secretion

5.1 - The Fasting and Postprandial Concentrations of GIP and GLP-1 are Higher in Intestinal Lymph than in Peripheral or Portal Plasma

The lymph fistula model is commonly used to study the transport of lipid and lipophilic compounds in a variety of animal models. However, the collected lymph from these models also contains material drained from the lamina propria, in addition to the lipids and lipophilic compounds. Because of the close proximity of the incretin-secreting enteroendocrine cells to the lacteals (Figure 1) and because the lymph has a lower flow rate than blood, it is plausible that the concentration of the incretin hormones may be higher in intestinal lymph than in peripheral or portal plasma.

To investigate this hypothesis, D’Alessio et al. (36) measured the concentration of several gastrointestinal hormones in Sprague-Dawley rats outfitted with an intestinal lymphatic duct cannula and either a portal or jugular vein catheter. Following an intragastric mixed meal bolus, lymph was continuously collected and portal or jugular venous blood was sampled for 4 h. Plasma and lymph samples were analyzed for insulin, GLP-1, and peptide tyrosine-tyrosine (PYY).

The results demonstrated that intestinal lymph contains measurable amounts of insulin, GLP-1, and PYY and that the lymphatic secretion profile of these hormones parallels that of plasma. However, D’Alessio et al. (36) found that the relative concentrations in lymph and plasma vary for the three hormones. The data suggest that insulin is not preferentially secreted into the lymph but rather enters the lymphatic system via capillary filtration. Interestingly, both the fasting and postprandial concentrations of the incretin hormone GLP-1 were higher in the intestinal lymph than in either peripheral (jugular) or portal plasma. Compared to insulin, GLP-1 has a higher lymph:plasma ratio, which indicates that GLP-1 is highly concentrated in the lymph compartment. Moreover, the data indicated that GLP-1 enters the lymphatic system directly rather than by capillary filtration. If the latter were correct then the lymphatic
Concurrent with their rationale, the data suggested that the high lymph:plasma ratio may be due to the close proximity of the lacteal to the basal end of the GLP-1-secreting L cells. To further test this idea, the concentration of PYY in intestinal lymph and peripheral plasma was measured.

PYY is a member of the PP-fold family, which includes pancreatic polypeptide (PP) and neuropeptide Y (NPY). Like GLP-1, PYY is also secreted from the enteroendocrine L cells following meal consumption (182). If the elevated lymph:plasma ratio for GLP-1 is only due to the proximity of the endocrine cell to the lymphatic capillary, then the concentration of PYY should also be higher in intestinal lymph than in peripheral plasma. Although postprandial lymphatic PYY concentrations were higher than plasma levels, the lymphatic concentrations were generally less than twice as large (36). The secretion profile of PYY is in between that of insulin and GLP-1, suggesting that the anatomical location of secretory cells to the lymphatic capillaries is not the reason for the elevated lymph:plasma GLP-1 ratio per se. Rather, D’Alessio et al. (36) argue that the higher lymphatic concentration of GLP-1 indicates targeted secretion of the hormone into the intestinal lymphatic system. Although the lymphatic system may not be the major route of transport for GLP-1 into the circulation, the higher lymphatic concentrations raise the possibility that GLP-1 may have specific physiological effects mediated in this compartment.

In a similar study, Lu et al. (100) compared the intestinal lymph and portal plasma concentrations of GIP following a duodenal mixed nutrient challenge. Both lymphatic and plasma GIP concentrations increased after the duodenal nutrient bolus; however, similar to GLP-1, the lymphatic GIP concentration was significantly higher than the portal plasma concentration. The dynamics of lymphatic and plasma GIP secretion were similar; however, GIP was more concentrated in the intestinal lymphatics than in the hepatic portal vein, which is presumably the area of circulation where the levels of GIP are the highest.
Less dilution by the fluid compartment and the apparent targeting of GIP and GLP-1 to the intestinal lymphatics results in the higher lymphatic concentration of the incretin hormones. The hormones also undergo less degradation by DPP-IV in the lymph. The activity of the incretin degrading enzyme DPP-IV was assessed in lymph and plasma following either an intraduodenal continuous lipid infusion (36) or an intraduodenal mixed nutrient bolus (99). In both studies the DPP-IV activity was significantly higher in plasma than in lymph. The data adds another piece to the puzzle: the concentration of the incretin hormones are higher in lymph not only because of less dilution and apparent targeting to the intestinal lacteals but also because of less degradation by DPP-IV.

5.2 - Using the Lymph Fistula Rat Model to Study the Secretion of GLP-1 and GIP to Lipid and Carbohydrate

From the data, both D’Alessio et al. (36) and Lu et al. (100) concluded that the conscious lymph fistula rat model is a novel, alternative method to study the secretion of both GIP and GLP-1. Utilizing the lymph fistula rat model provides increased sensitivity for detecting changes in response to various stimuli, e.g. nutrients. Accordingly, Lu et al. (99, 100) used the model to characterize the secretion of GLP-1 and GIP to individual and combination of nutrients. In both studies, lymph fistula rats were provided with isocaloric and isovolumetric boluses of either lipid or carbohydrate. A third group of animals was provided an isocaloric, isovolumetric combined nutrient bolus.

The individual nutrient doses, as well as the combined nutrient dose, stimulated intestinal lymphatic secretion of GLP-1 (99). Despite peaking at different times, lipid and carbohydrate induced comparable increases in GLP-1 secretion. The authors state that the carbohydrate bolus was thicker and more viscous than the lipid bolus, and, thus, argue that the delay in dextrin-induced GLP-1 secretion was due to the differences in the physical form of the two nutrient boluses. The combination of nutrients induced a peak in GLP-1 secretion that was additive, compared to the individual lipid- and carbohydrate-induced peaks.
Similar to GLP-1, the individual and the combined nutrient doses stimulated GIP release (100). The GIP peak produced by carbohydrate was twice as large as that produced by lipid. Interestingly, and unlike GLP-1, the combination of nutrients elicited a peak in GIP secretion that was significantly larger than the sum of the lipid and carbohydrate peak, suggesting that products of lipid and carbohydrate digestion are synergistic in stimulating GIP secretion from the enteroendocrine K cells.

In summary, the lymph fistula rat is an alternative model to study the regulation and the secretion of GIP and GLP-1. The concentrations of both hormones are approximately 3 to 6 fold higher in lymph than in peripheral or portal plasma. The elevated concentrations are due in part to the reduced degradation by DPP-IV and also due to less dilution by the circulating fluid; not only does lymph have a smaller fluid pool size than blood but the flow rate of lymph is also slower. Additionally, this model allows the continuous collection of lymph from conscious animals, eliminating any potential side effects on lymph flow and gastrointestinal function due to anesthesia. The volume of fluid collected from the animals is sufficient to perform a number of assays beyond the measurement of GIP and GLP-1, thus removing the restrictive factor of blood volume. Furthermore, additional collecting cannulae can be surgically inserted to simultaneously sample pancreatic juices, bile, and portal/peripheral blood. Most importantly, the concentration in the intestinal lymph provides a more accurate representation of the local milieu within the intestinal mucosa where the hormones may interact with other enteroendocrine cells and signal through the enteric or autonomic neurons. As the majority of both incretin hormones have been degraded (up to 80% of the initial secreted output) before entering the peripheral circulation, it is plausible to suspect that the greater part of GIP and GLP-1 action is not effected via traditional endocrine signaling but rather through stimulation of neurons innervating the hepatic portal vein and the intestinal mucosa. Indeed, recent findings have demonstrated the presence of the GLP-1 receptor on enteric neurons and signaling through these neurons regulates intestinal motility (4); therefore, it is possible that signaling
through the GLP-1 receptors on these enteric neurons may play important roles in food intake and glucose sensing.

6 - Obesity, Diabetes, and Incretins

The prevalence of obesity in the United States and the global landscape is rapidly rising. Obesity and its associated co-morbidities, e.g. type 2 diabetes mellitus, cardiovascular disease, and non-alcoholic fatty liver disease, threaten to replace undernutrition and infectious disease as the most significant contributors to ill health in the developing world. Of the mentioned co-morbidities, type 2 diabetes is characterized by a dysregulation in insulin sensitivity and glucose homeostasis. Several factors are involved in regulating insulin secretion and sensitivity; of growing importance is the role of the incretin hormones. As mentioned previously, the primary function of both GIP and GLP-1 is to enhance insulin secretion following meal ingestion. GLP-1 additionally regulates glucose homeostasis by regulating food intake and decreasing gastrointestinal motility; both functions decrease the amount of nutrients delivered to the circulation and peripheral tissues. Although it plays a prominent role in stimulating insulin release, signaling through the GIP receptor also promotes the expansion of adipose tissue. GIP signaling leads to stimulation of lipoprotein lipase activity and an increase in fatty acid and triglyceride synthesis.

Given its role in glucose homeostasis and adipocyte development, it is not surprising that impairments in the incretin system have been reported in patients with type 2 diabetes. Impairments in the incretin response have been well-documented in individuals with type 2 diabetes. Several studies (119, 169, 172) have reported decreased postprandial GLP-1 secretion. In contrast, GIP secretion from enteroendocrine cells in type 2 diabetic patients is normal or slightly elevated. These patients do however present with impaired insulinotropic effects of GIP at the pancreatic β-cells (40, 116, 119). The attenuated GLP-1 secretion and compromised GIP function contribute to the pathology of type 2 diabetes. Because the glucoregulatory properties of GLP-1 are still functional in insulin resistant individuals unlike that of GIP,
therapeutic strategies have focused on the development of GLP-1 receptor agonists. Exenatide, the only approved GLP-1 receptor agonist, is a synthetic form of exendin-4, a 39-amino acid peptide originally isolated from the venom of the *Heloderma suspectum* lizard (Gila monster) (49). Exendin-4 is a potent agonist of the GLP-1 receptor and is not susceptible to DPP-IV degradation.

Despite the well-documented relationship between type 2 diabetes and the impairments in the incretin system, the dynamics of postprandial incretin secretion in diet-induced obesity are not clearly defined. GIP secretion is either increased (53, 174) or unaffected (131, 132, 171) and GLP-1 secretion appears to be decreased (1, 51, 53, 131, 174) or unchanged (101, 131, 132, 163, 171) in obese, non-diabetic individuals. The reasons for these variations are complex and may be partly due to the wide array of immunoassays and test meals used in the studies. There is also no strong evidence to suggest that the degree of obesity influences the level of incretin secretion impairment. Additionally, the nutritional status of the subjects prior to experimentation is rarely stated; therefore, it is difficult to determine if prior eating habits have any potential effects on incretin secretion. From this data, it is currently unclear if, and how, incretin secretion is affected by obesity. Furthermore, it is unclear if the alterations are caused by obesity or the high-energy diet that often induces obesity.

Along these lines, Bailey *et al.* (7) challenged obese hyperglycemic (ob/ob) mice with either a high-fat (HF), high-carbohydrate (HC), or chow diet for 8 weeks. Compared to chow-fed lean mice, the ob/ob mice had a higher body weight and were hyperphagic, hyperglycemic, and hyperinsulinemic. The chow-fed obese mice also had significantly higher plasma GIP levels, which was attributed to increased intestinal GIP content. Within the ob/ob groups, animals challenged with the HF diet had elevated plasma GIP levels compared to ob/ob mice fed the HC or chow diets. Both high energy diets (HF and HC) induced K cell hyperplasia above that of the chow-fed group; however, the HF diet additionally increased intestinal GIP concentration and content, which resulted in the elevated plasma GIP levels. While this data suggests that obesity may be having an effect on GIP production and secretion (chow-fed
lean vs. ob/ob animals), it also indicates that the particular diet consumed can further exacerbate the hypersecretion of GIP. Gniuli et al. (61) recently reported that 30-day feeding of a diet rich in saturated fat to male Wistar rats significantly elevated GIP levels following an oral glucose challenge. In a follow-up study (60), the authors report that HF-feeding stimulates duodenal proliferation of endocrine cells which differentiate into GIP-secreting K cells; although the study was unable to establish a causal relationship, the authors argue that the high-fat diet elevates circulating GIP levels, which precedes the development of obesity. The authors also report significantly attenuated plasma GLP-1 levels in HF-fed animals; however, in this case, the authors suggest that the alteration was not due to a direct effect of the diet but to a consequence (hyperinsulinemia) of obesity (this is merely speculation and was not supported by study data).

Regardless of the lack of agreement surrounding the secretion of incretins in the obese state, many investigators have tried to elucidate the cause of the impaired incretin response in the development of obesity. Because GLP-1 has been shown to be an important regulator of satiety and food intake, it has been argued that decreased levels of circulating GLP-1 enhance excess body weight gain. However, mice lacking expression of the GLP-1 receptor (GLP-1R) do not show evidence of abnormal body weight nor feeding patterns despite exhibiting increased blood glucose levels and decreased circulating insulin concentrations (152). In a similar study, GLP-1R and leptin deficient mice did not display increased body weight or total food intake compared with ob/ob mice with a functional GLP-1 receptor (153). Obesity-associated leptin resistance has also been implicated as a key factor in the attenuated secretion of GLP-1. Leptin receptors are found on rodent and human intestinal L cells, and leptin can stimulate GLP-1 release in three commonly used L cell models (FRICs, murine GLUTag cells, and human NCI-H716 cells). Anini and Brubaker (5) have also shown that intraperitoneal injections of leptin in ob/ob mice increase GLP-1 secretion. As mentioned previously, both hyperinsulinemia and hyperglycemia may negatively affect GLP-1 secretion. Lim et al. (95) demonstrated that acute insulin treatment has a direct stimulatory effect on GLP-1-secreting L cells and that insulin resistance due to hyperinsulinemia significantly
impaired GLP-1 release in vitro and in vivo. GLP-1 secretion is also attenuated by hyperglycemia (175). The mechanism underlying this reduction is not clear; however, the authors suggest that the effect may be partly attributed to direct negative effects of circulating glucose on the L cell and to a deceleration in gastric emptying.

Others have associated GIP signaling with the etiology of obesity. Mice with a null mutation in the GIP receptor gene are protected from obesity and insulin resistance when placed on a high-fat diet. Additionally, ob/ob:GIPR−/− mice gain less weight and have less adiposity than ob/ob controls (108). Disrupting the GIP signal via a GIP vaccination (55) or targeted ablation of the GIP-secreting K cells (3) also ameliorates the obese condition in HF-fed mice. Zhou et al. (184) demonstrated that under high-fat conditions, GIP signaling in adipocytes is required for effective accumulation of nutrients, and without the GIP receptor, there is a switch from fat storage to fat oxidation. It has also been argued that obese individuals have increased DPP-IV activity, which would ultimately decrease the circulating levels of active GIP and GLP-1. Morbidly obese individuals have increased DPP-IV concentrations before biliopancreatic diversion (101), and DPP-IV deficient mice are protected against obesity and insulin resistance (28).

Despite this knowledge, the following questions remain to be answered: How does obesity affect incretin secretion? Does impaired incretin secretion precede obesity or is altered secretion a consequence of obesity? Is the impaired secretion a primary genetic defect or a secondary effect from consuming a high-energy diet? These questions will be addressed in this dissertation.
AIMS OF THE PRESENT INVESTIGATION

Obesity is an expanding global health problem. It is estimated worldwide that 1.6 billion adults are overweight (body mass index [BMI] > 25 kg/m²) and 400 million additional adults are obese (BMI > 30 kg/m²). Between the years 1980 and 2004, the prevalence of obesity in the US has increased from 15 to 33% in adults and from 6 to 19% in children (122). Obesity is associated with several co-morbidities (71); many of these conditions are mediated through insulin resistance and glucose intolerance, which can be partially attributed to an altered incretin response. The two incretin hormones GIP and GLP-1 enhance postprandial insulin secretion. Ingestion of nutrients strongly promote the release of both GIP and GLP-1 from the enteroendocrine cells K and L cells, respectively; however, the mechanisms underlying these processes are largely unknown. Additionally, the relationship between diet-induced obesity and the postprandial release of GIP and GLP-1 has not been clearly demonstrated. It is unclear if the alterations are caused by obesity or the consumption of a high-energy diet that often induces obesity. To gain a better understanding of the etiology and thus treatment of the defects in the incretin response in obese individuals, it is important to first understand the physiology of how the secretion of incretins is regulated by various macronutrients and how this process is altered following the chronic feeding of a high-fat diet.

The aims of this dissertation were thus to investigate the effects of acute nutrient stimulation and chronic high-fat feeding on the secretion of GIP and GLP-1 using the lymph fistula rat model. We hypothesized that there would be a dose dependent effect on incretin secretion in response to lipid, carbohydrate, and protein and that chronic feeding of a high-fat diet would result in aberrant incretin secretion. As discussed in the Introduction, we chose to use the lymph fistula model to measure incretin secretion for several reasons. First, the concentrations of both incretin hormones are higher in intestinal lymph than in plasma. Second, the model allows for the continuous collection of fluid from conscious animals. Third, the lymphatic incretin concentrations more accurately represent the concentrations within the intestinal mucosa where the hormones may interact with other cells or neurons.
**Aim 1:** To identify the relationships between increasing amounts and different types of dietary macronutrients on the secretion of GIP and GLP-1 using the lymph fistula rat model (I and II).

The primary stimulus for the secretion of the incretin hormones is the ingestion of nutrients. However, the dose-response relationships between the amount and type of ingested macronutrient and the secretion of incretins are not well-defined. Previous reports investigating nutrient-stimulated incretin secretion have examined either individual macronutrients or mixed meals over a limited range of doses. Using the lymph fistula rat model, we examined if increasing duodenal doses (0.275, 0.55, 1.1, 2.2, and 4.4 kcal) of lipid, carbohydrate, and protein had dose-dependent effects on the secretion of GIP and GLP-1. We further determined if the amount of incretin secretion varied depending on the type of macronutrient and if there was a difference between GIP and GLP-1 secretion for each individual nutrient.

**Aim 2:** To investigate the effects of chronic high-fat feeding on incretin secretion using the lymph fistula rat model and to further determine if these effects are due to increased consumption of calories or to the increased percentage of fat in the diet (III).

The incretin hormones, GIP and GLP-1, enhance postprandial insulin secretion, promote adipogenesis, and regulate gastrointestinal motility and food intake. To date, a consensus on how the incretin response is altered in obesity is lacking. We investigated the effects of chronic high-fat-feeding on incretin secretion in the lymph fistula rat model. Rats were provided a high-fat (HF) or low-fat (LF) diet *ad libitum* for 3 or 13 weeks; a high-fat pair-fed (HF-PF) group was included as a control during the 3-week feeding trial. Energy intake, body weight, and body composition were regularly monitored. At the culmination of the feeding period, all animals were challenged with a duodenal mixed meal to measure lymphatic incretin secretion.
EXPERIMENTAL FINDINGS AND DISCUSSION

The following section summarizes the results from the three papers located at the end of this dissertation and discusses the implications of those results within the field of incretin biology. The papers will be referred to by their roman numerals (see Copyright Notice for more details). Please consult these papers for more detailed information regarding methodology and results.

1 – The Effect of Acute Lipid, Carbohydrate, and Protein Meals on Incretin Secretion

As discussed in the Introduction, the incretin hormones GIP and GLP-1 have been reported to respond to a variety of nutrients, either individually or in combination. However, it is unclear if there is a dose-dependent response between the amount of nutrient ingested and the secretion of the hormones in vivo. In this dissertation, we have demonstrated using the lymph fistula rat model that both GIP and GLP-1 respond dose dependently to increasing doses of dietary lipid (I) and carbohydrate (II); however, increasing doses of protein had no effect on the secretion of either hormone (II).

To investigate the effects of increasing amounts and different types of dietary macronutrients on incretin secretion, we infused lipid, carbohydrate, or protein as individual nutrient boluses into male Sprague-Dawley rats that had been surgically outfitted with a duodenal feeding tube. Additionally, the mesenteric lymphatic duct of these animals was cannulated to allow for the collection of intestinal lymph. Previously (and as discussed in the Introduction), we reported that the concentration of both incretin hormones is higher in intestinal lymph than in peripheral or portal plasma (36, 99, 100). The lymph fistula rat model provides increased sensitivity for detecting changes in response to various stimuli, such as nutrients; thus, for this reason (and others mentioned in the Introduction), we have chosen to use this model to study the incretin changes in response to acute nutrient stimuli.
Following an overnight recovery, the rats were infused with a 3-ml bolus of normal saline or increasing caloric amounts of lipid (Liposyn II 20%), carbohydrate (dextrin), or protein (whey protein); the doses ranged from 0.275 kcal to 4.4 kcal. Liposyn II 20% is an intravenous fat emulsion that contains a 50:50 blend of safflower and soybean oil; dextrin is a glucose polymer; and whey protein is a mixture of globular proteins isolated from whey (a by-product of cheese production). Liposyn II 20%, dextrin, and whey protein were chosen as the initial infusates because they each required additional intestinal digestive steps prior to absorption (i.e. hydrolysis of triglycerides to fatty acids, dextrin to glucose, and protein to either amino acids or small peptides). Furthermore, we had successfully demonstrated GIP and GLP-1 secretion in response to 4.4 kcal duodenal boluses of Liposyn II 20% or dextrin (99, 100). We chose whey as our initial protein source because previous reports had documented incretin secretion following whey-supplemented meals (52, 170); one study, in particular, used whey protein from the same source as our own study and had demonstrated enhanced incretin secretion following whey-supplementation (66).

In this dissertation, we have demonstrated that both GIP and GLP-1 respond dose dependently to increasing doses of lipid (I) and carbohydrate (II). Consistent with our present findings, α-linolenic (73), oleic (75), and palmitoleic (144) acids have all been found to have a dose-dependent stimulatory effect on GLP-1 secretion in vitro. Glucose also stimulates dose-dependent incretin secretion from the GLUTag cell line (64, 133) and primary K (124) and L (134) cells. Additionally, GLP-2, a hormone produced in the L cell and co-secreted with GLP-1, is released in a calorically-dependent manner following either a lipid or carbohydrate challenge in vivo (183). In regards to a mixed nutrient meal, Vilsbøll et al. (174) reported that both GIP and GLP-1 secretion was higher in lean humans following a larger meal compared to an identical meal of smaller caloric value. These studies, in conjunction with our results, strongly support the model that the magnitude of incretin secretion is dependent on the intestinal caloric load.
In spite of observing dose-dependent trends for both lipid- and carbohydrate-induced incretin release, we did not detect whey protein-induced GIP and GLP-1 secretion for any of the five doses that was significantly different from saline or from each other (II). Similarly, GLP-2 did not respond to a protein-only meal, despite having calorically-dependent response to both carbohydrate and lipid (183). However, peptides (29) and amino acids (135) stimulate GLP-1 secretion dose dependently from STC-1 and GLUTag cells, respectively. Moreover, several studies have demonstrated successful in vivo incretin release following a protein-only meal (24, 63, 80). In these studies, the protein meals were ingested orally, whereas we provided our protein doses as bolus meals via an intraduodenal feeding tube. Although we chose a protein source (whey) that was easily digested and absorbed (14, 38), it is possible that bypassing the stomach decreased the amount of protein hydrolysis and subsequently reduced the amount of peptide and amino acids available for intestinal absorption and subsequent incretin secretion. To test this hypothesis, we infused increasing amounts (ranging from 0.275 to 4.4 kcal) of casein hydrolysate into the duodenum of rats and analyzed the lymphatic incretin secretion (II). We were unable to obtain whey protein hydrolysate from a reputable source; therefore, we opted to use casein as our protein hydrolysate. Whey protein and casein are both derived from milk, and although casein is considered a ‘slow’ protein in regards to digestion and absorption (14, 38), it has been enzymatically hydrolyzed, thus bypassing any concern of its ‘slow’ protein characteristics. Again, we were unable to detect incretin secretion that was greater than saline for any of the doses tested. Additionally, the incretin secretion produced by the hydrolysate doses was not greater than that produced by whey protein doses of equal caloric value. In a recent study, Dailey et al. (35) continuously infused glucose, casein hydrolysate, or linoleic acid into the jejunum of male Sprague-Dawley rats for 7 h/day for five consecutive days. At the end of the five day study period, the authors detected significantly elevated GLP-1 plasma levels in the animals that received the glucose or the linoleic acid infusions but not the casein hydrolysate treatment.
Our results (II) and the study by Dailey et al. (35) indicate that protein is not a potent secretagogue; however, previous studies (discussed in the Introduction) suggest otherwise. When comparing the latter studies to the former, three key differences are apparent: 1) Intestinal versus oral delivery of nutrients, 2) Individual nutrient challenge versus mixed nutrient challenge, and 3) In vitro versus in vivo models.

We originally suspected that bypassing the stomach impaired the digestion and absorption of the whey protein bolus, which subsequently resulted in decreased incretin secretion. Previous reports of protein-induced incretin secretion had provided the nutrients via an oral route rather than an intestinal route, as in our study and the study by Dailey et al. However, even when the protein was provided in a digested form (i.e. casein hydrolysate) via the duodenum (II) or the jejunum (35), incretin secretion was insignificant. An exception to this observation is the work published by Hira et al. (72). Here, ileal infusions of zein hydrolysate (derived from corn) stimulated GLP-1 secretion compared to control (water) or a glucose infusion (72); this would suggest that hydrolyzed protein infused directly into the intestine is sufficient to stimulate GLP-1 secretion. However, not all protein hydrolysates were able to induce GLP-1 release; in a subsequent study (109), only zein hydrolysate (compared to meat hydrolysate) was capable of stimulating secretion of GLP-1. These results, alongside those presented earlier, suggest that incretin release may be dependent on the type of protein ingested and/or the stomach may have a role in regulating the release of the incretin hormones. Further investigations are needed to clarify these issues.

Supplementing mixed meals with protein, rather than providing protein as an individual macronutrient, is another difference between our study and previous reports. Although ingesting protein solely has been documented to induce incretin release, the majority of studies employing whey as the protein source have used it in combination with other macronutrients (52, 66, 170). It is possible that whey protein alone does not provide a sufficient stimulus to induce GIP and GLP-1 release; however, in combination with other nutrients, it may amplify the signal generated by carbohydrate and/or lipid. As mentioned in the Introduction, Lu et al. (100) reported that the combination of lipid and carbohydrate had a greater GIP
response than either nutrient alone, indeed suggesting a potentiation effect when nutrients are combined. If and how this occurs with protein is an interesting future research question.

Finally, the model employed to investigate protein-induced incretin secretion may complicate interpretation of the results. Work aimed at understanding the mechanisms behind protein-induced incretin release has been accomplished using in vitro models. As discussed in the Introduction, cell lines are the best cell culture model currently available, since the development of a primary cell culture system has been plagued with difficulties. Although two of the three GLP-1-secreting cell lines (GLUTag and NCI-H716) release the hormone in response to either amino acids or peptides (29, 135), no results have been published using the primary K and L cells models developed by Parker et al. (124) and Reimann et al. (134), respectively; thus it is unclear if the cell lines respond similarly to primary enteroendocrine cells or in vivo systems.

In summary, this dissertation has demonstrated that both GIP and GLP-1 respond dose dependently to increasing amounts of either acute carbohydrate or lipid (I and II) in chow-fed Sprague Dawley rats. However, there was minimal incretin response to dietary protein (II). This lack of response is not due to incomplete nutrient digestion, as a protein hydrolysate also failed to simulate incretin secretion. Further work is needed to determine the role of dietary protein in the regulation of incretin secretion.

2 - Differential Secretory Responses of GIP and GLP-1 to Lipid and Carbohydrate

Although both GIP and GLP-1 respond dose dependently to increasing amounts of dietary lipid and carbohydrate, the sensitivities of the hormones to each macronutrient is different. In this dissertation, we have demonstrated that the GLP-1-secreting L cells are more sensitive than the GIP-secreting K cells to changes in intraluminal lipid content (I), whereas the GIP-secreting K cells are more responsive than the GLP-1-secreting L cells to increases in intestinal carbohydrate content (II). After normalizing the data to
saline levels, we plotted the 3-h cumulative GIP and GLP-1 outputs as a function of the infused nutrient dose. The slopes of the generated best-fit lines correspond to the amount of incretin secreted per infused calorie of nutrient during the 3-h study period. By comparing the slopes of each best-fit line, we can determine which hormone is more responsive to each macronutrient. Figure 1 displays a graphical summary of the results from these studies.

The best-fit lines generated from the lipid data produced slopes of 0.474 for GIP and 0.657 for GLP-1 (I). On the other hand, the slopes of the best-fit lines generated from the carbohydrate data were 1.634 for GIP and 0.611 for GLP-1 (II). The steeper slope for the GLP-1 lipid data suggests that the GLP-1-secreting L cells are more sensitive to changes in the intestinal lipid content, whereas the larger slope for the GIP carbohydrate data indicates that the GIP-secreting K cells are more responsive to increasing amounts of infused dietary carbohydrate. In previous studies using only one dose of carbohydrate or lipid, we observed a similar trend (99, 100). In response to a 4.4 kcal lipid bolus, we found that the peak stimulation for GIP was 4 fold greater than fasting levels versus 10 fold higher than fasting levels for GLP-1. Then, when animals were challenged with a 4.4 kcal bolus of carbohydrate, the peak stimulation for GIP was 11 fold greater than fasting levels versus 9 fold greater than fasting levels for GLP-1. This data, along with the results from this dissertation, support the conclusion that the K cells are more sensitive than the L cells when challenged with carbohydrate; whereas, the L cells respond more than the K cells when challenged with lipid.

One concern we had during the course of study was that different portions of intestine were not being exposed to the same nutrient bolus, i.e. the distal gut would be exposed to a different nutrient concentration and composition than the proximal small intestine. We chose to use a duodenal infusion in the current studies to increase the likelihood that nutrients would reach the distal portion of the intestine. Although we did not track the progress of the lipid bolus, previous studies (17, 147) have detected radiolabeled lipid in the ileum within 15 min following an intragastric dose of triolein as small as 10 mg
Cumulative lymphatic GIP (A) and GLP-1 (B) output plotted as a function of nutrient dose. Five lipid (●), five carbohydrate (□), and five whey protein (○) doses were tested (0.275, 0.55, 1.1, 2.2, 4.4 kcal for each nutrient). Values (fold amounts above saline) are means + SE. The best-fit lines generated from the lipid data produced slopes of 0.474 for GIP and 0.657 for GLP-1. The slopes of the best-fit lines generated from the carbohydrate data were 1.634 for GIP and 0.611 for GLP-1.
(0.9 kcal) and a 1 kcal intragastric dose of Intralipid; therefore, we presume that our intraduodenal doses of lipid would reach the ileum in the same or less amount of time.

We were more concerned about inadequate nutrient exposure in the distal small intestine when interpreting the carbohydrate results, as this could be one possible explanation for the smaller GLP-1 response. However, the GIP response was still greater than the GLP-1 response when carbohydrate was delivered directly into the distal small intestine (Slope of best-fit lines: GIP – 0.752 vs. GLP-1 – 0.559) (II). Additionally, the slopes for ileal and duodenal GLP-1 data (0.559 vs. 0.526) are almost identical, suggesting that the L cells are equally responsive to either ileal or duodenal carbohydrate; therefore, the distally-located L cells are sufficiently exposed to nutrient during the duodenal infusions. The results of the ileal-infusions further support that the K cells are more responsive than the L cells to increasing doses of dietary carbohydrate.

When comparing the GIP or GLP-1 responses to each macronutrient, we observed that carbohydrate is more effective than lipid at stimulating GIP secretion (Slope of best-fit lines: Carbohydrate – 1.634 vs. Lipid – 0.474). The enhanced sensitivity of the GIP-producing K cells to changes in carbohydrate intake, compared to changes in intestinal lipid content, reflects the insulinotropic potential of the infused nutrient. Both glucose and fatty acids are capable of stimulating insulin release (30; 121); however, fatty acids induce moderate insulin secretion and are not considered primary insulin secretagogues (185). When challenged with a lipid-based meal, the need to produce insulin is low, and the added insulinotropic effect of GIP-signaling is not essential. In contrast, when provided with a carbohydrate-based meal, regulating glucose homeostasis is necessary; in this scenario, the enhancement of glucose-stimulated insulin secretion via GIP is advantageous. Then, why does lipid stimulate GIP secretion – if the need for insulin is low, why would a hormone involved in glucose homeostasis be secreted at all? Although the primary function of both incretin hormones is to enhance glucose-dependent insulin secretion, both hormones play additional important physiological roles. GIP enhances adipogenesis by stimulating lipoprotein lipase
activity, enhancing fatty acid synthesis and incorporation into triglycerides, and down-regulating glucagon-stimulated lipolysis (58), all of which promote fat deposition rather than mobilization. Although the need to produce insulin may be low when provided a fat-meal, the lipogenic properties of GIP, however, do promote storage of the ingested lipid in adipocytes.

Whereas carbohydrate appears to the more potent GIP secretagogue, we observed that both carbohydrate and lipid are equally effective at stimulating GLP-1 secretion (Slope of best-fit lines: Carbohydrate – 0.611 vs. Lipid – 0.657). Along with its effects on the pancreas, GLP-1 is involved in regulating gastric emptying and intestinal motility – the ileal brake reflex. The ileal brake is a distal-to-proximal feedback system that slows the intestinal transit of nutrients to aid digestion and absorption (102). Interestingly, Nauck et al. (120) demonstrated that the insulinotropic effects of GLP-1 are outweighed by the hormone’s ability to inhibit gastric emptying. Although we did not measure the effect of different macronutrients on intestinal motility in this dissertation, we propose that carbohydrate and lipid may be contributing equally to the ileal brake reflex via a GLP-1-based mechanism. As nutrients reach the distal portion of the gut, GLP-1 is secreted in a dose-dependent manner to reduce intestinal transit and enhance proximal nutrient absorption. Layer et al. (90) have studied the effects of both carbohydrate and lipid on the ileal brake reflex and reported that these responses may not be specific for particular food components but rather a non-specific effect to the presence of unabsorbed nutrients in the distal small intestine. Further studies are needed to clarify how lipid and carbohydrate specifically contribute to the ileal brake reflex and whether or not GLP-1 is indeed the mediating factor.

In summary, this dissertation has demonstrated in chow-fed Sprague-Dawley rats that a) GIP-secreting K cells are more responsive then GLP-1-secreting L cells to dietary carbohydrate, b) GLP-1-secreting L cells are more sensitive to dietary lipid than GIP-secreting K cells, c) carbohydrate is the more potent GIP secretagogue, and d) lipid and carbohydrate are equally effective at stimulating GLP-1 release (I and II). Together, we propose that the similar GLP-1 responses to lipid and carbohydrate support the hormone’s
role in the ileal brake reflex, whereas the much larger effect of carbohydrate on GIP secretion reflects the insulinotropic potential of the infused nutrient.

3 – The Effect of Chronic High-Fat Feeding on the Secretion of GIP and GLP-1

As alluded to in the Introduction, the role of incretin secretion in obesity is not clearly defined. In obese, non-diabetic individuals, GIP secretion is either increased (51, 174) or unaffected (131, 132, 171), while GLP-1 secretion is decreased (1, 51, 53, 131, 174) or unchanged (101, 131, 132, 163, 171). However, from these studies it is not clear which occurs first, obesity or incretin secretion impairments, which prompts the following questions: Does an obese state lead to impaired incretin secretion or does impaired incretin secretion lead to an obese state? If the latter is more accurate, what causes the primary defect in incretin secretion?

Obesity is associated with hyperleptinemia, hyperinsulinemia, and hyperglycemia, all three of which have been linked to impairments in incretin secretion. Anini and Brubaker (5) demonstrated that GLP-1 secretion was augmented in ob/ob mice that received injections of leptin. Chronic hyperglycemia (175) and hyperinsulinemia (ultimately resulting in insulin resistance) (95) both cause decreased GLP-1 secretion. These data suggest that chronic changes in incretin secretion may be a secondary consequence of obesity, arising after the metabolic defects of obesity have occurred and further exacerbating the obese condition. On the other hand, altered incretin secretion may be a causative agent in the development of obesity. Changes in incretin concentration could lead to perturbed insulin secretion, gastric emptying and intestinal motility, food intake, and adipose tissue growth, all of which would favor the development of obesity. Initial alterations in incretin secretion could be caused by genetic factors or by environmental factors, such as diet. In studies that investigate the effects of obesity on incretin secretion, the nutritional status of the subjects prior to experimentation is rarely stated; therefore, it is difficult to determine if prior
eating habits, such as consumption of a high-energy diet, have any potential effects on incretin secretion. Thus, the relationship between incretin secretion and obesity is unclear.

In I and II, we studied the effects of acute nutrient stimulation on the secretion of GIP and GLP-1. In III, we investigated how chronic nutrient stimulation affected incretin secretion. To accomplish this, we fed male Sprague-Dawley rats either a HF or LF diet for 3 or 13 weeks. Following the designated feeding period, the animals were challenged with a mixed meal [Ensure: 3.125 kcal/animal – 0.075 g fat (21.6%), 0.5 g carbohydrate (64.0%), 0.1125 g protein (14.4%)] following the insertion of an intestinal lymphatic duct cannula to measure incretin secretion. Interestingly, despite demonstrating no signs of obesity beyond hyperphagia, both the 3-week and 13-week HF-fed animals had elevated lymphatic GIP and GLP-1 concentrations compared to animals fed a LF diet following an identical mixed meal challenge.

We modeled our first study after that published by Woods et al. (181). We used the same HF and LF diets and also chose 10 weeks as our initial feeding period. Woods et al. (181) reported successful use of this particular diet in inducing obesity in male and female Long-Evans rats. In their study, the HF-fed animals weighed 10% more and had 50% more body fat than the LF-fed group following 10 weeks of HF or LF feeding. By 10 weeks, there was still no difference in body weight and only a small difference in body composition between our HF and LF groups, so we extended the feeding period by an additional 3 weeks. At the end of the 13-week feeding period, the animals underwent the lymph fistula procedure. As previously stated, despite demonstrating no signs of obesity beyond excess food intake, the HF-fed animals had significantly elevated GIP and GLP-1 levels compared to the LF-fed group.

Following the data from our 13-week experiment, we next investigated whether or not a shorter period of HF-feeding could induce similar changes in incretin secretion. Several studies have observed significant changes in intestinal morphology and function following only 2-4 weeks of high-fat feeding (94); we thus chose an intermediate feeding period of 3 weeks. Similar to the data from the 13-week feeding period,
the only difference between 3-week HF-fed animals and LF-fed animals was increased energy intake. Following a duodenal mixed meal challenge, the 3-week HF-fed animals once again had significantly increased concentrations of lymphatic GIP and GLP-1.

We were surprised that there was no difference in body weight and only a modest difference in percent body fat between the two feeding groups, despite the consumption of excess energy by the HF group for up to 13 weeks. We also observed no difference in energy expenditure (measured at 8 weeks following initiation of diet study), oral glucose tolerance tests, insulin tolerance tests, or fasting insulin and leptin levels between the 13-week-fed HF and LF groups. Using the same diet and feeding protocol, Woods et al. (181) successfully induced obesity in rats following 10 weeks of HF-feeding. The primary difference between the two studies is the use of Sprague-Dawley rats in our study over Long Evans rats in the work by Woods et al. (181). The segregation of Sprague-Dawley animals into diet-induced obesity prone and diet-induced obesity resistant groups has been reported previously (25, 92, 93, 140). Individual weight curves, however, do not suggest the stratification of our HF-fed animals into two distinct groups. Further investigations are needed to understand the mechanism behind the resistance to diet-induced obesity in these animals. Regardless, the animal model allowed us to eliminate obesity and any secondary consequences of obesity, such as hyperleptinemia, hyperinsulinemia, and hyperglycemia, as potential mechanisms underlying the elevated GIP and GLP-1 concentrations. The results from III suggest that alterations in incretin secretion may occur prior to the development of obesity. Thus, the elevated levels of GIP and GLP-1 are consequences of either hyperphagia (increased energy intake) or the consumption of a HF diet (increased percentage of dietary fat) and not due to obesity.

We acknowledge that stating the elevated incretin levels may lead to obesity is premature using our current data. Our animals did not develop an obese phenotype, so therefore we cannot conclude that the increased GIP and GLP-1 levels would eventually lead to obesity. Further studies are needed in either a
diet-induced susceptible rat strain or in Sprague-Dawley animals fed the HF diet for longer than 13 weeks to verify this statement.

4 – Differential Responses of GIP and GLP-1 to Aspects of a High-Fat Diet

The chronic feeding of a HF diet for as little as 3 weeks resulted in elevated levels of GIP and GLP-1 secretion following a duodenal mixed meal challenge. As previously discussed, the animals did not become obese despite consuming more energy per day than the corresponding LF group. Although surprising, the absence of a weight difference between the two groups allowed us to determine the effect of the diet alone on incretin secretion without obesity or weight gain as a confounding factor. The HF-fed animals not only consumed a diet richer in fat than the LF group, they also consumed more calories per day. To clarify if the alterations in incretin secretion were due to the increased energy intake or the increased dietary fat percentage, we included a high-fat pair-fed (HF-PF) group during the 3-week feeding study (III). The HF-PF animals received the same diet as the HF-fed animals, but they were restricted to the amount of calories consumed ad libitum by the LF group. At the end of the 3-week feeding period, there was no difference in body weight between the HF-PF group and the HF or LF group and only a small difference in percent body fat between the HF-PF and the LF groups. The HF-PF animals were also challenged with a duodenal mixed meal [Ensure: 3.125 kcal/animal – 0.075 g fat (21.6%), 0.5 g carbohydrate (64.0%), 0.1125 g protein (14.4%)] to analyze their lymphatic incretin secretion. Interestingly, the GIP secretion profile for the HF-PF group more closely aligned with the HF group, whereas the HF-PF and LF groups produced similar GLP-1 secretory responses. The data suggested that, following the consumption of a HF diet, the increased GIP secretion is driven by the greater percentage of fat in the diet, while the increased GLP-1 secretion is driven by the excess caloric intake.
The intestine adapts during the consumption of a HF diet to accommodate the digestion and absorption of the larger lipid load. Intestinal transit is increased, and small intestinal morphology and function is modified (94, 150). Singh et al. (155) observed elevated levels of jejunal and ileal mucosal enzymes involved in lipid absorption and a subsequent increased uptake of oleic acid following 4 weeks on a chow diet supplemented with 20% lard. Data from Balint et al. (9) suggest that this increase in oleic acid uptake is additionally due to cellular hypertrophy in the ileum. Is it possible that the enteroendocrine cells are also undergoing adaptations following chronic HF feeding? Along these lines, ob/ob mice challenged with a HF diet had elevated plasma GIP levels compared to ob/ob mice fed a high carbohydrate (HC) or chow diet (7). Both high energy diets (HF and HC) induced K cell hyperplasia; however, the HF diet additionally increased intestinal GIP concentration and content, which resulted in the elevated plasma GIP levels. Whether or not the ob/ob mice had a stronger propensity for K cell hyperplasia than the lean mice following a chronic high-energy diet was never addressed in the study, thus making the results difficult to interpret for diet-induced obese animals without genetic disruptions. Regardless, the data do suggest that consumption of a HF diet increases production and secretion of GIP. Gniuli et al. (61) recently reported that 30-day feeding of a diet rich in saturated fat to male Wistar rats significantly elevated GIP levels following an oral glucose challenge; in a follow-up study, the authors report that HF-feeding stimulates duodenal proliferation of endocrine cells which differentiate into GIP-secreting K cells (60). However, GIP secretion was reduced below pre-diet levels after 90 days on the HF diet due to increased apoptosis in the duodenal epithelium (61). We did not observe a drastic reduction in GIP secretion following 13 weeks (compared to 3 weeks) on a HF diet. Differences in our results versus those reported by Gniuli et al. (60, 61) are not due to the type of fat in the diet, since palmitic acid is the primary fatty acid in both HF diets. However, the strain of rat (Wistar vs. Sprague-Dawley) used in the two studies may be a contributing factor to the conflicting results. Gniuli et al. (60, 61) observed a significant increase in the body weight of the HF-fed Wistar rats compared to the control group, whereas our HF-fed Sprague-Dawley animals weighed the same as our LF-fed control group at the end of the study. The greater susceptibility of Wistar rats to diet-induced obesity (22) may have accelerated the lipotoxic effects of the
HF diet on the intestinal epithelium. Regardless, the data do suggest that consumption of a HF diet increases production and secretion of GIP. These studies, in combination with our data, demonstrate that consumption of a HF diet results in elevated GIP levels. Furthermore, the fat in the diet may be elevating GIP levels via K cell hyperplasia and increasing intestinal GIP concentration and content.

Unlike the GIP data, the consumption of a diet higher in fat alone does not explain the elevated GLP-1 levels. The HF-PF group did not have increased lymphatic GLP-1 concentrations despite consuming a diet with a higher fat content, suggesting that the increased energy intake of the HF group is causing the rise in GLP-1 secretion. Although difficult to explain at this time, given the complexity of the GLP-1 secretory system, this observation is of interest. The excess caloric intake could be causing hypertrophy and/or hyperplasia of the distal small intestine epithelium, sensitizing the GLP-1-secreting L cells to nutrient stimuli, and/or altering the endocrine and neural signaling on the L cell. Future studies are needed to understand the mechanism behind the hyperphagia-induced elevated GLP-1 secretion.

Since increased energy intake is driving the elevated GLP-1 secretion following the consumption of a HF diet, is it possible that excess consumption of any diet would also result in elevated GLP-1 secretion, thus making this a nutrient-independent effect? Given this thought, it is tempting to speculate that there is a connection between these data and the data presented in Discussion Point 2 (Lipid and carbohydrate are equally potent GLP-1 secretagogues). Is it possible that in a setting of acute nutrient stimulation, similar machinery is being employed by lipid and carbohydrate to induce GLP-1 release and that this same machinery is being altered when chronically stimulated by a HF (or potentially a HC) diet? As discussed in the Introduction, the mechanisms underlying lipid-induced incretin secretion are not well-established. Because of this, answering the above-posed questions becomes challenging, yet intriguing nonetheless.

In summary, this dissertation has demonstrated that GIP and GLP-1 secretion is increased following chronic consumption of a HF diet for 3 or 13 weeks in Sprague-Dawley rats. Furthermore, the elevated
GIP secretion is driven by the higher percentage of fat in the HF diet (compared to the LF diet), whereas the increased GLP-1 levels are due to the increased consumption of energy. Further studies are needed to understand how these particular aspects of the diet affect incretin production and secretion.
GENERAL SUMMARY AND CONCLUDING REMARKS

In summary, this dissertation has explored aspects of the physiology of incretin secretion. Specifically, we have investigated the effects of acute nutrient stimulation and chronic high-fat (HF) feeding on the release of GIP and GLP-1 in Sprague-Dawley rats using the lymph fistula rat model. The results of this dissertation have led to the following important conclusions:

1. In chow-fed Sprague-Dawley rats, GIP and GLP-1 respond dose dependently to increasing acute amounts of dietary lipid and carbohydrate. However, there was no effect on GIP and GLP-1 secretion to protein doses of equal caloric value. (I and II)

2. In chow-fed Sprague-Dawley rats, carbohydrate is more effective than lipid at stimulating GIP release following an acute nutrient challenge. Under the same conditions, lipid and carbohydrate are equally potent GLP-1 secretagogues. (I and II)

3. Chronic HF-feeding increases lymphatic GIP and GLP-1 secretion following a mixed nutrient challenge in Sprague-Dawley rats, in spite of the fat mass increase and weight gain generally associated with consumption of a HF diet. (III)

4. Following chronic feeding of a HF diet, the increased GIP secretion is driven by the greater percentage of fat intake, whereas the increased GLP-1 secretion is caused by the excess caloric intake. (III)

Together, the findings from this dissertation have added important information to the field of incretin biology. These results may have important bearings on the nutritional and pharmacological regulation of
GIP and GLP-1 secretion. Currently, GLP-1 mimetics and DPP-IV inhibitors are used for the treatment of type 2 diabetes. An additional potential therapeutic avenue is stimulating the release of the incretin hormones at the intestinal level; this would allow individuals to produce and secrete their own hormones, ideally replicating the physiological secretion time course and response to various nutrients. To accomplish this task, a greater understanding of the physiological and cellular mechanisms underlying nutrient-induced incretin secretion is needed. The findings from this dissertation have provided steps in that direction. Furthermore, in order to provide incretin-based treatment to obese and type 2 diabetic patients, it is important to first understand the physiological defects in incretin secretion for both of these conditions. It is unclear in the literature whether alterations in incretin secretion are primary causes or secondary consequences of the disease. In regards to obesity, our findings suggest that mere consumption of a high-fat diet (for as little as 3 weeks) can result in significant changes in incretin secretion following an acute mixed nutrient challenge. Future studies are needed to determine how the HF diet produces these changes and what would be the long-term effects of chronic elevated incretin levels. This dissertation also further verifies the utility of the lymph fistula rat model to study the secretion of GIP and GLP-1. Through our work, we have demonstrated that this model could be used to investigate the changes in gastrointestinal hormones in obesity and type 2 diabetes and potentially following bariatric surgery.
FUTURE DIRECTIONS

In light of the findings from this dissertation, several interesting research questions are revealed. Here, a few of these questions and their corresponding experimental approaches are addressed.

**Question 1: Does dietary protein potentiate the acute incretin response to a lipid or carbohydrate-based meal? If so, what is the mechanism underlying this response?**

In II, we demonstrated that both whey protein and casein hydrolysate were not potent incretin secretagogues; however, as mentioned in the Discussion, other investigators have successfully demonstrated successful GIP and GLP-1 secretion following whey protein-supplemented meals. This suggests that protein by itself is incapable of stimulating substantial incretin secretion, but when in combination with other nutrients, protein may amplify the signal generated by carbohydrate and/or lipid. To test this possibility, lymph fistula rats would be provided isovolumetric doses of carbohydrate, lipid, or protein alone. The incretin responses following these nutrient challenges would be compared to the response following a carbohydrate + protein or lipid + protein bolus. Protein alone has little to no effect on the stimulation of incretin secretion; therefore, if a response greater than that produced for carbohydrate or lipid is generated, then the addition of dietary protein is indeed potentiating the incretin response.

As the mechanisms underlying both carbohydrate and lipid-induced incretin secretion are not clear, the second part of this question becomes more difficult. The protein may be elevating incretin levels by a) increasing production of the hormones, b) stimulating more secretion of the hormones, c) reducing degradation of the hormones once secreted, or d) any combination of the three components. Although the levels of DPP-IV are significantly reduced in the lymph, degradation of the hormones can occur prior to entering the lymph compartment, i.e. in the lamina propria. It is possible that the addition of protein may
inhibit the degradation of both GIP and GLP-1. Gunnarsson et al. (66) demonstrated that supplementation of whey protein to a glucose challenge had no effect on total GIP concentration but did increase intact GIP levels, suggesting a reduction in hormone degradation; they also noted a significant reduction in DPP-IV activity in the proximal small intestine. Therefore, it would be of interest to assess the ratio of intact to total incretin hormone following protein supplementation and to evaluate the DPP-IV activity in the different sections of the small intestine. Although our proposed study is similar to that described by Gunnarsson et al. (66), the authors did not provide isocaloric test meals; thus, it is unclear if the increase in incretin secretion is due to an intrinsic property of the whey protein or merely due to the increased caloric load. To measure incretin production and secretion, the intestinal GIP and GLP-1 mRNA and protein level should also be monitored.

**Question 2: Do impairments in incretin secretion precede obesity?**

In III, we demonstrated that chronic feeding of a HF diet resulted in elevated GIP and GLP-1 secretion. Despite consumption of a high-energy diet for up to 13 weeks, the animals did not increase in body weight or body fat. Together, these data suggest that elevated GIP and GLP-1 levels may occur prior to the development of obesity. As mentioned in the Discussion, we are hesitant to make this claim since our animals did not become obese during the feeding period. To test this hypothesis, the study should be replicated in an obese-prone rat strain, such as Long Evans or Obese-Prone Sprague-Dawley rats. Incretin secretion should be monitored over the course of the high-fat feeding period; characteristics of obesity, such as substantial weight gain, accumulation of body fat, hyperinsulinemia, and hyperleptinemia, should be noted in relation to the changes in incretin secretion. If increased concentrations of GIP and GLP-1 indeed precede an obese phenotype, elevated postprandial incretin levels could be considered a risk factor for the development of obesity.
Question 3: What is the mechanism underlying the elevated GIP secretion following chronic stimulation of dietary fat?

The results from the mixed nutrient challenge in the HF-PF group suggest that the fat component of the HF diet drives the increased GIP secretion observed following chronic consumption of a HF diet. As mentioned in the Discussion, the fat in the diet may be elevating GIP levels via K cell hyperplasia and increasing intestinal GIP concentration and content, as suggested by the studies from Bailey et al. (7) and Gniuli et al. (60, 61). Preliminary studies to measure intestinal incretin content in our 3-week HF- and LF-fed animals were inconclusive. In this experiment, duodenal, jejunal, and ileal segments were collected from three HF-fed and three LF-fed animals following a 4-h fast. Unlike the results from the lymph fistula model, we did not see any difference in either GIP or GLP-1 content between the two feeding groups (Figure 1). It is possible that differences in intestinal content may not be apparent under fasting conditions. It is unclear from the studies by Bailey et al. (7) and Gniuli et al. (60, 61) if the intestines were collected under a fasted or fed state; therefore, it would be constructive to measure intestinal incretin content under both fasting and fed conditions, as well as during the lymph fistula nutrient challenge. In addition to measuring intestinal incretin content, it is also necessary to calculate the number of GIP-positive staining cells within the isolated intestinal segment. Determining if more GIP is made per K cell or if the GIP content per cell is constant and there is merely an increase in the number of K cells is possible with the availability of both sets of data.

The methodology used in the preliminary study would only account for localized hyperplasia – rather than an increase in the number of K cells in the particular section of intestine collected for the study. It is possible that endocrine cell hyperplasia may be occurring over a longer length of the intestine, i.e. in a region that normally does not contain an abundance of GIP-secreting K cells. Therefore, larger portions of the small intestine should be used to make these measurements. If, indeed, there is an increase in length of intestine populated with K cells, increased gastrointestinal transit, as a result of the chronic
Percentage GIP (A) and GLP-1 (B) protein content was determined after 3 weeks on either a HF (n=3) or LF (n=3) diet in Sprague-Dawley rats. Intestinal segments were taken from the uppermost portion of the duodenum, just distal of the pylorus; the mid-section of the jejunum; and the lowermost portion of the ileum, just proximal of the ileal-cecal junction. Data are presented as means ± SE.
consumption of a high-fat diet, may be one of the mechanisms involved. Increased gastrointestinal transit has been reported following consumption of a high-fat diet (19, 33, 94). Exposure to nutrients in more distal portions of the gut could lead to the hyperplasia of GIP-secreting K cells; thus, gastric emptying and intestinal transit should be measured in both the HF- and LF-groups.

Increased sensitivity of the K cells to lipid is another potential explanation for the elevated GIP levels observed after chronic HF-consumption. To test this hypothesis, HF- and LF-fed rats should be challenged with both a lipid-only bolus and a carbohydrate-only bolus. If the K cells of the HF-fed rats are more sensitive to lipid, then the HF-fed animals should have a higher lipid-induced GIP response than the LF-fed animals; the fold increase between the feeding groups for the lipid-only bolus should be larger than the fold increase for the carbohydrate-only bolus. Ideally, an additional way to test this hypothesis is to measure the level of the cellular components involved in lipid-induced GIP secretion; however, as this pathway has not been entirely unraveled, an alternate approach would be to measure the message and protein level of the G-protein coupled receptors involved in the process, such as GPR119 and GPR120.

**Question 4: Will any high-energy diet, regardless of the primary nutrient source, result in elevated GLP-1 secretion?**

The results from III imply that the elevation in GLP-1 secretion following the chronic feeding of a HF diet is not due to the increased consumption of dietary fat but rather due to the increased intake of energy. These findings suggest that any diet, regardless of the primary nutrient source, *if consumed in excess* will result in elevated GLP-1 levels. The LF diet utilized in III is essentially also a high-carbohydrate (HC) diet, while the HF diet is a LC diet. However, the LF (HC) diet did not result in elevated GLP-1 secretion because it was not consumed in excess compared to the HF (LC) diet. To circumvent this issue, one group of animals should be provided the LF diet *ad libitum* for 3 weeks; the second group of animals should also receive the LF diet for the same time period but the caloric amount consumed should be
restricted. Following the feeding period, the animals will undergo the lymph fistula surgical procedure to test their incretin response to a mixed meal. If increased energy intake results in elevated GLP-1 secretion, then the calorie-restricted LF-fed group should have a smaller GLP-1 response than the group fed the diet *ad libitum*. Interestingly, Chen *et al.* (26) demonstrated that GLP-1 secretion and L cell number was markedly reduced following 4 weeks of food restriction (60% less than *ad libitum*-fed rats). Four weeks after the food restriction period was halted, GLP-1 secretion and L cell number increased towards that of control animals. This data suggests that the amount of energy consumed does significantly affect the GLP-1 response. As the rats generally do not overconsume the LF diet, an alternative approach would be to daily provide the animals via oral gavage the appropriate amount of calories. One group of animals would receive the caloric equivalent of a normal *ad libitum* meal, while the other group would be provided the diet in excess. Following the duodenal mixed meal challenge, the animals receiving the diet in excess should have a larger GLP-1 response if the elevated GLP-1 levels were indeed caused by an excess consumption of calories.
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Stimulation of Incretin Secretion by Dietary Lipid: Is It Dose-Dependent?

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ABSTRACT

Following the ingestion of nutrients, the secretion of the incretin hormones glucose-dependent insulinotrophic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) by the enteroendocrine cells increases rapidly. Previous studies have shown that oral ingestion of fat stimulates secretion of both incretins; however, it is unclear if there is a dose-dependent response between the amount of lipid ingested and the secretion of the hormones in vivo. Recently, we have found that the concentration of the incretin hormones is higher in intestinal lymph than in peripheral or portal plasma. We therefore used the lymph fistula rat model to test if a dose-dependent trend exists between the secretion of GIP and GLP-1 and dietary lipid. Under isoflurane anesthesia, the major mesenteric lymphatic duct of male Sprague-Dawley rats was cannulated. Each animal received a single, intraduodenal bolus of saline or varying amounts of the fat emulsion Liposyn II (0.275, 0.55, 1.1, 2.2, 4.4 kcal). Lymph was continuously collected for 3 h and analyzed for triglyceride (TG), GIP, and GLP-1 content. In response to increasing lipid calories, the secretion of TG, GIP, and GLP-1 into lymph increased dose dependently. Interestingly, the GLP-1-secreting cells were more responsive than the GIP-secreting cells to changes in intraluminal lipid content. The differing sensitivities of the two cell types to changes in intestinal lipid support the concept that separate mechanisms may underlie lipid-induced GIP and GLP-1 secretion. Furthermore, we speculate that the increased sensitivity of GLP-1 to intestinal lipid content reflects the hormone’s role in the ileal brake reflex. As lipid reaches the distal portion of the gut, GLP-1 is secreted in a dose-dependent manner to reduce intestinal motility and enhance proximal fat absorption.
**INTRODUCTION**

Several investigators in the 1960s observed that the plasma insulin response to an intravenous glucose load was 30-40% lower than that seen after an oral glucose load. Accordingly, an alimentary mechanism, in addition to circulating blood glucose levels, was suggested to regulate insulin release from pancreatic β-cells (26). The postprandial enhancement of insulin secretion by gut factors was termed the *incretin effect*. Over the next 40 years, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) were determined to be the hormones involved in the incretin effect. GIP is released from enteroendocrine K cells, which are located primarily in the duodenum and proximal jejunum, and is secreted in response to the absorption of nutrients. GLP-1 is secreted by intestinal L cells, located mainly in the distal ileum and colon, in response to a variety of nutrient, neural, and endocrine factors (8). Additionally, it has been reported that a small subset of duodenal endocrine cells contain both GIP and GLP-1 (23, 31).

As an incretin hormone, one of the primary functions of GIP is to enhance postprandial insulin secretion from pancreatic β-cells. GIP also up-regulates β-cell insulin gene transcription and biosynthesis (34), stimulates β-cell proliferation, and reduces β-cell apoptosis. Of additional importance are the anabolic effects of GIP on adipocytes. Signaling through the GIP receptor increases fatty acid synthesis and incorporation into triglycerides and down-regulates lipolysis (1). GLP-1 exhibits similar insulinotropic effects by enhancing insulin secretion, stimulating β-cell proliferation, and decreasing β-cell apoptosis. GLP-1 additionally improves glycemic control by decreasing gastrointestinal motility via the ileal brake reflex, thereby reducing delivery of absorbed nutrients to the circulation over time (17, 20, 29, 30). Interestingly, Nauck and colleagues (25) demonstrated that the insulinotropic effects of GLP-1 are outweighed by the hormone’s ability to inhibit gastric emptying. Finally, it has been documented that exogenous administration of GLP-1 induces satiation (16, 32) and satiety (11, 24).
The primary stimulus for incretin secretion is the ingestion of nutrients. Carbohydrate, fat, and protein alone, as well as mixed meals, have all been documented to induce the release of GIP and GLP-1 from enteroendocrine cells. However, the mechanisms underlying nutrient-induced incretin secretion are not clear. Studies suggest that nutrient absorption is required for the secretion of GIP, whereas the presence of nutrients in the lumen is sufficient to induce GLP-1 secretion. For example, GIP, but not GLP-1, secretion was affected in patients with intestinal malabsorption (2) and in studies using pharmacological agents that impede nutrient uptake (9, 10). Additionally, several G-protein coupled receptors involved in sugar sensing and fatty acid signaling have been detected on GLP-1-secreting cells (6, 12, 14, 21).

Although known that the ingestion of nutrients stimulates GIP and GLP-1 release, it is unclear whether or not there is a dose-dependent relationship between the amount of nutrient ingested and the secretion of the incretin hormones in vivo. Furthermore, it is not well-defined if the dose-response patterns are affected by the type of ingested macronutrient. Using the lymph fistula rat model, the present study was undertaken to specifically investigate how GIP and GLP-1 output is affected by increasing doses of intraduodenally infused lipid.
MATERIALS AND METHODS

Animals: Adult, male Sprague-Dawley rats weighing 250-350 g were purchased from Harlan Laboratories (Indianapolis, IN). During a two week acclimation period, the animals were allowed free access to water and standard chow (Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories). The animals were housed in a room with a 12:12-h light-dark cycle (lights on/off at 6 am/6 pm); humidity (50%) and temperature (21°C) were maintained. The University of Cincinnati Institutional Animal Care and Use Committee (Cincinnati, OH) approved all procedures.

Surgical Procedure and Recovery: Prior to surgery, the animals were fasted overnight with free access to water. Under isoflurane anesthesia, the superior mesenteric lymphatic duct was cannulated with polyvinyl chloride tubing (0.5 mm inner diameter, 0.8 mm outer diameter, Tyco Electronics, Castle Hill, Australia), with slight modifications to the procedure described by Bollman and colleagues (4). Instead of suture, the cannula was secured with a drop of cyanoacrylate glue (Krazy Glue, Columbus, OH). A silicone feeding tube (1.02 mm inner diameter, 2.16 mm outer diameter, VWR International, West Chester, PA) was introduced into the fundus of the stomach and advanced approximately 2 cm beyond the pylorus into the duodenum. The feeding tube was secured by a purse-string ligature in the stomach. Both the lymph cannula and the duodenal feeding tube were exteriorized through the right flank. After surgery, the animals were placed in Bollman restraint cages (3) and allowed to recover overnight (18-22 h); the animals were kept in a temperature-regulated chamber (24°C) to prevent hypothermia. To compensate for fluid and electrolyte loss due to lymphatic drainage, a 5% glucose-saline solution was infused into the duodenum at 3 ml/h for 6-7 h, followed by an overnight infusion at 3 ml/h of saline only.

Lipid Infusion: To test the effects of dietary fat on GIP and GLP-1 secretion, a 3-ml mixture bolus of lipid emulsion (Liposyn II 20%, Hospira, Lake Forest, IL) and 0.9% saline was provided as a single meal via the duodenal feeding tube. Five experimental doses (0.275, 0.55, 1.1, 2.2, 4.4 kcal) were studied.
(Table 1). The caloric amount of the full dose (4.4 kcal) is equivalent to half the total daily fat intake of the rat. Liposyn II 20% contains a 50:50 blend of safflower and soybean oil and a caloric content of 2 kcal/ml. A group of control animals were provided with a 3-ml bolus of 0.9% saline.

**Lymph Collection:** Lymph was collected in a conical centrifuge tube on ice for 1 h to establish fasting lymph output and triglyceride (TG), GIP, and GLP-1 secretion. The animals (n = 50, 8-9 animals per group, Table 1) were then given the 3-ml lipid/saline bolus. Thirty minutes following the nutrient bolus, a 0.9% saline infusion was provided at 3 ml/h for the remainder of the study period. Lymph samples were collected on ice at 0.5, 1, 2, and 3 h following the lipid bolus. Each sample contained 10% by volume of an anti-proteolytic cocktail (0.25 M EDTA, 0.80 mg/ml aprotinin, 80 U/ml heparin).

**TG Measurement:** TG concentrations were determined using an assay kit which measures the amount of glycerol released from the hydrolysis of triglycerides (Randox, Crumlin, UK). After a 1:10 dilution with sterile water, 2 μl diluted lymph sample was combined with 200 μl reagent and incubated for 10 min at 21°C. The optical absorbance was read at 500 nm. Final concentrations (mg/dl) were calculated using standards provided by Randox.

**GIP and GLP-1 Measurements:** GIP and GLP-1 concentrations were determined using commercially available ELISA kits (LINCO Research, St. Charles, MO). The GIP ELISA measures both active GIP(1-42) and non-active GIP(3-42). To a microtiter plate pre-coated with anti-GIP monoclonal antibodies, 10 μl lymph sample was added to each well. After a 1.5-h incubation, 100 μl detection antibody (biotinylated anti-GIP polyclonal antibody) was added to the captured molecules, followed by a 1-h incubation and addition of 100 μl enzyme solution (streptavidin-horseradish peroxidase conjugate). The immobilized antibody-enzyme conjugates were quantified by monitoring the horseradish-peroxidase activity at 450 and 590 nm in the presence of 100 μl substrate solution (3, 3’, 5, 5’ – tetramethylbenzidine) and 100 μl stop solution (0.3 M HCl). The final concentrations (pg/ml) were
calculated using standards from LINCO. The GIP concentrations were then converted to pM (1 pM GIP = 4.4 pg/ml GIP).

The GLP-1 ELISA measures biologically active GLP-1(7-37) and GLP -1(7-36)NH₂ and does not cross-react with glucagon, GLP-2, and inactive GLP-1(9-37) and GLP-1(9-36)NH₂. To a microtiter plate pre-coated with anti-GLP-1 monoclonal antibodies, 100 μl lymph sample was added and incubated overnight at 4°C. To each well, 200 μl detection conjugate (anti-GLP-1 alkaline phosphatase conjugate) was added, followed by a 2-h incubation. The antibody-enzyme conjugates were quantified by monitoring the fluorescent product formation after addition of 200 μl diluted substrate (4-methylumbelliferyl phosphate). The phosphatase activity was measured at excitation/emission wavelengths of 355/460 nm in the presence of 50 μl stop solution. The final concentrations (pM) were calculated using standards provided by LINCO.

**Data and Statistical Analysis:** Hourly outputs were calculated by multiplying together the hourly lymph volume and TG, GIP, or GLP-1 concentrations. Hourly lymph, TG, GIP, and GLP-1 outputs were analyzed using a two way ANOVA. The Bonferroni t-test was used as the post-test analysis. Differences were considered significant if p < 0.05. Total 3-h secretion for each parameter was determined by summing the hourly outputs for TG, GIP, or GLP-1 above the fasting level. Total lymph output was examined using a one way ANOVA, while total TG, GIP, and GLP-1 outputs were analyzed using a Kruskal-Wallis one way ANOVA on ranks with Dunn’s method as the post-test analysis. Differences were considered significant if p < 0.05. Additionally, cumulative GIP and GLP-1 outputs were plotted as a function of infused lipid dose after normalizing the data to saline levels. For each data set, best-fit lines were generated and subjected to linear regression analysis. Slopes were considered significantly different from zero if p < 0.05 (SigmaPlot, version 10.0).
RESULTS

Effect of Lipid Dose on Lymph Flow: The lymph flow rate for the five experimental lipid doses and the saline control is shown in Figure 1. The fasting lymph flow ranged from $2.10 \pm 0.18$ to $2.53 \pm 0.23$ ml/h and was similar for the five experimental groups, as well as the saline control. All the infusions, including the saline control, increased lymph flow slightly above fasting levels. Only the lymph flow for the 2.2 kcal lipid dose at 2 h ($3.76 \pm 0.53$ ml/h) was statistically different from saline ($2.55 \pm 0.18$ ml/h). However, there was no statistically significant difference in the total 3-h lymph output for any of the lipid groups (data not shown). The total lymph output ranged from $6.81 \pm 0.50$ to $9.20 \pm 0.56$ ml over the 3-h collection period.

Effect of Lipid Dose on TG Secretion: Lymph TG output was examined in response to increasing caloric doses of lipid infused into the duodenum. All five doses raised the level of lymphatic TG above that of the saline control at both the 1- and 2-h time points (Figure 2A). TG secretion peaked at 1 h for lipid doses 0.275 and 0.55 kcal ($20.23 \pm 1.91$ and $21.61 \pm 5.54$ mg/h, respectively) but peaked later at 2 h for lipid doses 1.1, 2.2, and 4.4 kcal ($21.55 \pm 3.18$, $40.73 \pm 7.80$, and $43.32 \pm 3.77$ mg/h respectively). TG output was significantly different from saline for all five doses at the time of peak secretion. By 3 h, TG secretion was decreasing toward baseline or had returned to baseline for all doses.

Cumulative TG secretion was calculated by summing the hourly TG outputs over the 3-h lymph collection period (Figure 3A). As expected, the total TG secretion increased in response to larger amounts of dietary lipid, ranging from $28.00 \pm 1.77$ mg for the 0.275 kcal lipid dose to $101.45 \pm 8.46$ mg for the 4.4 kcal lipid dose. Multiple comparison analysis detected five significant differences among the saline and lipid dose groups: 4.4 kcal vs. saline, 4.4 kcal vs. 0.275 kcal, 4.4 kcal vs. 0.55 kcal, 2.2 kcal vs. saline, and 1.1 kcal vs. saline. As the caloric content of each lipid dose increased, the cumulative TG secretion also increased, signifying a dose-dependent relationship.
**Effect of Lipid Dose on GLP-1 Secretion:** Hourly lymphatic GLP-1 output was computed by multiplying together the lymph flow rate by the hourly GLP-1 concentration (Figure 2B). All lipid doses stimulated GLP-1 release above baseline. For lipid dose 0.275 kcal, GLP-1 secretion peaked at 0.5 h (0.14 ± 0.03 pmole/h). Lipid doses 0.55 and 1.1 kcal produced peak GLP-1 release at 1 h (0.18 ± 0.05 and 0.23 ± 0.03 pmole/h, respectively). GLP-1 output peaked at 2 h for the largest two lipid doses, 2.2 kcal (0.30 ± 0.06 pmole/h) and 4.4 kcal (0.35 ± 0.07 pmole/h). GLP-1 secretion was significantly different from saline at 2 h (peak output) for lipid doses 2.2 and 4.4 kcal and was returning or had returned to baseline for all lipid doses by 3 h.

Summing the hourly GLP-1 outputs over the collection period yielded the total 3-h GLP-1 output as depicted in Figure 3B. Total GLP-1 secretion increased contemporaneously as the caloric amount of lipid increased, ranging from 0.33 ± 0.04 pmole for the 0.275 kcal lipid dose to 0.85 ± 0.17 pmole for the 4.4 kcal lipid dose. GLP-1 secretion was approximately 1.5 fold and 4 fold higher than that for the saline dose (0.20 ± 0.06 pmole) for the lowest and the highest lipid dose, respectively. Multiple comparisons were made to determine if there were statistical differences in the cumulative GLP-1 secretion among the five doses and the saline control. The total GLP-1 secretion for lipid dose 4.4 kcal was significantly different from the total secretion for lipid dose 0.275 kcal and the saline control; additionally, the total GLP-1 secretion for lipid dose 2.2 kcal (0.64 ± 0.09 pmole) was significantly different from that for the saline control. Although significant differences were not detected for each dose comparison, cumulative GLP-1 output increased as the caloric content of lipid increased, suggestive of a dose-dependent trend.

**Effect of Lipid Dose on GIP Secretion:** To test the effects of varying lipid doses on GIP secretion, the product of the hourly GIP concentration and lymph flow rate was calculated (Figure 2C). All five lipid doses stimulated GIP secretion above baseline. By 0.5 h, GIP secretion had peaked and was decreasing to baseline levels for lipid doses 0.275 kcal (0.16 ± 0.02 pmole/h) and 0.55 kcal (0.15 ± 0.03 pmole/h). On
the other hand, GIP secretion for the remaining three lipid doses peaked at later time points before returning to baseline. At 1 h, lipid dose 1.1 kcal generated a peak in GIP secretion that was significantly greater than saline (0.26 ± 0.04 pmole/h). Lipid doses 2.2 and 4.4 kcal yielded peaks at 2 h (0.26 ± 0.04 and 0.31 ± 0.05 pmole/h, respectively). GIP output was significantly different from saline at 2 h for both lipid doses 2.2 and 4.4 kcal.

Total 3-h GIP secretion was calculated by summing together the hourly GIP output values (Figure 3C). GIP secretion ranged from 0.31 ± 0.03 pmole for the 0.275 kcal lipid dose to 0.76 ± 0.13 pmole for the 4.4 kcal lipid dose. Multiple comparisons allowed further analysis of the differences among the lipid doses. Five significant differences were detected: 4.4 kcal vs. saline, 4.4 kcal vs. 0.275 kcal, 4.4 kcal vs. 0.55 kcal, 2.2 kcal vs. saline, and 1.1 kcal vs. saline. Similar to GLP-1 secretion, total GIP secretion appeared to follow a dose-dependent trend in response to increasing amounts of lipid.

**Comparison of GLP-1 to GIP Secretion:** To compare the secretory ability of GLP-1-producing cells to GIP-producing cells, the cumulative output data were plotted as a function of infused lipid calories after first being normalized to saline levels. The equations for the best-fit lines generated for GLP-1 and GIP are $y = 0.657x + 1.528$ ($R^2 = 0.347$; $p < 0.001$) and $y = 0.474x + 1.253$ ($R^2 = 0.350$; $p < 0.001$), respectively. Although both lines have slopes significantly greater than zero, indicative of a dose-dependent relationship, the steeper slope for the GLP-1 data suggests that the GLP-1-secreting cells are more sensitive to changes in intraluminal lipid content.
DISCUSSION

The incretin hormones GIP and GLP-1 are secreted from the enteroendocrine K and L cells, respectively, and enhance postprandial insulin secretion. The primary stimulus for the secretion of the incretin hormones is the ingestion of nutrients. Carbohydrate, fat, and protein alone, as well as mixed meals, have all been reported to induce the release of GIP and GLP-1 (8, 15). Whether or not there are dose-response relationships between the amount and type of macronutrient ingested and the secretion of incretins in vivo, however, has not been well-defined.

The incretin hormones are typically measured in the systemic blood; however, the concentration of both GIP and GLP-1 in plasma is low due to portal dilution and rapid degradation by dipeptidyl peptidase-IV (DPP-IV) (22). Additionally, incretin measurements in plasma are made difficult due to the limited volume of blood that can be removed from an animal. Recently, we have found that intestinal lymph is an alternative fluid compartment in which to measure the incretin hormones (7, 18, 19). The lymph fistula rat has a catheter inserted into the superior mesenteric lymphatic duct, which allows for drainage from the entire gastrointestinal tract (4). The concentration of both GIP and GLP-1 is higher in intestinal lymph than it is in portal or peripheral plasma. The high concentration is due, in part, to less DPP-IV degradation. Also, since lymph has a lower flow rate than portal blood, the hormones are less diluted once secreted from the enteroendocrine cells, thereby raising the concentration of GIP and GLP-1. The lymph fistula rat model is an excellent tool to study incretin hormone release because the measured concentrations more closely mimic the amount of hormone sensed by the enteric neurons. In addition, the elevated concentrations allow for sensitive detection of incretin secretion changes in response to external stimuli, such as nutrients.

Using the lymph fistula rat model, we investigated the effects of increasing doses of lipid on postprandial GIP and GLP-1 secretion over a 3-h time period. Here, we have shown that both GIP and GLP-1
cumulative 3-h outputs, as well as peak hourly outputs, respond dose dependently to increasing doses of dietary lipid ranging from 0.275 kcal to 4.4 kcal. Consistent with our present findings, it has been previously suggested that GLP-1 secretion is dependent on the caloric size of the ingested meal (1). In vitro, α-linolenic (12), oleic (13), and palmitoleic (27) acids have all been found to have a dose-dependent stimulatory effect on GLP-1 secretion. Additionally, Vilsbøll and colleagues (33) reported that both GIP and GLP-1 secretion was higher in lean and obese humans, as well as in type 1 and type 2 diabetic patients, following a large mixed meal, compared to an identical meal of smaller caloric value. Furthermore, GLP-2, a proglucagon-derived peptide co-secreted with GLP-1, appears to be secreted in a calorically-dependent manner (35).

Although both hormones responded dose dependently to increasing caloric amounts of lipid, the GIP secretion pattern was not as well-defined as that for GLP-1. As shown in Figure 3C, total GIP secretion seemed to exhibit a trend in which there was a fairly constant response within a certain dose range. The lower two lipid doses (0.275 and 0.55 kcal) induced the same amount of cumulative GIP release, whereas the larger three lipid doses (1.1, 2.2, and 4.4 kcal) stimulated a similar level of GIP secretion that was approximately 2.3 fold higher than that produced by the lower two doses. It is tempting to suggest that an all-or-nothing phenomenon is occurring, in which only those lipid doses containing 1.1 kcal or above are capable of stimulating a substantial and prolonged GIP response; however, the statistical analysis is not clear enough to make this conclusion. We are currently unaware of any data that suggests that the secretion of GIP to nutrients follows an all-or-nothing pattern.

Additionally, our data demonstrated that the GLP-1-secreting L cells may be more sensitive than the GIP-secreting K cells to changes in intraluminal lipid content. Plotting the cumulative 3-h outputs against the amount of infused lipid generated best-fit lines with slopes of 0.657 for GLP-1 and 0.474 for GIP. The larger slope for the GLP-1 data suggests that the GLP-1-secreting cells are more responsive to changes in the amount of dietary lipid. Indeed, in previously published reports from our laboratory (18, 19), we
found that the peak stimulation for GLP-1 was 10 fold greater than fasting levels versus 4 fold higher than fasting levels for GIP when challenged with a 4.4 kcal lipid bolus, further signifying that the GLP-1-secreting cells respond more robustly to intestinal lipid loads. Although supported in the literature, this result is somewhat surprising. Since fat absorption occurs predominately within the jejunum, it would be expected that less lipid would reach the distal portion of the small intestine where the majority of L cells are located. Less available lipid would therefore mean less nutrient stimulation and lower levels of GLP-1 release. However, this explanation is based upon the assumption that the regulation of GIP and GLP-1 secretion is the same. It is commonly accepted that the secretion of GIP is dependent on nutrient absorption, whereas the presence of the nutrients in the intestinal lumen is sufficient to stimulate GLP-1 secretion, indicating that the underlying mechanisms behind GIP and GLP-1 secretion may differ. For instance, in observations from patients with intestinal malabsorption (2) and studies using pharmacological agents that impede nutrient uptake (9, 10), GIP secretion was diminished, while the concentration of GLP-1 was unaffected. Due to the differences in nutrient sensing, it is reasonable to suggest that the K cells and L cells may have different levels of responsiveness to a given amount of lipid.

The enhanced sensitivity of GLP-1 secretion to increasing lipid loads reflects the hormone’s role in the ileal brake reflex (17, 20, 25, 29, 30). The ileal brake is a distal-to-proximal feedback loop that slows the gastrointestinal transit of nutrients to aid digestion and absorption. The presence of nutrients in the ileum induces a signal to the proximal gut to delay gastric emptying and reduce intestinal motility. Although the identity of the ileal brake mediators is not entirely clear, GLP-1 is considered a strong candidate. In the present experiment, to eliminate the effects of variable rates of nutrient entry due to gastric emptying, we infused the lipid doses intraduodenally. The duodenal infusion increases the likelihood that fat would reach the distal portion of the gut and subsequently indicates to the body that nutrients have not yet been absorbed. Previous studies (5, 28) have detected radiolabeled lipid in the ileum within 15 min following an intragastric dose of triolein as small as 10 mg (0.9 kcal) and a 1 kcal intragastric dose of Intralipid. While we cannot be certain that the lipid is reaching the ileum in a dose-dependent manner, we
hypothesize that, as the infused lipid dose increases, more fat reaches the ileum and the secretion of GLP-1 increases consequently. The elevated levels of GLP-1 would then, in turn, act on the proximal gut by slowing intestinal transit, thereby promoting increased fat digestion and absorption.

In summary, we have demonstrated that in response to increasing lipid calories, the secretion of TG and the incretin hormones GIP and GLP-1 into the lymph increased dose dependently over a 3-h time period. Additionally, we have shown that the GLP-1-secreting cells are more responsive than GIP-secreting cells to changes in intraluminal lipid content. The differing sensitivities of the K cells and the L cells to intestinal lipid changes support the concept that separate mechanisms may underlie lipid-induced GIP and GLP-1 secretion. Furthermore, we speculate that the increased sensitivity of GLP-1 to intestinal lipid content reflects the hormone’s role in the ileal brake reflex. As lipid reaches the distal portion of the gut, GLP-1 is secreted in a dose-dependent manner to reduce intestinal motility and enhance proximal fat absorption. Whether or not this paradigm is dependent on the type of macronutrient and will be altered in diet-induced obese or type 2 diabetic animals is of considerable interest to basic and clinical scientists.

ACKNOWLEDGEMENTS

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REFERENCES


TABLE 1

Each dose contains half the caloric amount of the previous dose. The caloric amount of the full dose (4.4 kcal) is equivalent to half the total daily fat intake of the rat.

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Hourly lymph flow rate following administration of a duodenal bolus of lipid emulsion or saline. Five lipid doses (0.275, 0.55, 1.1, 2.2, 4.4 kcal) were tested. Data are presented as means + SE. *p < 0.05 vs. saline.
FIGURE 2

Lymphatic TG (A), GLP-1 (B), and GIP (C) output following a duodenal bolus of lipid emulsion or saline plotted as a function of time. Five lipid doses (0.275, 0.55, 1.1, 2.2, 4.4 kcal) were tested. Data are presented as means ± SE. *p < 0.05 vs. saline at time of peak secretion.
Cumulative lymphatic TG (A), GLP-1 (B), and GIP (C) output following a duodenal bolus of lipid emulsion or saline. Five lipid doses (0.275, 0.55, 1.1, 2.2, 4.4 kcal) were tested. Cumulative secretion was calculated by summing together the hourly TG, GLP-1, or GIP output values over the 3-h collection period. Data are presented as means ± SE. *p < 0.05 vs. saline; †p < 0.05 vs. lipid 0.275 kcal; ‡p < 0.05 vs. lipid 0.55 kcal.
Cumulative lymphatic GLP-1 (A) and GIP (B) output plotted as a function of infused lipid dose. Five lipid doses (0.275, 0.555, 1.1, 2.2, 4.4 kcal) were tested. Data are presented as fold amounts above saline and depicted as means + SE. The equations for the best-fit lines generated for GLP-1 and GIP are $y = 0.657x + 1.528$ ($R^2 = 0.347; p < 0.001$) and $y = 0.474x + 1.253$ ($R^2 = 0.350; p < 0.001$), respectively. The slopes are both significantly greater than zero ($p < 0.001$) and differed between the two lines.
Differential Responses of the Incretin Hormones GIP and GLP-1 to Increasing Doses of Dietary Carbohydrate but not Dietary Protein in Lean Rats

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ABSTRACT

Previous studies have shown that oral ingestion of nutrients stimulates secretion of the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1); however, it is unclear if there is a dose-dependent response between the amount of nutrient ingested and the secretion of the hormones in vivo. Using our lymph fistula rat model, we previously demonstrated that both GIP and GLP-1 responded dose dependently to increasing amounts of infused dietary lipid and that the GLP-1-secreting cells were more sensitive to changes in intestinal lipid content. In the current study, we investigated the dose-dependent relationships between incretin secretion and the two remaining macronutrients, carbohydrate and protein. To accomplish this objective, the major mesenteric lymphatic duct of male Sprague-Dawley rats was cannulated. Each animal received a single bolus (3 ml) of saline, dextrin, whey protein, or casein hydrolysate (0.275, 0.55, 1.1, 2.2, 4.4 kcal) via a surgically-inserted duodenal or ileal feeding tube. Lymph was continuously collected for 3 h and analyzed for GIP and GLP-1 content. Both GIP and GLP-1 outputs responded dose dependently to increasing amounts of dietary carbohydrate but not protein. Additionally, we found that the GIP-secreting cells were more sensitive than the GLP-1-secreting cells to changes in intestinal carbohydrate content.
INTRODUCTION

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are intestinal incretin hormones produced from enteroendocrine K cells and L cells, respectively. Although it has been reported that a small subset of duodenal endocrine cells produce both GIP and GLP-1 (25, 34), the majority of the GIP-secreting K cells are located in the proximal small intestine, while the GLP-1-secreting L cells are primarily located in the ileum. As incretin hormones, one of the primary roles for these molecules is to enhance postprandial insulin secretion. Beyond this, GIP and GLP-1 have several additional physiological functions. Both hormones up-regulate insulin gene transcription and biosynthesis, stimulate pancreatic β-cell proliferation, and inhibit β-cell apoptosis (1, 19, 37). GLP-1 also maintains glucose homeostasis by regulating gastrointestinal motility and food intake (23, 26, 33). On the other hand, GIP enhances lipogenesis by stimulating lipoprotein lipase activity, enhancing fatty acid synthesis and incorporation into triglycerides, and down-regulating glucagon-stimulated lipolysis, all of which promote fat deposition rather than mobilization (14). In fact, mice lacking the receptor for GIP are resistant to diet-induced obesity (24, 40), making GIP an attractive target for anti-obesity therapy.

GIP secretion is primarily stimulated by nutrients, whereas GLP-1 secretion is stimulated by a combination of nutrient, neural, and hormonal influences (1). Although we have an understanding of the factors that induce incretin secretion, the mechanisms underlying this process still remain to be elucidated. Whether or not all three macronutrients utilize the same cellular machinery to stimulate hormone release and if the GIP-secreting K cells and the GLP-1-secreting L cells respond in a similar fashion to the same stimuli are questions yet to be answered. Currently, it is suggested that nutrient absorption is required for GIP release, whereas the presence of nutrients in the intestinal lumen is sufficient to stimulate GLP-1 secretion (12, 13).
Typically, the incretin hormones are measured in systemic blood; however, the concentration of both active GIP and GLP-1 in plasma is low due to portal dilution and rapid degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV). The half-life of GLP-1 is less than 2 min, while the half-life for GIP is 7 min in humans but less than 2 min in rodents (1). Recently, we have demonstrated that intestinal lymph is an alternative fluid compartment for the collection and measurement of the incretin hormones. In our lymph fistula rodent model, a catheter is inserted into the major mesenteric lymphatic duct, allowing for continuous lymphatic drainage from the entire gastrointestinal tract (intestine, stomach, pancreas, and portions of the liver) of conscious animals. We found that the concentration of both incretin hormones is higher in intestinal lymph than in either peripheral or portal plasma (9, 21, 22). The higher incretin concentrations are likely due to less dilution by the circulating fluid and less degradation by DPP-IV. There is also evidence to suggest specific targeting of GLP-1 to the lymph compartment (9). The lymph fistula model is an excellent tool to study incretin biology because small changes in secretion can be detected over time. Additionally, the measured concentrations may more accurately mimic the concentration of incretin hormones sensed by the enteric neurons.

Using the lymph fistula model, we had previously shown that both GIP and GLP-1 responded dose dependently to increasing amounts of infused dietary lipid. Additionally, we found that the GLP-1-secreting L cells were more sensitive than the GIP-secreting K cells to changes in the intraluminal lipid content (39). In the current study, we investigated the dose-dependent relationships between incretin secretion and the two remaining macronutrients, carbohydrate and protein.
MATERIALS AND METHODS

Animals: Adult, male Sprague-Dawley rats weighing 250-350 g (Harlan Laboratories, Indianapolis, IN) were acclimated to their environment for two weeks prior to experimentation. During acclimatization, the animals were allowed free access to water and standard chow (Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories). The animals were housed in a room with controlled humidity (50%) and temperature (21°C) and a 12-h-light/dark cycle. The University of Cincinnati Institutional Animal Care and Use Committee approved all procedures.

Surgical Procedure and Recovery: Prior to surgery, the animals were fasted overnight with free access to water. Under isoflurane anesthesia, a midline laparotomy was performed. The major mesenteric lymphatic duct was cannulated with polyvinyl chloride tubing (0.5 mm inner diameter, 0.8 mm outer diameter, Tyco Electronics, Castle Hill, Australia), with slight modifications to the procedure described by Bollman et al. (5). Instead of suture, the cannula was secured with a drop of cyanoacrylate glue (Krazy Glue, Columbus, OH). A silicone feeding tube (1.02 mm inner diameter, 2.16 mm outer diameter, VWR International, West Chester, PA) was introduced into the stomach via a small gastrotomy and advanced 1-2 cm beyond the pylorus into the duodenum. The feeding tube was secured with a purse-string ligature in the stomach. For a subset of animals, a smaller silicone feeding tube (0.64 mm inner diameter, 1.19 mm outer diameter, Braintree Scientific, Braintree, MA) was inserted directly into the small intestine, rather than the stomach, approximately 25 cm proximal of the ileal-cecal junction, and secured with a purse-string ligature. Both the lymph cannula and the duodenal or ileal feeding tube were exteriorized through the right flank; the abdomen was then closed in two layers. After surgery, the animals were placed in Bollman restraint cages (4) and allowed to recover overnight (18-22 h); the animals were kept in a temperature-regulated chamber (24°C) to prevent hypothermia. To compensate for fluid and electrolyte loss due to lymphatic drainage, a 5% glucose-saline solution was infused into the duodenum at 3 ml/h for 6-7 h, followed by an overnight infusion at 3 ml/h of saline.
Nutrient Doses and Lymph Collection: To initially test the effect of dietary carbohydrate and protein on incretin secretion, a 3-ml bolus mixture of dextrin (Sigma-Aldrich, St. Louis, MO) plus 0.9% saline or whey protein (100% AnyWhey, Optimum Nutrition, Aurora, IL) plus 0.9% saline was provided as a single meal via the duodenal feeding tube. As previous reports had documented incretin secretion following a whey-supplemented meal (11, 17, 35), we chose whey as our initial protein source. For each nutrient, five experimental doses were tested (0.275, 0.55, 1.1, 2.2, 4.4 kcal; Table 1 – carbohydrate doses; Table 2 – protein doses). Three additional groups of animals received a 3-ml bolus mixture of casein hydrolysate (N-Z-Case Plus, Sigma-Aldrich, St. Louis, MO) plus 0.9% saline (0.275, 1.1, 4.4 kcal; Table 2). A group of control animals (n = 14) were given a 3-ml bolus of 0.9% saline. To test the effects of ileal carbohydrate exposure on incretin secretion, a 3-ml mixture of dextrin and 0.9% saline or saline alone was infused as a single bolus via the ileal feeding tube; three doses were tested (0.275, 0.55, 1.1 kcal; Table 1). In our previous study that investigated incretin secretion following increasing doses of dietary lipid (39), we also used lipid doses ranging from 0.275 kcal to 4.4 kcal. The caloric amount of the highest dose (4.4 kcal) is equivalent to half the daily fat intake of the rat. To make comparisons to our previous data, we chose to use the same doses for the current study. The highest dose (4.4 kcal) is equivalent to a third of the daily protein intake and a tenth of the daily carbohydrate intake of the rat.

The morning after surgery, lymph was collected in a conical centrifuge tube on ice for 1 h to establish fasting lymph, GIP, and GLP-1 outputs. The animals (n = 111, 4-10 animals per group, Tables 1 and 2) were then given the 3-ml bolus of carbohydrate/saline, protein/saline, protein hydrolysate/saline, or saline alone. Thirty minutes following the nutrient bolus, all animals were maintained on a saline infusion at 3 ml/h for the remainder of the collection period. Lymph samples were continuously collected on ice for 0.5, 1, 2, and 3 h following the nutrient bolus. Each sample contained 10% by volume of an antiproteolytic cocktail (0.25 M EDTA, 0.80 mg/ml aprotinin, 80 U/ml heparin).
**Measurement of GIP and GLP-1 Concentration:** GIP and GLP-1 concentrations were determined using commercially available sandwich ELISA kits (LINCO Research, St. Charles, MO). The GIP ELISA is specific to active GIP(1-42) and non-active GIP(3-42) and does not cross-react with glucagon, oxyntomodulin, GLP-1, or GLP-2. As reported by LINCO, both the intra-assay and inter-assay coefficients of variance (CV) are 3.5%. The final concentrations (pg/ml) were calculated using standards from LINCO; the GIP concentrations were then converted to pM (1 pM GIP = 4.4 pg/ml GIP). The GLP-1 ELISA measures biologically active GLP-1(7-37) and GLP-1(7-36)NH₂ and will not detect glucagon, GLP-2, and inactive GLP-1(9-37) and GLP-1(9-36)NH₂. As reported by LINCO, the intra-assay CV is 7.4% and the inter-assay CV is 8.0%. The final concentrations (pM) were calculated using standards provided by LINCO.

**Data and Statistical Analysis:** Data are presented as means ± SE. Hourly outputs were calculated by multiplying together the hourly lymph volume and GIP or GLP-1 concentrations. Hourly lymph, GIP, and GLP-1 outputs were analyzed using a two way ANOVA. The Bonferroni t-test was used as the post-test analysis. Differences were considered significant if p < 0.05. Cumulative 3-h secretion for each parameter was determined by summing the hourly outputs for GIP or GLP-1 above the fasting level. Total lymph, GIP, and GLP-1 outputs were examined using a one way ANOVA with the Bonferroni t-test as the post-test analysis. Protein and protein hydrolysate total GIP and GLP-1 outputs were compared using a student’s t-test. Differences were considered significant if p < 0.05. Additionally, cumulative GIP and GLP-1 outputs were plotted as a function of infused nutrient dose after normalizing the data to saline levels. For each data set, best-fit lines were generated and subjected to linear regression analysis. Slopes were considered significantly different from zero if p < 0.05 (SigmaPlot, version 10.0).
RESULTS

Effect of Dietary Carbohydrate and Whey Protein on Lymph Flow: Lymph flow rates for the carbohydrate and protein doses are shown in Figures 1A and 1B, respectively. The fasting lymph flow rates did not differ among the five carbohydrate, five protein, or saline doses. The lymph flow rate for the 0.55 and 1.1 kcal carbohydrate doses (4.80 ± 0.87 and 4.46 ± 0.45 ml/h, respectively) and the 0.55 kcal protein dose (3.77 ± 0.32 ml/h) was significantly different from saline (2.47 ± 0.34 ml/h) thirty minutes following the nutrient bolus. Additionally, at 1 h, the lymph flow rate for the 2.2 kcal carbohydrate dose (4.30 ± 0.58 ml/h) was significantly different from saline (2.46 ± 0.26 ml/h). Cumulative 3-h lymph flow was similar for all ten nutrient doses and was not significantly different from saline (data not shown).

Effect of Dietary Carbohydrate and Whey Protein on GIP Secretion: Hourly and cumulative 3-h lymphatic outputs were analyzed to determine the effect of dietary carbohydrate and protein on GIP secretion (Figure 2). All five carbohydrate doses stimulated GIP secretion above the saline control, and by the end of the 3-h collection period, GIP secretion had returned or was returning to baseline for each carbohydrate dose (Figure 2A). GIP secretion peaked at 0.5 h for the 0.275, 0.55, and 1.1 kcal carbohydrate doses and was significantly greater than saline (0.06 ± 0.01 pmole/h) for the 0.55 and 1.1 kcal doses (0.37 ± 0.04 and 0.91 ± 0.17 pmole/h, respectively). For the largest two carbohydrate doses (2.2 and 4.4 kcal), GIP secretion peaked significantly at 1 and 2 h, respectively (1 h: 2.2 kcal carb – 0.57 ± 0.11 pmole/h, saline – 0.07 ± 0.01 pmole/h; 2 h: 4.4 kcal carb – 1.16 ± 0.34 pmole/h, saline – 0.09 ± 0.01 pmole/h). Cumulative 3-h lymphatic GIP secretion ranged from 0.35 ± 0.05 (0.275 kcal carb) to 2.08 ± 0.44 pmole (4.4 kcal carb) (Figure 2B). The total GIP output for the 4.4 kcal carbohydrate dose was significantly different from the 0.275 and 0.55 kcal carb doses and saline (0.55 kcal carb – 0.39 ± 0.05 pmole; saline – 0.24 ± 0.03 pmole). Total GIP secretion for carbohydrate doses 2.2 and 1.1 kcal (0.72 ± 0.11 and 0.77 ± 0.19 pmole, respectively) was also significantly different from saline.
In contrast to the hourly secretion pattern in response to carbohydrate, the experimental doses of whey protein did not significantly stimulate GIP secretion above the saline control (Figure 2C). The cumulative lymphatic GIP secretion ranged from $0.46 \pm 0.06$ (4.4 kcal protein) to $0.55 \pm 0.08$ pmole (0.55 kcal protein) and paralleled the trends observed in the hourly output data (Figure 2D).

**Effect of Dietary Carbohydrate and Whey Protein on GLP-1 Secretion:** Hourly and 3-h cumulative lymphatic GLP-1 secretion was examined in response to increasing caloric doses of dietary carbohydrate and protein (Figure 3). All carbohydrate doses stimulated GLP-1 secretion above baseline (Figure 3A). GLP-1 secretion peaked at 0.5 h for the four lowest carbohydrate doses (0.275, 0.55, 1.1, and 2.2 kcal carb); the peak secretion values for the 1.1 and 2.2 kcal carbohydrate doses ($0.52 \pm 0.10$ and $0.57 \pm 0.11$ pmole/h, respectively) were significantly greater than saline ($0.12 \pm 0.02$ pmole/h). GLP-1 release peaked later at 2 h for the 4.4 kcal carbohydrate dose ($0.67 \pm 0.26$ pmole/h) and was significantly different from saline ($0.13 \pm 0.01$ pmole/h). By 3 h, GLP-1 levels had returned to baseline for all five doses.

Cumulative lymphatic GLP-1 secretion was calculated by summing the hourly GLP-1 outputs over the 3-h collection period (Figure 3B). The total GLP-1 secretion increased in response to larger amounts of dietary carbohydrate, ranging from $0.32 \pm 0.03$ pmole for the 0.275 kcal carbohydrate dose to $1.29 \pm 0.27$ pmole for the 4.4 kcal carbohydrate dose. The total GLP-1 secretion for the 4.4 kcal carbohydrate dose was significantly different from the 0.55 and 0.275 kcal carbohydrate doses and the saline control (0.55 kcal carb - $0.34 \pm 0.03$ pmole; saline - $0.36 \pm 0.03$ pmole); additionally the total GLP-1 secretion for the 2.2 kcal carbohydrate dose was significantly different from the 0.275 kcal carbohydrate dose (2.2 kcal carb - $0.75 \pm 0.11$ pmole).

Similar to the hourly GIP response, none of the five whey protein doses significantly raised GLP-1 levels above the saline control (Figure 3C). The cumulative lymphatic GLP-1 secretion ranged from $0.36 \pm 0.02$ (0.275 kcal protein) to $0.56 \pm 0.10$ pmole (2.2 kcal protein) (Figure 3D); however, the changes were not significantly different from saline or each other.
Comparison of GIP and GLP-1 Cumulative Secretion in Response to Dietary Carbohydrate and Whey Protein: Cumulative GIP and GLP-1 outputs were plotted as a function of infused nutrient dose after first normalizing the data to saline levels to compare the secretory ability of GIP- and GLP-1-producing cells following increasing doses of carbohydrate or protein (Figure 4). The equations for the best-fit lines generated for the GIP data are $y = 1.634x + 0.835$ ($R^2 = 0.503; p < 0.001$) for carbohydrate and $y = 0.107x + 1.686$ ($R^2 = 0.0302; p = 0.209$) for protein (Figure 4A). The equations for the best-fit lines produced for the carbohydrate and protein GLP-1 data are $y = 0.611x + 0.825$ ($R^2 = 0.467; p < 0.001$) and $y = 0.057x + 1.156$ ($R^2 = 0.0278; p = 0.228$), respectively (Figure 4B). The slopes for both the protein GIP and GLP-1 data are not significantly different from zero; on the other hand, the slopes for the carbohydrate GIP and GLP-1 data are significantly greater than zero, indicating that both GIP- and GLP-1-secreting cells responded dose dependently to increasing amounts of dietary carbohydrate but not protein.

Effect of Ileal Carbohydrate Exposure on Incretin Secretion: The steeper slope for the carbohydrate GIP data following the duodenal infusions suggests that the GIP-secreting K cells are more sensitive than the GLP-1-secreting L cells to caloric changes in dietary carbohydrate. Since less distal exposure to nutrients is one explanation for the difference in K cell and L cell responsiveness, the use of ileal infusions would allow exposure of the distal L cells to the same nutrient concentration as the proximal K cells during the duodenal infusions. Accordingly, incretin secretion was monitored after ileal infusions of the three lowest carbohydrate doses (3-ml bolus: 0.275, 0.55, and 1.1 kcal).

The hourly and 3-h cumulative lymphatic GIP and GLP-1 secretion was analyzed following increasing caloric doses of ileal-infused carbohydrate (Figure 5). The two highest doses stimulated GIP secretion above the saline control (Figure 5A). GIP secretion peaked significantly at 0.5 h for the 0.55 kcal dose and at 1 h for the 1.1 kcal dose (0.5 h: 0.55 kcal carb – 0.22 ± 0.03 pmole/h, saline – 0.07 ± 0.02 pmole/h;
1 h: 1.1 kcal carb - 0.21 ± 0.06 pmole/h, saline – 0.06 ± 0.01 pmole/h). Likely due to bypassing the large majority of the GIP-secreting K cells, GIP secretion following the ileal infusions is substantially lower than that following duodenal carbohydrate doses of equal caloric value. All three ileal carbohydrate doses elicited peaks in GLP-1 secretion (Figure 5B) that were significantly greater than saline at 0.5 h (0.275 kcal carb - 0.20 ± 0.04 pmole/h, 0.55 kcal carb - 0.28 ± 0.04 pmole/h, 1.1 kcal carb - 0.22 ± 0.04 pmole/h, saline - 0.11 ± 0.02 pmole/h).

As before, cumulative GIP and GLP-1 outputs were plotted as a function of infused nutrient dose after first normalizing the data to saline levels to compare the secretory ability of the GIP- and GLP-1-producing cells to ileal-infused (Figure 5C) or duodenal-infused carbohydrate (Figure 5D). Since the three lowest doses were used for the ileal infusions, only the responses to the three lowest duodenal-infused doses are shown in Figure 5D. The slope for the ileal GIP data (0.752) is lower than that for the duodenal GIP data (1.903); as stated previously, this is most likely due to the large bypass of GIP-secreting cells. On the other hand, the slopes for ileal and duodenal GLP-1 data (0.559 vs. 0.526) are almost identical, suggesting that the L cells are equally responsive to either ileal or duodenal carbohydrate; therefore, the distally-located L cells are sufficiently exposed to nutrient during the duodenal infusions.

**Effect of Hydrolyzed Protein on GIP and GLP-1 Secretion:** As stated previously, the five whey protein doses did not stimulate significant GIP or GLP-1 secretion. In our study, the nutrient boluses are infused directly into the small intestine; it is possible that bypassing the stomach decreases the amount of protein hydrolysis and subsequently reduces the amount of peptide and amino acids available for intestinal absorption and incretin stimulation. To test this, lymph-fistula rats were given 3-ml boluses of casein hydrolysate (0.275, 1.1, 4.4 kcal). Cumulative incretin output produced by the casein hydrolysate was compared to that following the whey protein challenges. The protein hydrolysate did not stimulate significant GIP (Figure 6A) or GLP-1 (Figure 6B) secretion above basal levels. Additionally, there was
no difference in cumulative GIP and GLP-1 output between the whey protein and casein hydrolysate infusions at the 0.275 and 1.1 kcal doses. At the 4.4 kcal dose, however, GIP and GLP-1 output was significantly lower for the casein hydrolysate infusions compared to the whey protein infusions (GIP: whey protein – 0.46 ± 0.06 pmole, casein hydrolysate – 0.19 ± 0.05 pmole; GLP-1: whey protein – 0.44 ± 0.06 pmole, casein hydrolysate – 0.27 ± 0.03 pmole).
DISCUSSION

In this study, we used the lymph fistula rat model to investigate the effects of increasing doses of carbohydrate and protein on GIP and GLP-1 secretion. Here, we demonstrated that both lymphatic GIP and GLP-1 cumulative 3-h outputs, as well as peak hourly outputs, respond dose dependently to increasing amounts of dietary carbohydrate (with the exception of GIP secretion for the 2.2 kcal dose). On the other hand, there was no effect on GIP and GLP-1 secretion to protein doses of equal caloric value. Additionally, our data suggest that the GIP-secreting K cells are more sensitive than the GLP-1-secreting L cells to changes in intraluminal carbohydrate content.

To our knowledge, this is the first report investigating the in vivo relationships between increasing doses of carbohydrate and protein on lymphatic incretin secretion. The benefits of using the lymph fistula rodent model to measure the secretion of GIP and GLP-1 are numerous. The concentration of both incretin hormones is higher in intestinal lymph than in portal or peripheral plasma due to less degradation by DPP-IV and less dilution by the smaller circulating fluid compartment (9, 21, 22). Additionally, this model allows the continuous collection of lymph from conscious animals, eliminating any potential side effects of anesthesia. Furthermore, the hormone concentrations measured in lymph may more accurately mimic the concentrations sensed by the enteric neurons.

In agreement with our carbohydrate data, other investigators have recorded dose-dependent increases in GLP-1 secretion following a glucose stimulus from the GLUTag cell line (16, 29). More recently, primary isolated K and L cells have been shown to respond dose dependently to a glucose challenge (28, 30). Xiao and colleagues (38) also demonstrated that GLP-2, a hormone produced in the L cell and co-secreted with GLP-1, is released in a calorically-dependent manner following a dextrose challenge. Furthermore, Schirra and colleagues (32) reported a dose-dependent trend in both glucose-induced GIP and GLP-1 secretion in humans. However, in both the Xiao and Schirra studies, only two nutrient doses
were tested. The present study, on the other hand, includes five carbohydrate doses, which provides a more accurate picture of the relationship between increasing carbohydrate loads and incretin hormone secretion.

Despite observing a dose-dependent trend for both carbohydrate-induced GIP and GLP-1 secretion, we did not detect whey protein-induced incretin secretion for any of the five doses that was significantly different from saline or from each other. Similarly, GLP-2 has been found not to respond to a protein-only meal, in spite of having calorically-dependent responses to both carbohydrate and fat (38). However, peptides (7) and amino acids (31) stimulate GLP-1 secretion dose dependently from STC-1 and GLUTag cells, respectively. Moreover, several studies have demonstrated successful in vivo incretin release following a protein-only meal (6, 15, 18). In these studies, the protein meals were ingested orally, whereas we provided our protein doses as bolus meals via an intraduodenal feeding tube. Despite choosing an easily digestible and absorbable protein source (whey) (3, 10), bypassing the stomach may have decreased the amount of protein hydrolysis and thus reduced the amount of peptides/amino acids available for intestinal absorption and incretin stimulation. Yet, when this hypothesis was tested with intraduodenal infusions of casein hydrolysate, the secretion of GIP and GLP-1 was not greater than saline for any dose nor was it increased compared to doses of whey protein of equal caloric value, further suggesting that protein is not a potent incretin secretagogue.

Although we did not observe differences between protein-induced GIP and GLP-1 secretion, our data did suggest that GIP-secreting K cells were more sensitive than GLP-1-secreting L cells to changes in the amount of dietary carbohydrate. Plotting the cumulative 3-h outputs against the amount of infused carbohydrate generated best-fit lines with slopes of 1.634 for GIP and 0.611 for GLP-1. The steeper slope for the GIP data suggests that the GIP-secreting cells are more responsive to changes in intraluminal carbohydrate content. In our previously published studies, there was a similar trend: in response to a 4.4
kcal carbohydrate bolus, the peak stimulation for GIP was 11 fold greater than fasting levels versus 9 fold greater than fasting levels for GLP-1 (21, 22).

Less distal exposure to nutrients is one explanation for the difference in K cell and L cell responsiveness to changes in dietary carbohydrate; however, the GIP response was still greater than the GLP-1 response to increasing doses of carbohydrate delivered directly into the distal small intestine (Slope of best-fit lines: GIP – 0.752 vs. GLP-1 – 0.559). Additionally, cumulative GLP-1 secretion was similar regardless if the carbohydrate boluses were infused via the duodenal or the ileal feeding tube (Slope of best-fit lines: Duodenal – 0.526 vs. Ileal – 0.559). Because the ileal infusions expose the distal L cells to the same nutrient concentration as the proximal K cells during the duodenal infusions, the results further support that the K cells are more sensitive than the L cells to changes in intraluminal carbohydrate content. In light of these results, the data suggest that different mechanisms may control the release of GIP and GLP-1 in response to carbohydrate. Currently, it is proposed that absorption of nutrients is required for GIP release, whereas the presence of nutrients in the intestinal lumen is sufficient to stimulate GLP-1 secretion. Compared to healthy controls, GIP secretion was reduced in patients with intestinal malabsorption (2). Moreover, inhibitors of carbohydrate digestion (12) and absorption (13) decreased GIP secretion without affecting GLP-1 levels. Wachters-Hagedoorn and colleagues (36) also found that the rate of glucose absorption was correlated with plasma concentrations of GIP but not GLP-1. Taken together, these studies and our data suggest that the differential responses of GIP and GLP-1 to increasing doses of dietary carbohydrate are most likely due to the different mechanisms regulating the release of these hormones.

Similar to the carbohydrate results, we have previously reported that both GIP and GLP-1 responded dose dependently to increasing amounts of dietary lipid. However, the GLP-1-secreting cells were more responsive than the GIP-secreting cells to changes in intraluminal lipid content (39). Comparing our current results to the previous study, it appears that both carbohydrate and lipid are equally potent GLP-1
secretagogues (Slope of best-fit lines: Carbohydrate – 0.611 vs. Lipid – 0.657), whereas carbohydrate is more effective than lipid at stimulating GIP secretion (Slope of best-fit lines: Carbohydrate – 1.634 vs. Lipid – 0.474). We had previously proposed that the enhanced sensitivity of GLP-1 to changes in dietary lipid reflects the hormone’s role in the ileal brake reflex (39). The ileal brake is a distal-to-proximal feedback system that slows the intestinal transit of nutrients to aid digestion and absorption, and GLP-1 is considered to be a putative mediator of this effect (23). Although we did not measure the effect of the nutrient doses on intestinal motility, the data suggests that, compared to lipid, carbohydrate may be contributing equally to the ileal brake reflex via a GLP-1-based mechanism. As lipid or carbohydrate reaches the distal portion of the gut, GLP-1 is secreted in a dose-dependent manner to reduce intestinal transit and enhance proximal nutrient absorption. Indeed, Layer and colleagues (20) have successfully demonstrated the effect of both carbohydrate and fat on the ileal brake reflex and indicate that these responses may not be specific for particular food components but rather a non-specific effect to the presence of unabsorbed nutrients in the distal small intestine.

Whereas carbohydrate and lipid appear to be equally effective at stimulating GLP-1 secretion, we found that carbohydrate is the more potent GIP secretagogue. The enhanced sensitivity of the GIP-producing K cells to changes in carbohydrate intake reflects the insulinotropic potential of the infused nutrient. Both glucose and fatty acids are capable of stimulating insulin release (8, 27). However, fatty acids induce moderate insulin secretion and are not considered primary insulin secretagogues (41). When challenged with a lipid-based meal, the need to produce insulin is low, and the added insulinotropic effect of GIP-signaling is not essential. In contrast, when provided with a carbohydrate-based meal, regulating glucose homeostasis is necessary; in this scenario, the enhancement of glucose-stimulated insulin secretion via GIP is advantageous. As stated in the introduction, GIP also plays an important role in adipogenesis. Whereas the need to produce insulin may be low when provided a fat-meal, the lipogenic properties of GIP, however, do promote lipid storage in adipocytes.
In conclusion, using our lymph fistula rat model, we have shown that both GIP and GLP-1 respond dose dependently to increasing amounts of dietary carbohydrate, but the incretin response following protein doses (intact whey protein or protein hydrolysate) of equal caloric value was no greater than the saline control. Additionally, we found that the GIP-secreting K cells were more sensitive than the GLP-1-secreting L cells to changes in intestinal carbohydrate content. To our knowledge, this is the first study that uses multiple nutrient doses to investigate in vivo lymphatic incretin secretion. In comparison with our previously published data, in which we investigated the simulation of lymphatic incretin secretion by increasing amounts of dietary lipid (39), we hypothesize that the similar GLP-1 responses to lipid and carbohydrate demonstrate the hormone’s role in the ileal brake reflex, whereas the much larger effect of carbohydrate on GIP secretion in rodents reflects the insulinoventropic potential of the infused nutrient.

ACKNOWLEDGEMENTS

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REFERENCES


## TABLE 1

Carbohydrate Doses

### Duodenal Carbohydrate

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### Ileal Carbohydrate

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TABLE 2

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FIGURE 1

Hourly lymph flow rate following a duodenal carbohydrate (A), whey protein (B), or saline bolus (0.275, 0.55, 1.1, 2.2, 4.4 kcal for each nutrient). Values are means ± SE. *p < 0.05 vs. saline.
FIGURE 2

Hourly (A,C) and cumulative (B,D) lymphatic GIP output following either a duodenal carbohydrate, whey protein, or saline bolus (0.275, 0.55, 1.1, 2.2, 4.4 kcal for each nutrient). Hourly output was determined by multiplying together the lymph flow rate by the hourly GIP concentration. Cumulative secretion was calculated by summing together the hourly GIP output values during the 3-h collection period. Values are means + SE. *p < 0.05 vs. saline at time of peak secretion (A). *p < 0.05 vs. saline, #p < 0.05 vs. carbohydrate 0.275 kcal, and ^p < 0.05 vs. carbohydrate 0.55 kcal (B).
Hourly (A,C) and cumulative (B,D) lymphatic GLP-1 output following either a duodenal carbohydrate, whey protein, or saline bolus (0.275, 0.55, 1.1, 2.2, 4.4 kcal for each nutrient). Hourly output was determined by multiplying together the lymph flow rate by the hourly GLP-1 concentration. Cumulative secretion was calculated by summing together the hourly GLP-1 output values during the 3-h collection period. Values are means + SE. *p < 0.05 vs. saline at time of peak secretion (A). *p < 0.05 vs. saline, #p < 0.05 vs. carbohydrate 0.275 kcal, and ^p < 0.05 vs. carbohydrate 0.55 kcal (B).
Cumulative lymphatic GIP (A) and GLP-1 (B) output plotted as a function of nutrient dose. Five carbohydrate (⚫) and five whey protein (□) doses were tested (0.275, 0.55, 1.1, 2.2, 4.4 kcal for each nutrient). Values (fold amounts above saline) are means + SE. Equations for best-fit lines generated for GIP and GLP-1 are shown. Only the slopes for the carbohydrate best-fit lines were significantly greater than zero (p < 0.001, carbohydrate GIP; p < 0.001, carbohydrate GLP-1; p = 0.209, protein GIP; p = 0.228, protein GLP-1).
Hourly lymphatic GIP (A) and GLP-1 (B) output following either an ileal carbohydrate (0.275, 0.55, 1.1 kcal) or saline bolus. Hourly output was determined by multiplying together the lymph flow rate by the hourly GIP or GLP-1 concentration. Values are means + SE. *p < 0.05 vs. saline at time of peak secretion. Cumulative lymphatic GIP and GLP-1 output plotted as a function of carbohydrate dose. Three ileal (C) and three duodenal (D) doses were tested (0.275, 0.55, 1.1 kcal). Values (fold amounts above saline) are means + SE. Equations for best-fit lines generated for GIP and GLP-1 are shown. All of the slopes for the best-fit lines were significantly greater than zero.
Cumulative lymphatic GIP (A) and GLP-1 (B) output following either a duodenal whey protein, protein hydrolysate, or saline bolus (0.275, 1.1, 4.4 kcal for each nutrient). Cumulative secretion was calculated by summing together the hourly GIP or GLP-1 output values during the 3-h collection period. Values are means + SE. *p < 0.05: protein vs. protein hydrolysate at that dose.
Chronic High-Fat Feeding Increases Both GIP and GLP-1 Secretion without Altering Body Weight

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ABSTRACT

The incretin hormones, GIP and GLP-1, enhance postprandial insulin secretion, promote adipogenesis, and regulate gastrointestinal motility and food intake. To date, a consensus on how the incretin response is altered in obesity is lacking. We investigated the effects of chronic high-fat-feeding on incretin secretion in the lymph fistula rat model. Male Sprague-Dawley rats (8 wk) were provided a high-fat (HF) or low-fat (LF) diet ad libitum for 3 or 13 wk; a high-fat pair-fed (HF-PF) group was included as a control during the 3-wk feeding trial. Energy intake, body weight, and body composition were regularly monitored. At the culmination of the feeding period, an intestinal lymphatic duct cannula and duodenal tube were surgically inserted. All animals were challenged with a 3-ml Ensure bolus (3.125 kcal/animal) to measure lymphatic incretin secretion. Despite a significantly higher energy intake, both the 3-wk and 13-wk HF-fed animals did not have an increase in body weight and only a slight increase in body fat compared to LF-fed rats. Following the duodenal mixed meal challenge, the 3-wk and 13-wk HF-fed rats had significantly greater lymphatic GIP secretion and GLP-1 secretion than the LF-fed animals. Additionally, the HF-PF group displayed a secretion profile similar to the HF-fed animals for GIP but a similar pattern to the LF-fed animals for GLP-1. The HF-PF data suggest that the increased GIP secretion is driven by the greater percentage of fat intake, whereas the increased GLP-1 secretion is driven by the excess caloric intake.
INTRODUCTION

Obesity is an expanding global health problem. It is estimated worldwide that 1.6 billion adults are overweight (body mass index [BMI] > 25 kg/m²) and 400 million additional adults are obese (BMI > 30 kg/m²). Between the years 1980 and 2004, the prevalence of obesity in the US increased from 15 to 33% in adults and from 6 to 19% in children (34). In addition to increasing health care costs (14), obesity is associated with several co-morbidities (20) – many of which are mediated through insulin resistance and glucose intolerance, such as type 2 diabetes, nonalcoholic fatty liver disease, cardiovascular disease, and metabolic syndrome. This impaired insulin response may be partially attributed to an altered incretin effect.

The incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted from the enteroendocrine K and L cells, respectively, in response to ingested nutrients. The main function of GIP and GLP-1 is to enhance postprandial insulin secretion. Both incretin hormones also up-regulate pancreatic β-cell insulin gene transcription and biosynthesis, stimulate β-cell proliferation, and reduce β-cell apoptosis (3, 22, 48). GLP-1 additionally improves glycemic control by regulating food intake (19, 42) and decreasing gastrointestinal motility (30, 33, 40), thereby reducing the delivery of absorbed nutrients to the circulation over time. On the other hand, signaling through the GIP receptor promotes fat deposition by enhancing adipogenesis (16).

Impairments of the incretin response in patients with type 2 diabetes have been well-documented. Several studies (32, 43, 45) have reported decreased postprandial GLP-1 secretion. In contrast, GIP secretion from enteroendocrine cells in type 2 diabetic patients is normal or slightly elevated. These patients do however present with impaired insulinotropic effects of GIP at the pancreatic β-cells (12, 31, 32). The attenuated GLP-1 secretion and compromised GIP function contribute to the pathology of type 2 diabetes.
Because the glucoregulatory properties of GLP-1, unlike those of GIP, are still functional in insulin resistant individuals, therapeutic strategies have focused on the development of GLP-1 receptor agonists.

Despite the established evidence supporting the impaired incretin response in diabetics, this effect is not wholly agreed upon in obesity. In obese, non-diabetic humans, GIP secretion has been reported to increase (15, 46) or not change (35, 36, 44), whereas GLP-1 secretion remains unchanged (1, 13, 15, 35, 46) or is attenuated (29, 35, 36, 41, 44). The reasons for these variations are complex and may be partly due to the wide array of immunoassays and test meals used in the studies. Additionally, the nutritional status of the subjects prior to experimentation is rarely stated; therefore, it is difficult to determine if prior eating habits have any potential effects on incretin secretion. From this data, it is currently unclear if, and how, incretin secretion is affected by obesity. Furthermore, it is unclear if the alterations are caused by obesity or the high-energy diet that often induces obesity. In this study, we tested the hypothesis that chronic high-fat feeding affects the secretion of the incretin hormones, GIP and GLP-1. To test this hypothesis, we measured GIP and GLP-1 levels following a mixed meal challenge using the lymph fistula rat model in Sprague-Dawley rats chronically fed a high fat diet for 3 or 13 weeks. We further determined if these effects were due to the increased consumption of calories or to the increased percentage of fat in the diet using a high-fat pair-fed group.
MATERIALS AND METHODS

**Animals:** Male, Sprague-Dawley rats (7 wk) (Harlan Laboratories, Indianapolis, IN) were housed individually and maintained in a temperature- (21°C) and humidity-controlled (50%) room with a 12-h light/dark cycle. The animals were allowed free access to water and standard chow (Harlan Teklad 7012 Mouse/Rat Sterilizable Diet, Harlan Laboratories, Indianapolis, IN) during a 1-wk acclimatization period prior to placement into dietary groups on the basis of comparable mean body weights. All animal procedures and protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee (Cincinnati, OH).

**Diets and Experimental Design:** The animals were provided one of two pelleted, semi-purified nutritionally complete experimental diets (AIN-93M) prepared by Dyets (Bethlehem, PA). The high-fat (HF) diet contains 20 g of fat/100 g of diet (19 g of butter oil and 1 g of soybean oil). The low-fat (LF) diet contains 4 g of fat/100 g of diet (3 g of butter oil and 1 g of soybean oil). The HF and LF diets differed only by fat and carbohydrate content as described previously (49). We selected a high-fat diet as our high-energy diet, since there is a significant positive correlation between dietary fat intake and the prevalence of obesity in both human and animal studies (8, 9, 21, 49). Moreover, studies in rodents also suggest that consumption of a high-fat diet alters gastrointestinal function and intestinal morphology (5, 25, 38, 39), both of which could ultimately lead to impaired incretin secretion.

In the first experiment, animals were provided *ad libitum* access to either the HF or LF diet for 13 wk. Body weight and energy intake were monitored every 2-3 d. Body composition was analyzed prior to initiation of the diet and then every other week until completion of the study. At the end of the 13-wk feeding period, the animals were challenged with a mixed meal following the insertion of an intestinal lymphatic duct cannula to measure incretin secretion.
In the second experiment, the feeding period was reduced from 13 wk to 3 wk. This second group of animals was provided *ad libitum* access to either the HF or LF diet. Because HF-fed and LF-fed rats consume different amounts of calories per day, a high-fat pair-fed (HF-PF) group was included as a control. The HF-PF animals were provided the HF diet but with a caloric content equivalent to the average daily consumption of the rats fed the LF diet *ad libitum*. The HF-PF group was included to clarify if changes in incretin secretion are due to increased total energy intake or increased percentage of dietary fat. Body weight and energy intake were monitored every 2-3 d, and body composition was analyzed prior to diet initiation and at the completion of the 3-wk feeding period. At the end of the study, the animals underwent a mixed meal challenge to measure the lymphatic secretion of the incretin hormones.

**Body Composition:** Body composition (lean and fat mass) was measured using an EchoMRI whole-body composition analyzer (Echo Medical Systems, Houston, TX). Body fat was calculated as percentage of body weight.

**Lymph Fistula Operation and Recovery:** Prior to surgery, the animals were fasted overnight with free access to water. Under isoflurane anesthesia, the major mesenteric lymphatic duct was cannulated with polyvinyl chloride tubing (0.5 mm inner diameter, 0.8 mm outer diameter, Tyco Electronics, Castle Hill, Australia) with slight modifications to the procedure described by Bollman (7). Instead of suture, the cannula was secured with a drop of cyanoacrylate glue (Krazy Glue, Columbus, OH). A silicone feeding tube (1.02 mm inner diameter, 2.16 mm outer diameter, VWR International, West Chester, PA) was introduced into the stomach and advanced slightly beyond the pylorus into the duodenum. The feeding tube was secured with a purse-string ligature in the stomach. Both the lymph cannula and the duodenal feeding tube were exteriorized through the right flank; the abdomen was then closed in two layers. After surgery, the animals were placed in Bollman restraint cages (6) and allowed to recover overnight (18-22 h). The animals were kept in a temperature-regulated chamber (24°C) to prevent hypothermia. To
compensate for fluid and electrolyte loss due to lymphatic drainage, a 5% glucose-saline solution was infused into the duodenum at 3 ml/h for 6-7 h, followed by an overnight infusion of saline only at 3 ml/h.

**Mixed Meal Challenge and Lymph Collection:** Following an overnight recovery, the animals underwent a mixed meal challenge to analyze incretin secretion. Lymph was collected in a conical tube on ice for one hour to establish fasting lymph output and incretin hormone secretion. The animals were then provided a 3-ml mixed meal bolus [Ensure, Abbott Nutrition, Columbus, OH; 3.125 kcal/animal – 0.075 g fat (21.6%), 0.5 g carbohydrate (64.0%), 0.1125 g protein (14.4%)]. Thirty minutes following the nutrient bolus, a 0.9% saline infusion was provided at 3 ml/h for the remainder of the study period. Lymph samples were continuously collected on ice every ten minutes for the first hour and then every hour thereafter following the mixed meal bolus. Each sample contained 10% by volume of an anti-proteolytic cocktail (0.25 M EDTA, 0.80 mg/ml aprotinin, 80 U/ml heparin).

**GIP and GLP-1 Measurements:** GIP and GLP-1 concentrations were determined using commercially available ELISA kits (LINCO Research, St. Charles, MO). The GIP ELISA measures both active GIP(1-42) and non-active GIP(3-42) and does not cross-react with glucagon, oxyntomodulin, GLP-1, or GLP-2. As reported by LINCO, both the intra-assay and inter-assay coefficients of variance (CV) are 3.5%. The final concentrations (pg/ml) were calculated using standards from LINCO; the GIP concentrations were then converted to pM (1 pM GIP = 4.4 pg/ml GIP). The GLP-1 ELISA measures biologically active GLP-1(7-37) and GLP -1(7-36)NH₂ and does not cross-react with glucagon, GLP-2, or inactive GLP-1(9-37) and GLP-1(9-36)NH₂. As reported by LINCO, the intra-assay CV is 7.4% and the inter-assay CV is 8.0%. The final concentrations (pM) were calculated using standards provided by LINCO.

**Statistical Analysis:** Energy intake, body weight, body composition, and hourly incretin concentrations were analyzed using two way repeated measures ANOVA. GIP and GLP-1 concentration area under the curve was calculated using the trapezoidal rule and analyzed using a Student’s t-test for Experiment 1 and
one way ANOVA for Experiment 2. Cumulative energy intake for Experiment 2 was also analyzed using one way ANOVA. The Tukey test was used for all ANOVA post-test analyses. Differences were considered significant if $p < 0.05$ (SigmaPlot, version 10.0).
RESULTS

Experiment 1 – 13-wk HF-feeding: Energy Intake, Body Weight, Body Composition: During the 13-wk feeding period, the HF-fed animals consumed daily more energy than the LF-fed animals (average daily energy intake: HF – 359.52 kJ/d vs. LF – 321.51 kJ/d); energy intake was significantly greater for the HF-fed animals at over 50% of the measured time points (Figure 1A). Despite an increase in energy intake, the HF-fed animals did not weigh significantly different from the LF-fed animals during the entire 13-wk feeding period (final body weight: HF – 458.24 ± 11.51 g vs. LF – 441.70 ± 7.22 g; p = 0.154) (Figure 1B). However, despite similar body weights, the HF group had a significantly greater body fat percentage than the LF group at all time points (final body fat measurement: HF – 10.38 ± 0.71% vs. LF – 8.10 ± 0.46%; p = 0.002) (Figure 1C).

Experiment 1 – 13-wk HF-feeding: Incretin Secretion following a Mixed Meal Challenge: After 13 wk of dietary intervention, both groups of animals underwent our lymph fistula procedure to measure postprandial incretin secretion in response to a mixed meal (Ensure, 3.125 kcal) bolus. The nutrient dose stimulated GIP secretion in both the HF- and LF-fed animals (Figure 2). GIP secretion peaked at 40 min for both groups (HF – 593.73 ± 135.61 pM vs. LF – 323.54 ± 41.11 pM; p < 0.001) and returned to basal levels 180 min following the nutrient challenge (Figure 2A). GIP concentration was significantly greater (approximately 1.75 times larger) at 30 and 40 min for the HF group compared to the LF group (30 min: HF – 535.50 ± 107.28 pM vs. LF – 319.10 ± 37.48 pM; p = 0.003). Although not significant, there was a trend towards increased overall (0-60 min) GIP secretion for the animals on the HF diet (HF – 19526.95 ± 3209.29 (pM)min vs. LF – 12737.21 ± 1186.90 (pM)min; p = 0.071) (Figure 2B).

Lymphatic GLP-1 secretion was also stimulated following the mixed meal challenge in both the HF- and LF-fed animals (Figure 3). GLP-1 secretion peaked at 30 min and had returned to basal levels by the end of the 180 min collection period. The HF group had a significantly greater concentration of GLP-1 at the
time of peak secretion compared to the LF group (HF - 510.82 ± 108.26 pM vs. LF – 363.76 ± 40.52 pM; p = 0.029). Similar to GIP, there appears to be a non-significant trend towards increased overall (0-60 min) GLP-1 secretion for the HF group (HF – 15769.51 ± 2532.36 (pM)min vs. LF – 13090.46 ± 1182.27 (pM)min; p = 0.357).

**Experiment 2 – 3-wk HF-feeding: Energy Intake, Body Weight, Body Composition:** Following the data from our 13-wk experiment, we next wanted to investigate whether or not a shorter period of HF-feeding could induce similar changes in incretin secretion. Several studies have observed significant changes in intestinal morphology and function following only 2-4 wk of high-fat feeding (25); we thus chose an intermediate feeding period of 3 wk. Similar to the data from the 13-wk feeding period, the 3-wk HF-fed animals also had a higher energy intake than the LF-fed animals at all measured time points (Figure 4A) (average daily energy intake: HF – 365.47 kJ/d vs. LF – 315.22 kJ/d). There was no difference in body weight (Figure 5A) or body fat percentage (Figure 5B) between the 3-wk HF- and LF-fed animals (final body weight: HF – 315.78 vs. 8.48 g; LF – 317.71 vs. 8.28 g; p = 0.969) (final body fat measurement: HF – 7.22 ± 0.33% vs. LF – 6.17 ± 0.24%; p = 0.771).

We additionally included a 3-wk high-fat pair-fed (HF-PF) group. Because the HF-fed animals are hyperphagic, they are not only consuming a diet that contains a higher percentage of fat but also consuming more energy overall. This makes it difficult to interpret if the changes in incretin secretion are due to the increased consumption of energy or the increased percentage fat content in the diet. The HF-PF group was provided the HF diet but with a caloric content equivalent to the average daily consumption of the rats fed the LF diet ad libitum. By definition, the LF and HF-PF groups cumulatively consumed the same amount of energy during the 3-wk period (Figure 4B), which was significantly different from the cumulative energy intake of the HF group. Additionally, there was no difference in body weight between the HF-PF group and the HF or LF group following 3 wk of feeding (Figure 5A). Although there was no change in body fat between the HF and HF-PF group at the end of the 3-wk feeding period,
there was a small difference between the LF and the HF-PF group (Figure 5B) (LF – 6.17 ± 0.24% vs. HF-PF – 7.62 ± 0.48%; p = 0.033).

Experiment 2 – 3-wk HF-feeding: Incretin Secretion following a Mixed Meal Challenge: Following the 3-wk feeding period, lymphatic GIP (Figure 6) and GLP-1 (Figure 7) secretion was measured, as described above, after duodenal administration of a mixed meal bolus (Ensure, 3.125 kcal). GIP secretion peaked at 40 min for the HF group (688.36 ± 84.52 pM) and at 30 min for the LF (435.90 ± 44.20 pM) and HF-PF (697.12 ± 71.05 pM) groups. Compared to the LF group, the HF group had significantly greater GIP secretion at 40, 50, and 60 min following the mixed meal challenge (Figure 6A); overall (0-60 min AUC) GIP secretion was also significantly greater for HF-fed than for the LF-fed animals (HF – 21208.80 ± 1694.71 (pM)min vs. LF – 14520.89 ± 1068.82 (pM)min; p = 0.020) (Figure 6B).

The duodenal mixed meal bolus also stimulated GLP-1 secretion in the 3-wk HF/LF-fed animals (Figure 7). Similar to GIP, GLP-1 secretion peaked at 40 min for the HF (485.06 ± 75.89 pM) group and at 30 min for the LF (380.35 ± 66.39 pM) and HF-PF (359.69 ± 36.97 pM) groups. GLP-1 secretion for the HF group was significantly larger than that for the LF group 40 and 50 min following the nutrient bolus (Figure 7A). Additionally, although not significant, there was a trend towards increased overall (0-60 min) GLP-1 secretion for the HF-fed group (HF – 16346.03 ± 2518.63 (pM)min vs. LF - 10713.96 ± 1279.43 (pM)min; p = 0.121) (Figure 7B).

As mentioned previously, we included the HF-PF group to differentiate whether or not the changes in incretin secretion due to consumption of a HF diet were because of the increased energy intake or the increased percentage of fat in the diet. Both the HF and the HF-PF groups received the same diet, whereas the LF and HF-PF groups consume the same amount of calories but from different diets. Interestingly, the HF-PF animals displayed a secretion profile similar to the HF-fed animals for GIP (Figure 6) but a similar pattern to the LF-fed animals for GLP-1 (Figure 7). Compared to the LF-fed
group, the HF-PF group had significantly greater GIP secretion at 40 and 50 min following the nutrient bolus. Although the GIP secretion profile for the HF-PF animals aligned more precisely with the HF-fed group, the GIP concentration for the HF-PF group was significantly different than that for the HF group at 20 and 30 min (Figure 6A). Regardless, the overall (0-60 min) GIP secretion was the same for the HF-fed and the HF-PF groups (HF – 21208.80 ± 1694.71 (pM)min vs. HF-PF – 22980.14 ± 2551.75 (pM)min; p = 0.767) and was increased for the HF-PF group compared to the LF-fed group (LF – 145209.89 ± 1068.82 (pM)min vs. HF-PF – 22980.14 ± 2551.75 (pM)min; p = 0.010) (Figure 6B). Conversely, GLP-1 secretion for the HF-PF group was similar to the LF group at all time points but significantly less than that for the HF group at 40 and 50 min following the nutrient challenge (Figure 7A). Though not significant, there was a trend for decreased overall (0-60 min) GLP-1 secretion for the HF-PF group compared to the HF group (HF – 16346.03 ± 2518.63 (pM)min vs. HF-PF – 11013.31 ± 1024.24 (pM)min; p = 0.249) (Figure 7B). GLP-1 AUC was not different between the LF-fed and the HF-PF groups (LF – 10713.96 ± 1279.43 (pM)min vs. HF-PF 11013.31 ± 1024.24 (pM)min; p = 1.000).
DISCUSSION

Despite the well-documented relationship between type 2 diabetes and the impairments in the incretin system, the dynamics of postprandial incretin secretion in diet-induced obesity are not clearly defined. We sought to determine if the alterations in GIP and GLP-1 secretion are secondary consequences of obesity or rather are induced by the over-consumption of high-energy diets that often perpetuate obesity.

In the current study, we investigated the effects of chronic high-fat feeding on the secretion of GIP and GLP-1 using the lymph fistula rat model. Recently, we found intestinal lymph to be a sensitive medium to study the secretion of the incretin hormones (11, 27, 28). Male Sprague-Dawley rats were provided either a HF or LF diet for 13 weeks in Study 1 or for 3 weeks in Study 2. Interestingly, despite demonstrating no signs of obesity beyond hyperphagia, both the 3-week and 13-week HF-fed animals had elevated lymphatic GIP and GLP-1 concentrations compared to animals fed a LF diet, suggesting that alterations in incretin secretion may occur prior to the development of obesity. Thus, the elevated levels of GIP and GLP-1 are consequences of either increased energy intake (hyperphagia) or increased percentage of dietary fat (HF diet) and not due to secondary consequences of obesity.

To clarify if the alterations are due to the increased energy intake or the increased dietary fat percentage, we included the HF-PF group. The HF-PF animals received the same diet as the HF-fed animals; however, they were restricted to the amount of calories consumed ad libitum by the LF group. At the end of the 3-week feeding period, there was no difference in body weight between the HF-PF group and the HF or LF group and only a small difference in percent body fat between the HF-PF and the LF groups. The GIP secretion profile for the HF-PF group more closely aligned with the HF group, whereas the HF-PF and LF groups produced similar GLP-1 secretory responses. This data suggest that, following the consumption of a HF diet, the increased GIP secretion is driven by the greater percentage of fat in the diet, while the increased GLP-1 secretion is driven by the excess caloric intake.
We were surprised that there was not a significant difference in body weight and only a modest difference in percent body fat between the two feeding groups, despite the consumption of excess energy by the HF group for up to 13 weeks. We also observed no difference in energy expenditure (measured at 8 weeks following initiation of diet study), oral glucose tolerance tests, insulin tolerance tests, or fasting insulin and leptin levels (data not shown). Woods and colleagues (49) previously reported successful use of this particular diet in inducing obesity in male and female Long-Evans rats. In their study, the HF-fed animals weighed 10% more and had 50% more body fat than the LF-fed group following 10 weeks of HF or LF feeding. The primary difference between the two studies is the use of Sprague-Dawley rats in our study over Long-Evans rats in the work by Woods and colleagues (49). The segregation of Sprague-Dawley animals into diet-induced obesity prone and diet-induced obesity resistant groups has been reported previously (10, 23, 24, 37). Individual weight curves, however, do not suggest the stratification of our HF-fed animals into two distinct groups. Although we feel that determining the cause behind the lack of weight gain and fat mass increase is of interest, it is beyond the scope of our current study. Regardless, the absence of a weight difference between the two groups allowed us to determine the effect of the diet alone on incretin secretion without obesity or weight gain as a confounding factor.

The intestine adapts during the consumption of a HF diet to accommodate the digestion and absorption of a larger lipid load. Intestinal transit is increased, and small intestinal morphology and function is modified (25, 38). Singh and colleagues (39) observed elevated levels of jejunal and ileal mucosal enzymes involved in lipid absorption and a subsequent increased uptake of oleic acid following 4 weeks on a chow diet supplemented with 20% lard. Data from Balint and colleagues (5) suggest that this increase in oleic acid uptake is additionally due to cellular hypertrophy in the ileum. Is it possible that the enteroendocrine cells are also undergoing adaptations following chronic HF feeding? Along these lines, Gniuli et al. (18) recently reported that 30-day feeding of a diet rich in saturated fat to male Wistar rats significantly elevated GIP levels following an oral glucose challenge; in a follow-up study, the authors report that HF-feeding stimulates duodenal proliferation of endocrine cells which differentiate into GIP-
secrting K cells (17). In a similar study, ob/ob mice challenged with a HF diet had elevated plasma GIP levels compared to ob/ob mice fed a high-carbohydrate (HC) or chow diet (4). Both high energy diets (HF and HC) induced K cell hyperplasia; however, the HF diet additionally increased intestinal GIP concentration and content, which resulted in the elevated plasma GIP levels. Whether or not the ob/ob mice had a stronger propensity for K cell hyperplasia than the lean mice following a chronic high-energy diet was never addressed in the study, thus making the results difficult to interpret for diet-induced obese animals without genetic disruptions. Regardless, the data do suggest that consumption of a HF diet increases production and secretion of GIP. These studies, in combination with our data, demonstrate that consumption of a HF diet results in elevated GIP levels. Furthermore, the fat in the diet is elevating GIP levels via K cell hyperplasia and increasing intestinal GIP concentration and content.

Unlike the GIP data, the consumption of a diet higher in fat alone does not explain the elevated GLP-1 levels. The HF-PF group did not have increased lymphatic GLP-1 concentrations despite consuming a diet with a higher fat content, suggesting that the increased energy intake of the HF group is causing the rise in GLP-1 secretion. Although difficult to explain at this time, given the complexity of the GLP-1 secretory system, this observation is of interest. The excess caloric intake could be causing hypertrophy and/or hyperplasia of the distal small intestine epithelium, sensitizing the GLP-1-secreting L cells to nutrient stimuli, and/or altering the endocrine and neural signaling on the L cell. Recent studies have demonstrated that hyperleptinemia, hyperinsulinemia, and hyperglycemia (all secondary consequences of obesity) affect the secretion of GLP-1 (2, 26, 47); however, in our study, we did not observe elevated leptin, insulin, or glucose levels in our HF-fed group that could explain the altered GLP-1 secretion. Future work is needed to understand the mechanism behind the hyperphagia-induced elevated GLP-1 secretion.

In conclusion, we found that rats chronically fed a HF diet had greater lymphatic GIP and GLP-1 responses to a mixed nutrient challenge; furthermore, these responses occurred without the increased fat
mass and weight gain generally associated with chronic feeding of a HF diet. The data suggests that the alterations in incretin secretion may occur prior to the development of obesity. Data from the HF-PF group in the 3 week feeding trial suggested that the driving forces behind the elevated incretin levels are different for GIP and GLP-1. The increased GIP secretion is driven by the greater percentage of fat intake, whereas the increased GLP-1 secretion is driven by the excess caloric intake. Further studies are needed to investigate the mechanisms underlying the effect of a high-fat diet on GIP secretion and of hyperphagia on GLP-1 production and secretion.

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REFERENCES


Energy intake (A), body weight (B), and body fat percentage (C) of rats fed a HF (n=8) or LF (n=8) diet ad libitum for 13 wk. Values are means ± SE. *HF vs. LF; p < 0.05.
FIGURE 2

Hourly lymphatic GIP concentration (A) following a duodenal mixed meal challenge (Ensure, 3.125 kcal) in rats fed a HF (n=7) or LF (n=7) diet for 13 wk. Lymph was collected continuously for 3 h. (B) represents the 0-60 min AUC. Values are means ± SE. *HF vs. LF; p < 0.05
FIGURE 3

Hourly lymphatic GLP-1 concentration (A) following a duodenal mixed meal challenge (Ensure, 3.125 kcal) in rats fed a HF (n=7) or LF (n=7) diet for 13 wk. Lymph was collected continuously for 3 h. (B) represents the 0-60 min AUC. Values are means + SE. *HF vs. LF; p < 0.05.
FIGURE 4

Daily energy intake (A) of rats fed a HF (n=14) or LF (n=16) diet *ad libitum* for 3 wk. Cumulative energy intake (B) of the HF (n=14), LF (n=16), and HF-PF (n=8) groups. Values are means ± SE. *HF vs. LF, #HF vs. HF-PF; p < 0.05.
FIGURE 5

Body weight (A) and body fat percentage (B) of the 3-wk-fed HF (n=14), LF (n=16), and HF-PF (n=8) groups. Values are means ± SE. ^LF vs. HF-PF; p < 0.05.
Hourly lymphatic GIP concentration (A) following a duodenal mixed meal challenge (Ensure, 3.125 kcal) for the 3-wk-fed HF (n=8), LF (n=8), and HF-PF (n=5) groups. Lymph was collected continuously for 3 h. *HF vs. LF, #HF vs. HF-PF, ^LF vs. HF-PF; p < 0.05. (B) represents the 0-60 min AUC. *p < 0.05 vs. LF. Values are means ± SE.
Hourly lymphatic GLP-1 concentration (A) following a duodenal mixed meal challenge (Ensure, 3.125 kcal) for the 3-wk-fed HF (n=8), LF (n=8), and HF-PF (n=5) groups. Lymph was collected continuously for 3 h. *HF vs. LF, #HF vs. HF-PF; p < 0.05. (B) represents the 0-60 min AUC. Values are means + SE.