Direct and Indirect Effects of Fatty Acids on Secretion of the Antidiabetic Hormone, Glucagon-Like Peptide-1

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Physiology
University of Toronto

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In Memory of My Zio Fiore Rocca

Dedicated to My Parents, Palmerino and Ambrosina, and Family, Aninna, Saverio, Cindy, David and Cathy
If you can keep your head when all about you
Are losing theirs, and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
    Or being hated, don't give way to hating,
And yet don't look too good nor talk too wise;
If you can dream and not make dreams your master,
If you can think, and not make thoughts your aim,
If you can meet with triumph and disaster,
And treat those two imposters just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools;
If you can make one heap of all your winnings,
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings,
And never breathe a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the will which says to them "Hold on!"
If you can talk with crowds and keep your virtue,
Or walk with kings—nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
    With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And—which is more—you'll be a Man, my Son!

Rudyard Kipling
Who gathers knowledge
gathers pain.

Ecclesiastes 1:18
DIRECT AND INDIRECT EFFECTS OF FATTY ACIDS ON SECRETION OF THE ANTIDIABETIC HORMONE, GLUCAGON-LIKE PEPTIDE-1

Department of Physiology, University of Toronto

ABSTRACT

Glucagon-like peptide-1 (GLP-1) is produced from the proglucagon molecule by tissue-specific post-translational processing within intestinal L cells. GLP-1 regulates fuel homeostasis through its ability to stimulate the glucose-dependent secretion of insulin and to inhibit the secretion of glucagon. Furthermore, GLP-1 is a potent inhibitor of gastric acid secretion and motility, is a central satiety factor, and augments insulin sensitivity in peripheral tissues. Therefore, GLP-1 holds great potential for the treatment of patients with diabetes. By fully understanding the mechanisms that govern the secretion of GLP-1, the therapeutic potential of this hormone may be realized through strategies designed to increase its endogenous levels. Given the biphasic response of GLP-1 secretion to fat ingestion, it was hypothesized that fat has both direct and indirect effects on the L cell. To demonstrate this, both in vivo and in vitro techniques were utilized in the rat model. Long-chain monounsaturated fatty acids directly stimulated GLP-1 secretion whereas shorter chain length- and saturated fatty acids were not stimulatory to secretion. An atypical isoform of protein kinase C, PKC-ζ, and a fatty acid binding protein were hypothesized to be involved in fat-induced GLP-1 secretion and their presence within L cells was confirmed by immunohistochemistry. Moreover, diets rich in monounsaturated fatty acids have been reported to improve glycemic tolerance in patients with type 2 diabetes. These diets were demonstrated to benefit glycemic control through increased secretion of GLP-1. The
glycemic improvement was shown to be specific to GLP-1, as the GLP-1 receptor antagonist, exendin\(^{39}\), completely abolished the benefit induced by a monounsaturated fatty acid diet. Finally, the indirect mechanism governing fat-induced GLP-1 secretion was demonstrated to involve an interaction between the intestinal hormone glucose-dependent insulinotropic peptide and the vagus nerve, with the role for the vagus nerve being predominant. Therefore, secretion of the antidiabetic hormone, GLP-1, involves a complex array of luminal, neural and endocrine elements, with ingested fat stimulating GLP-1 secretion through both direct and indirect pathways. In the future, these regulatory mechanisms may be modulated to increase the secretion of GLP-1 as a therapeutic approach to the treatment of patients with type 2 diabetes.
ACKNOWLEDGMENTS

A project of this magnitude cannot be done without the help and encouragement from many people. I would like to begin by expressing my gratitude to the Department of Physiology and the Medical School at the University of Toronto for allowing me to continue my studies even though the course did not always follow the designated path. I would especially like to thank Judy Irvine for her patience and visionary commitment to my endeavours. The studies could not have been undertaken without the generous financial support from a number of sources including the Department of Physiology, the Banting and Best Diabetes Centre, the Canadian Diabetes Association and the Santalo and Hunt estates. During the course of any doctoral project long periods of lows and glooms are encountered. These were made bearable and brighter by the many supportive faces around the Medical Sciences Building. I would therefore like to thank all those at the security department, radiation protection department, teaching labs and cafeteria for their encouragement through the years.

I have received a great deal of thoughtful advice from my supervisors over the course of my studies. I would like to thank the members of my supervisory committee, Drs. Giacca, Steiner, and Gaisano for their commitment to the education of graduate students. Furthermore, Dr. DeBonì, and my teachers in the pre-clerkship years of medical school, Drs. Taylor, Wiley and Murray, provided inspiration, encouragement and friendship, which is greatly appreciated. Dr. Silverman and the MD/PhD program have given me the challenging and exciting opportunity to combine clinical medicine with basic science, and for this I am truly thankful.
Over the course of my studies, I have been extremely fortunate to work with some of the most entertaining and interesting students. Their support and camaraderie did wonders for enduring the rigors of the scientific method. Karen Gronau, Savita Dhanvantari and Devin Tucker first welcomed me into the lab and showed me that doing science can be fun. Jackson Huang, Ilias Iliopolous, Julie Kalitsky, Sari Cooper, Lilianne Dableh, Anoush Migirdicyan, Jonathon LaGreca and Patrick MacDonald all provided support mixed with lots of laughs along the way. The “Vranic Boys” consisting of Julian Mathoo, Simon Fisher, Mike Lekas and Rich McCaul provided advice and a sense of companionship early on. Jamie Joseph, Wendy Tavares, Kirk Fischer, Feisal Adatia, Natalie Walsh and Marie-Claude L’Heureux helped make the experience more enjoyable and I am thankful for the thoughtful conversations and coffee breaks we shared. Mark “Cremaster” Dacambra is truly a great friend and I am grateful for the opportunity to know him. Angelo Izzo is the backbone of the laboratory, the keystone holding everything in place. The years of laughter, his sense of humor, as well as his shrewd advice and strong friendship have helped me in more ways than I can think of.

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Above all, I have received immense support from my family. David and Cindy have always been ready with a funny story to divert my attention when it was needed and Saverio was always there to help me out when experiments ran late. My grandmother always had kind words, love and support ready. The Ernies were always there to cheer me up. I owe everything to my parents, Ambrosina and Palmerino, without their love and support I would be nowhere. My mother’s thoughtfulness and patience combined with my father’s creativity and perseverance have given me the tools needed to succeed in this endeavour. My greatest friend, Cathy has been with me from the start. She began as my friend, then became my fiancé and now my wife. She has shown the utmost patience with me and the demands that this work has placed on me and my time. Her forgiving nature and loyalty through the years is immeasurable and cannot be described. For this, I am entirely grateful and love her with all my heart and then some. I will always “wait and hope” with her.
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<td>ANOVA</td>
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<td>ATP</td>
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<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>Cholecystokinin</td>
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<td>CO</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>FRIC</td>
<td>Fetal rat intestinal cell</td>
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<td>gGLI</td>
<td>gut Glucagon-like immunoreactivity</td>
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<td>Glucose-dependent insulinotropic peptide</td>
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<td>GRP</td>
<td>Gastrin-releasing peptide</td>
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<td>Glicentin-related pancreatic polypeptide</td>
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<td>GUE</td>
<td>Proglucagon gene upstream enhancer</td>
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<td>HIT</td>
<td>Hamster insulin tumor cells</td>
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<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<td>intracerebroventricular</td>
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<td>i.v.</td>
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<td>Immunoglobulin G</td>
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<td>IRG</td>
<td>Immunoreactive glucagon</td>
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<td>K⁺</td>
<td>Potassium</td>
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<td>kb</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>OO</td>
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<td>PAM</td>
<td>Peptidyl-glycine α-amidating monooxygenase</td>
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<td>PBS</td>
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PC2: Prohormone convertase-2
PGDPs: Proglucagon-derived peptides
PKA: Protein kinase A
PKC: Protein kinase C
PLC: Phospholipase C
PMA: Phorbol 12-myristate 13-acetate
PPAR: Peroxisome proliferator-activated receptors
PYY: Peptide YY
RIA: Radioimmunoassay
s.c.: subcutaneous
SEM: Standard error of the mean
SFA: Saturated fatty acid
SNARE: Soluble NSF attachment protein receptors
STC-1: Secretin tumour cell-1
TFA: Trifluoroacetic acid
VIP: Vasoactive intestinal peptide
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1 INTRODUCTION

The discovery of Glucagon-like peptide-1 (GLP-1) has inspired a tremendous amount of scientific interest as this peptide hormone offers the potential to become a novel therapy for the epidemic of the present era. GLP-1, produced in the intestine, possesses a variety of functions that can effectively combat the major pathogenic factors that characterize type 2 diabetes. This extremely prevalent disease endangers the health and welfare of people living in both industrialized as well as emerging nations. Type 2 diabetes impacts the functioning of almost every organ system in the body, but is mainly characterized by the presence of a relative insulin secretory defect in the face of insulin resistance. Produced from the proglucagon molecule, GLP-1 has the potential to benefit the diabetic condition on several fronts. This hormone can effectively direct the metabolism of fuel at a fundamental level by altering the secretion rates of the two primary controllers of fuel fluxes, namely insulin and glucagon. Furthermore, the wide-ranging effects of GLP-1 can be utilized to regulate the rate of nutrient absorption, assimilation, and ingestion. However, there are several serious limitations to the prospects of utilizing this peptide hormone for diabetic therapy. First, the body metabolizes GLP-1 extremely rapidly, thus greatly impacting the effectiveness of this hormone. Additionally, efforts to maintain appropriate circulating levels, at present, require inconvenient and invasive injection therapy. An alternative approach to achieving therapeutic levels is to enhance the secretion of GLP-1, capitalizing on the fact that GLP-1 is an endogenous hormone. In order for this approach to be successful, the mechanisms that govern the secretion of GLP-1 must be fully characterized. The following body of work reviews the existing literature concerning the antidiabetic hormone GLP-1 and describes
several investigations into the mechanisms that regulate the secretion of this important peptide.

1.1 Proglucagon Gene Expression

GLP-1 is produced from the proglucagon molecule. In humans, the single gene encoding this prohormone is located on the long arm of chromosome 2 (1,2) and consists of 6 exons and 5 introns. The structure of the human proglucagon gene is demonstrated in figure 1-1. Exon 1 encodes the 5' untranslated region and exon 2 encodes the signal sequence, while exons 3, 4, and 5 encode the sequences of 3 bioactive peptides which are proteolytically cleaved from proglucagon to form glucagon, GLP-1, and glucagon-like peptide-2 (GLP-2), respectively (3). In contrast, other strategies for generating this diversity of regulatory peptides are utilized in lower organisms. For example, in chicken and trout, alternative mRNA splicing is responsible for the expression of glucagon and GLP-1 in the pancreatic α cell and glucagon, GLP-1 and GLP-2 in the intestinal L cell (4). In addition to alternative proteolytic cleavage and mRNA splicing, a third mechanism with the potential to generate diversity in the production of the proglucagon-derived peptides (PGDPs) is used in the sea lamprey. This organism produces two proglucagon mRNA transcripts that possess differing coding potential; the first encodes glucagon and GLP-1 while the second encodes GLP-2 and a potential glucagon (5). Finally, three distinct GLP-1-like peptides are encoded by the proglucagon gene in Xenopus, in addition to glucagon and GLP-2 (6).
Figure 1-1  The Proglucagon-derived Peptides

In mammals, the proglucagon gene is expressed in several tissues, including the endocrine pancreas, brain, and intestine, under the control of several DNA elements located in the 5' untranslated region. Specific DNA sequences have been identified that are important for control of proglucagon promoter activity in these tissues. Elements important for proglucagon gene expression within islet cells are located within 300 bp upstream of the transcriptional start site (7,8). These have been designated G1-G4. G3 is located between -274 and -234 bp and enhances the expression of the proglucagon gene in islet cells (8). It is made up of two subdomains termed G3A and G3B. G3A, but not G3B, has been demonstrated to bind nuclear proteins found in islet cells and enhance the transcription of the proglucagon gene (9). The second enhancer element is termed G2 and forms three distinct complexes with several islet nuclear proteins (8). The enhancer elements, G2 and G3 are capable of independently enhancing transcription of both heterologous and homologous promoters in α cells (8). Expression of the proglucagon gene in the pancreatic α cell is regulated by the two additional elements of the proglucagon promoter, G4 and G1. G4 is capable of binding nuclear proteins from insulin and glucagon-producing cells, thereby directing expression to islet cells (10). Sequences found within G1 are also important for islet proglucagon gene expression and are critical for directing expression of the proglucagon gene specifically to α cells (7,8,10,11). The critical nucleotides for α cell expression within G1 have been further delineated to nucleotides -118 and -60 (11). Disruption of complexes formed within the G1 element is associated with a decrease in proglucagon gene transcription. Given its low intrinsic transcriptional activity, G1 is considered to be a classical promoter (11).
In contrast to the α cell, very little is known about the DNA elements that are necessary for proglucagon gene expression in the brain or the intestinal L cell. In attempts to produce transgenic mice expressing SV40 large T antigen under the control of the proglucagon promoter, it was discovered that the transgene was expressed only in the pancreas and brain when 1.3 kb of the rat proglucagon gene 5' flanking sequence was fused to the large T antigen coding sequence (12). This implies that the elements important for proglucagon gene expression in the α cell and brain, but not the L cell, are contained within the proximal 1.3 kb of the 5' flanking sequence (7). However, when 2.3 kb of the proglucagon gene 5' flanking sequence was used, expression of the transgene was detected in the intestine, as well as the brain and pancreas, suggesting that the sequences necessary for intestinal proglucagon gene expression are located in a region spanning -2252/-1252 bp (13). Further investigation revealed that the proglucagon gene upstream enhancer, termed GUE, is located in this region and is essential for intestinal proglucagon gene expression (13,14). These sequences may also regulate α cell proglucagon gene expression, as they are capable of binding nuclear proteins from islet cell lines. These findings indicate that the GUE sequences are complex, containing several subdomains that are capable of binding islet- and enteroendocrine-cell nuclear proteins (14). Despite this recent progress, the factors that regulate intestinal proglucagon gene expression remain relatively unknown.

Several transcription factors are known to bind to and modulate the activities of the specific elements that regulate proglucagon transcription in the pancreas and/or intestine. The caudal-related gene, cdx-2/3, encodes a homeodomain protein that is expressed in both the pancreatic α cell and the intestinal L cell (15). This transcription factor can bind to the
G1 promoter and enhances gene expression in both pancreatic and intestinal cells (15,16). The homeobox protein isl-1 has also been demonstrated to bind to the G1 element and enhance gene expression specifically in the pancreatic α cell (17). A family of DNA-binding proteins that are found in abundance in liver, termed the Hepatocyte-Nuclear Factors (HNF), bind to the major proglucagon gene enhancer G2 and regulate proglucagon gene transcription. HNF-3α,-3β and -3γ are known to interact with the proglucagon gene. In fact, knockout mice lacking HNF-3α are hypoglycemic due to decreased production of glucagon associated with a 70% reduction in the expression of the proglucagon gene (18). Three isoforms of HNF-3β are also present in glucagon-producing cells and competitively interact with the G2 enhancer to regulate proglucagon gene transcription. HNF-3β1 is the only member of this family that is capable of repressing gene transcription (19). Finally, the transcription factor, pax-6, is essential for expression of the proglucagon gene in intestinal L cells (20).

Expression of the proglucagon gene in the pancreatic α cell and the intestinal L cell is differentially regulated by a number of extra- and intracellular signals. For example, the islet hormone insulin modifies proglucagon gene transcription in a hamster islet cell line (21). This effect is mediated by an insulin-responsive element in the proglucagon gene that corresponds to the G3A enhancer subdomain (22). Two islet-specific nuclear proteins interact with this G3A element, as well as with the G1 promoter, and are therefore potentially involved in the inhibitory effects of insulin on proglucagon gene transcription (23). In a similar study, elevation of plasma glucose levels for 5 days was associated with significant decreases in proglucagon mRNA levels (24). Evidence for the involvement of one of the
PGDPs in the suppression of proglucagon gene transcription was derived from experiments in which GLUTag (proglucagon-large T antigen) enteroendocrine L cell tumors were transplanted subcutaneously in nude mice. Elevated plasma levels of the PGDPs, including glucagon, in this animal model were associated with marked suppression of pancreatic proglucagon gene transcription, indicating that one of the PGDPs acts as a negative feedback signal on the pancreatic α cell (25). The specific identity of this PGDP remains to be established. Pancreatic proglucagon gene expression also appears to be mediated by a variety of classical second messenger systems. Protein kinase A (PKA) phosphorylates a transcription factor named CREB (cAMP-response element binding protein) which then dimerizes and binds specifically with the cAMP (cyclic Adenosine monophosphate) response element (CRE) that is located at −292 bp in the 5' untranslated region of the proglucagon gene (26). Phosphorylated CREB is capable of interacting with CREB-binding protein (CBP), which acts as a transcriptional coactivator, bridging the CRE and the basal transcriptional machinery (27). Binding of CREB to the proglucagon CRE is negatively modulated by CREB-associated proteins thereby decreasing the responsiveness of the CRE to stimulation by cAMP (28). Furthermore, activation of protein kinase C (PKC) by phorbol esters in αTC2 mouse islet cell line and in a cell line derived from a rat pancreatic islet cell tumor is similarly associated with increased proglucagon gene transcription (29,30). A PKC response element has been mapped to the G2 enhancer region and requires HNF-3β for enhancement of transcription (29). Intracellular Ca++ has also been demonstrated to influence proglucagon gene expression at the level of CREB binding to the proglucagon CRE (31,32).
In contrast to the pancreatic α cell, the control of L cell proglucagon gene transcription is relatively unknown. The effect of insulin on intestinal proglucagon gene expression has recently been suggested to be inhibitory, as levels of proglucagon gene mRNA transcripts were increased in streptozotocin-treated rats, and this was reversed upon treatment with insulin (33). The effect of the intestinal products of proglucagon on proglucagon gene transcription has not been documented. However, Glucose-dependent Insulinotropic Peptide (GIP), a hormone released from the upper gastrointestinal tract, induced significant increases in the content of PGDPs in a fetal rat intestinal cell (FRIC) culture model (34), implying that proglucagon mRNA levels are also increased by GIP. GIP is known to act through a 7-transmembrane spanning G protein-coupled receptor (GPCR), therefore increasing cellular levels of cAMP upon receptor activation. This second messenger increases the levels of proglucagon mRNA in FRIC cultures (35) and enhances proglucagon gene expression in the GLUTag enteroendocrine cell line (36). Gastrin-releasing peptide (GRP), an enteric neuropeptide, also stimulates transcription of the proglucagon gene in the GLUTag cell line (37). As GRP operates through a PKC-linked second messenger system, the role of PKC in intestinal proglucagon gene transcription has been evaluated. Unlike in pancreatic α cells, phorbol esters which are known activators of PKC, do not affect proglucagon gene expression in models of the intestinal L cell such as FRIC cultures (35) or the GLUTag cell line (38). Interestingly, GRP also leads to increases in cellular Ca++ levels; however, the effects of Ca++ on proglucagon gene expression in the L cell are not known.

Proglucagon gene expression in pancreatic α cells and intestinal L cells is differentially affected by several important physiological processes. For example, fasting
and hypoglycemia have been demonstrated to increase pancreatic proglucagon mRNA levels (39), whereas levels of proglucagon mRNA in jejunum and ileum are decreased by fasting (40). Although the L cell is found predominantly in the ileum and colon (41), jejunal but not ileal levels of proglucagon mRNA return to control levels upon refeeding. These findings indicate that the jejunum may be a smaller but more sensitive site for synthesis of proglucagon mRNA (40). Although the intestinal L cell is exposed to both circulating and luminal nutrients, the latter are believed to have a more important effect on proglucagon gene expression. Peptones have been found to increase intestinal proglucagon gene expression as observed in intestinal cell lines but not in pancreatic cell lines (42). Dietary fiber is also associated with increases in intestinal proglucagon gene expression (43,44). This may be due, in part, to increases in the levels of short chain fatty acids as a result of colonic fermentation. In support of this hypothesis, intraluminal infusion of long chain triglycerides increases proglucagon mRNA in jejunum but not ileum (40). Interestingly, intestinal proglucagon gene expression seems to exhibit a degree of adaptation to altered nutrient fluxes, as jejunoileal resection leads to increases in colonic proglucagon gene expression independent of caloric load (45).

1.2 Post-translational Products of Proglucagon

A single proglucagon molecule is translated in the brain, pancreas, and intestine (46,47). However, the PGDPs that are synthesized in and secreted from these tissues result from differential cell-specific post-translational processing of proglucagon. The major products of proglucagon processing in the pancreas are glucagon and the major proglucagon fragment (MPGF), but are glicentin, oxyntomodulin, GLP-1 and GLP-2 in the intestinal L cell (47) (Fig.1-1). Recently, the mechanisms responsible for such differential post-
translational processing of prohormones have been elucidated. The cloning of the endoprotease Kex2 in yeast led to the subsequent discovery of several mammalian homologues including enzymes such as prohormone convertase-1 (PC1), PC2 and furin. These are similar to the subtilisin family of Ca++-dependent endoproteases (48,49). Furin is ubiquitously expressed (50,51), whereas PC1 and PC2 expression is restricted to endocrine and neuroendocrine tissues (52,53). A number of related endoproteases have recently been discovered, however, PC1 and PC2 are responsible for the processing of numerous prohormones, such as proopiomelanocortin (54), proinsulin (55), and prosomatostatin (56), by endoprotease-mediated cleavage at pairs of basic amino acids. Localization of PC1 in intestinal L cells (57) and PC2 in pancreatic α cells (58) suggests that these endoproteases are responsible for the production of the intestinal and pancreatic PGDPs, respectively. Furthermore, a correlation study performed by analyzing PC1 and PC2 mRNA levels in several cell lines (InR1-G9, RIN 1056A and STC-1) indicates that the presence of PC1 correlates with the intestinal pattern of proglucagon processing whereas PC2 expression correlates with the production of pancreatic glucagon (59). PC1 and PC2 enzyme levels are also co regulated with levels of the prohormone. For example, PC1 and PC2 expression correlates with proopiomelanocortin regulation by dopamine (60). Whether this is the case with PC2 and proglucagon in the α cell remains to be demonstrated, however, elevation of cellular levels of cAMP stimulates parallel increases in PC1 and proglucagon gene expression in the GLUTag cell line (61). Thus, the tissue specificity of proglucagon processing appears to be mediated by tissue-specific expression of the PCs in pancreas and intestine.
1.2.1 Pancreatic Proglucagon Processing

Proglucagon processing within the pancreatic α cell results in the production of glicentin related pancreatic polypeptide (GRPP), intervening peptide-1 (IP-1), glucagon and MPGF, as well as small amounts of glicentin, oxyntomodulin, and GLP-1 (47,59,62) (Fig. 1-1). As discussed above, PC2 is implicated in the production of glucagon from proglucagon by its immunolocalization to the α cell (58). Furthermore, in αTC1-6 cells, a transformed α cell line that produces glucagon, high levels of PC2 but not PC1 mRNA levels were found. The expression of antisense PC2 mRNA drastically altered proglucagon processing in these cells, leading to a decrease in the production of glucagon as well as glicentin (63). However, in antisense PC2 studies conducted in InR1-G9 cells (64) and in αTC1-6 cells (65), production of glucagon was not altered by a >62% decrease in PC2 mRNA levels. In agreement with previous studies (63), however, is the finding that antisense PC2 expression caused a significant decrease in levels of glicentin (64,65). Furthermore, in vitro processing of proglucagon by PC1 and/or PC2 does not result in the production of glucagon (66), and co-expression of PC2 and proglucagon in heterologous endocrine and non-endocrine cells does not yield glucagon, but does result in the production of glicentin (67). Overexpression of proglucagon and PC2 with PACE4, PC5a or PC5b also does not lead to synthesis of glucagon (67). Nonetheless, knockout of the PC2 gene leads to a deficiency in pancreatic glucagon levels (68). Therefore, the pancreatic α cell may utilize combinations of known and/or unknown PC-like endoproteases to completely process proglucagon to glucagon.

1.2.1.1 Glucagon

Glucagon functions in concert with insulin in the regulation of bodily glucose fluxes. This 29 amino acid hormone acts to prevent hypoglycemia by stimulating hepatic glucose
production through the augmentation of gluconeogenesis and glycogenolysis (69,70). Binding of glucagon to its receptor on hepatocytes results in an increase in the cellular levels of cAMP, and the subsequent activation of PKA leads to the phosphorylation of key enzymes that regulate glycolysis, gluconeogenesis and glycogenolysis (71). As a result, net hepatic glucose production occurs. Furthermore, the inhibition of glycolysis induced by glucagon action leads to the suppression of fatty acid synthesis in favour of ketone production. Therefore, glucagon serves as a prime regulator of fuel fluxes in that it maintains an adequate fuel source for cerebral utilization. Glucagon may also stimulate lipolysis in the adipocyte (72). In addition to these effects, glucagon is a potent regulator of insulin secretion, acting in the pancreatic network to increase the secretion of insulin (69,70). The functions of the other pancreatic PGDPs are not known at the present time.

The mechanisms that govern the secretion of glucagon are extremely complex and only partially understood. Regulation of glucagon secretion is mediated, in part, by intrasislet mechanisms. Insulin is capable of inhibiting the secretion of glucagon; as well, somatostatin, released upon elevations in plasma glucagon, inhibits the secretion of glucagon in a negative-feedback manner (69,70,73). In addition, the α-adrenergic system stimulates the secretion of glucagon (69). However, the secretion of glucagon is primarily regulated by the ambient glucose concentration. In hypoglycemia, the secretion of glucagon is increased while the secretion of insulin is decreased. This effect on glucagon secretion likely occurs via two mechanisms. Hypoglycemia may directly act on α cells to increase the secretion of glucagon. However, the fall in insulin levels associated with hypoglycemia can also release the tonic inhibition of glucagon secretion mediated by insulin. Finally, during high protein intake in combination with low levels of carbohydrate, amino acid-induced secretion of
glucagon acts to prevent the hypoglycemia induced by the concomitant secretion of insulin (69).

### 1.2.2 Brain Proglucagon Processing

The proglucagon gene is expressed in the hypothalamus and brainstem, suggesting that the PGDPs may serve as neuromodulators or neurotransmitters in certain neural processes (46). The proglucagon gene is expressed at 100-fold higher levels in the brainstem compared with the hypothalamus (46). GLP-1 immunoreactive neurons have been detected in the dorsal motor nucleus of the vagus nerve as well as in the paraventricular nucleus. Glucagon staining was not detected in the hypothalamus but weak staining was present in the brainstem suggesting that the post-translational processing of proglucagon in the brain differs depending on the neural region involved (46). Radioimmunoassay of the adult rat hypothalamus, indicated that glicentin, oxyntomodulin, and low levels of glucagon are present. Therefore, the processing of proglucagon in hypothalamus seems to be intermediate between that of the pancreas and intestine (74). Furthermore, ontogeny experiments demonstrated that the processing of proglucagon undergoes significant changes during development. The ratio of glicentin/oxyntomodulin to glucagon increases from 3 to 46 throughout development, so that the adult hypothalamus contains a predominance of glicentin/oxyntomodulin (74,75). Whether this is due to changes in the expression of the PC enzymes is not known. Interestingly, activation of PKA stimulates both the synthesis and secretion of the PGDPs (76), while the excitatory neurotransmitter, glutamate, significantly increases the secretion but not the synthesis of the PGDPs from fetal rat hypothalamic cultures through the metabotropic glutamate receptor (77). The function of the other PGDPs present in the hypothalamus and brainstem is unknown, however, GLP-1, expressed in
specific neurons of the hypothalamus, may play a role in the regulation of food intake (refer to section 1.3.4).

1.2.3 Intestinal Proglucagon Processing

Cleavage of the proglucagon molecule in the intestine results in a number of peptides including several forms of the antidiabetic hormone GLP-1, as well as glicentin, oxyntomodulin, intervening peptide-2 (IP-2) and GLP-2 (47) (Fig. 1-1). Several studies demonstrate that, unlike the pancreatic processing of proglucagon, the intestinal PGDPs are formed entirely through the actions of PC1. Vaccinia virus-mediated expression of PC1 and proglucagon in heterogeneous endocrine cell lines is both necessary and sufficient to produce the intestinal PGDPs (67). This finding is confirmed by an in vitro study of proglucagon processing within αTC1-6 cells (66) as well as a PC1 antisense study (65). Overexpression of PC1 in an islet cell line similarly leads to increased production of glicentin, oxyntomodulin and GLP-2 (64). Curiously, the production of GLP-1^{7-36NH2} was not increased in this study, indicating that PC1 may not cleave the first six amino acids from the full length GLP-1^{1-36NH2} molecule to produce the truncated form of GLP-1 (64). This truncation is crucial for the bioactivity of GLP-1. GLP-1 is also amidated at the carboxyl terminal end through the actions of peptidyl-glycine α-amidating monooxygenase (PAM) (34). However, this amidation is not important in conferring biological activity, as both GLP-1^{7-37} and GLP-1^{7-36NH2} are equipotent in terms of insulin stimulation (78). Nonetheless, the majority of circulating GLP-1 is the bioactive amidated form, GLP-1^{7-36NH2} (79). In summary, therefore, the proglucagon molecule is predominantly cleaved by PC1 in the intestine to liberate a number of PGDPs with varying functions including glicentin, oxyntomodulin, GLP-2, and GLP-1.
1.2.3.1 *Glicentin*

Glicentin is a 69 amino acid peptide encoded in the amino terminal end of proglucagon. It encompasses the complete sequence of glucagon as well as oxyntomodulin and IP-1 (Fig. 1-1). A specific function for this member of the intestinal PGDPs is not defined, however, varied reports indicate that it may possess some biological activities. An early study indicated that glicentin inhibits the secretion of gastric acid stimulated by pentagastrin (80). A recent study of the growth promoting effects of the intestinal PGDPs also demonstrated a small, but significant role for glicentin in mediating growth of the intestinal mucosa (81). However, a receptor specific for glicentin has not been discovered.

1.2.3.2 *Oxyntomodulin*

Oxyntomodulin consists of the glucagon sequence plus IP-1. In contrast to glicentin, oxyntomodulin is well documented as possessing several biological functions. It is a potent inhibitor of both basal (82) and hormonally stimulated gastric acid secretion (82-86). However, when compared to the inhibition effected by intestinal nutrients, oxyntomodulin only partially inhibits gastric acid secretion, suggesting that a mixed meal stimulates the secretion or activity of other enterogastrones (85). The acid-suppressing activity of oxyntomodulin is confined to the carboxy-terminal fragment (residues 19-37), as this peptide suppresses acid-secretion to the same extent as full-length oxyntomodulin (86). Oxyntomodulin-mediated inhibition of acid secretion is accompanied by increases in cellular cAMP suggesting that this second messenger system is responsible for the biological effects of oxyntomodulin (84). However, in a purified rat parietal cell preparation, oxyntomodulin causes increased cAMP levels as well as an increase in acid secretion. This function of oxyntomodulin was found to be mediated by activation of the GLP-1 receptor, as
oxyntomodulin-stimulated acid secretion was inhibited by the GLP-1 receptor antagonist, exendin\(^{9-39}\) (87). This is consistent with the finding that no receptors for oxyntomodulin have been cloned to date. Therefore, oxyntomodulin may act *via* an indirect mechanism to cause the inhibition of acid secretion in the whole organism. Consistent with this hypothesis is the fact that oxyntomodulin can stimulate the secretion of the inhibitory peptide somatostatin *in vitro* in somatostatin-secreting RIN T3 cells (88) as well as in primary fundic D cells (89). Therefore, the inhibitory actions of oxyntomodulin on acid secretion may be indirect, through somatostatin-mediated inhibition of the cAMP pathway. Finally, a novel function for oxyntomodulin has recently been described, that being stimulation of glucose uptake in the ileum (90,91).

### 1.2.3.3 GLP-2

A function for this 33 amino acid intestinal PGDP has been recently elucidated. For years, increased levels of the PGDPs have been linked with growth and adaptation of the intestinal mucosa, as two patients described to have glucagon-producing tumors had associated villous enlargement in the small intestine (92,93) which regressed upon tumor resection (92). Many correlative studies have since shown increased intestinal PGDPs in parallel with gut growth (94,95). Finally, when analyzed by subcutaneous (s.c.) injection of synthetic PGDPs, GLP-2 was found to markedly induce the growth of the small intestine (81), in association with increased crypt cell proliferation and decreased rates of villus tip apoptosis (81,96). The newly developed mucosal layer is characterized by normal to enhanced function in terms of nutrient absorption (97) and the GLP-2-induced growth of the intestine may also be associated with acute increases in the rate of glucose uptake (98). Further evidence for the growth-promoting effect of GLP-2 is demonstrated by the finding
that GLP-2 levels are increased in models of intestinal adaptation such as streptozotocin-induced diabetes in rats (33). Investigations of the potential role of GLP-2 to act as an enterogastrone have also recently been performed, and demonstrate that intravenous (i.v.) infusions of this peptide inhibit insulin hypoglycemia-induced antral motility in pigs (99) and sham feeding-induced gastric acid secretion in humans (100). These findings are consistent with the presence of the GLP-2 receptor throughout the gastrointestinal tract (101). Thus, GLP-2, cleaved from proglucagon in the intestinal L cell represents an important mediator of intestinal adaptation that may prove to have substantial clinical benefits in the future (102).

1.3 Antidiabetic Functions of Glucagon-like Peptide-1

GLP-1 possesses several biological actions that are important in the regulation of fuel fluxes. Most importantly, GLP-1 acts to increase the secretion of insulin in a glucose-dependent manner while inhibiting the release of glucagon. Therefore, by manipulating the plasma levels of the principle mediators of fuel metabolism, GLP-1 is capable of greatly influencing energy storage mechanisms. Furthermore, the ability of GLP-1 to slow the rate of gastric emptying and possibly, to increase the feeling of satiety, greatly reduces the load of nutrients that are being delivered to the absorptive surfaces of the gastrointestinal tract. Additionally, the peripheral uptake of digested nutrients may also be enhanced by GLP-1 action. Therefore, the many functions of GLP-1 encompass an effective range through which fuel metabolism can be modified. Thus, the roles of GLP-1 can be potentially manipulated in order to augment the treatment of type 2 diabetes. The multiple effects of GLP-1 are mediated through a specific receptor found in various tissues, the activation of which enhances the processes involved in efficiently storing energy sources.
1.3.1 GLP-1 Receptor

1.3.1.1 Structure and Function

The cDNA for the human islet GLP-1 receptor has been cloned (103,104) and found to belong to a subclass of the 7 transmembrane, GPCR superfamily which includes receptors for glucagon, GIP, secretin, calcitonin, vasoactive intestinal peptide (VIP) and parathyroid hormone (105). The 463 amino acid GLP-1 receptor shares 47% amino acid homology with the receptor for glucagon (103). The correct tertiary structure of the amino-terminal of the receptor is crucial for ligand binding, however, additional domains of the receptor are also involved since the amino-terminal domain demonstrates a lower binding affinity for GLP-1 compared with the full length receptor (106). N-glycosylation of this sequence is also important for ligand binding, as well as for correct membrane insertion (107). Elements important for signal transduction have also been examined by scanning mutagenesis, revealing that the third intracellular loop is critical for coupling to the adenylyl cyclase second messenger system, a major signaling mechanism for GLP-1 receptor activation (see section 1.3.1.3). Several specific residues within the third intracellular domain are implicated in adenylyl cyclase activation and may contribute to an α-helix domain that is necessary for directly coupling to the G-protein (108,109).

1.3.1.2 Receptor Localization

Although first cloned from an islet cDNA library, the presence of GLP-1 receptors on β cells has been confirmed by Northern and Western blotting studies, as well as by immunohistochemistry (110). Furthermore, GLP-1 receptors have also been localized on a somatostatin-secreting cell line (111) as well as on a sub population of α cells as demonstrated by autoradiography and immunohistochemistry (110). GLP-1 receptor mRNA
transcripts are also located in the stomach (112), and have been specifically localized to the parietal cell (113). This pattern of GLP-1 receptor expression is consistent with a role for GLP-1 in gastric acid regulation. GLP-1 binding and receptor mRNA transcripts have also been described in rat brain (112,114). Furthermore, GLP-1 receptor mRNA transcripts are also located extensively throughout the hypothalamus in the rat, consistent with a role in satiety (see section 1.3.4). Specific areas with dense localization include supraoptic, paraventricular, and arcuate nuclei. Other areas identified by in situ hybridization include medial and lateral preoptic areas, lateral hypothalamus, and dorsomedial nucleus (115). Evidence suggests that circulating GLP-1 is capable of mediating inhibitory effects on gastric motility through interaction with central brain regions (116). This concept is supported by the fact that circulating GLP-1 specifically binds to GLP-1 receptors expressed in circumventricular organs that are outside of the blood-brain barrier, including the subfornical organ and the area postrema (117). GLP-1 receptors are also found in the lung (112,114,118) and kidney (112,114) and have been detected by RT-PCR in liver as well as the small and large intestine (114). Although the role of GLP-1 receptors in the liver is controversial, with one report indicating that GLP-1 does not influence second messenger systems in this organ (119), GLP-1 has been reported to stimulate intestinal somatostatin secretion in vitro (120). GLP-1 receptor mRNA has also been localized to the heart, however, the physiological significance of this expression remains unknown (121). Interestingly, the presence of GLP-1 receptors has also been detected in skeletal muscle (122,123). Furthermore, GLP-1 receptors have been identified in adipose tissue from rat (124) as well from depancreatized dogs (122). Although several studies have established the presence and activity of the GLP-1 receptor in these tissues, these findings remain quite controversial as several negative studies
have also been reported for these tissues (103,114,119,125) Therefore, there is evidence supporting the presence of receptors in established targets of GLP-1 as well as in more controversial tissues such as the liver, muscle, and fat.

1.3.1.3 Signal Transduction

Expression of the GLP-1 receptor in several cell lines suggests that the receptor is capable of activating multiple second messenger systems including the adenylyl cyclase-cAMP pathway and the phospholipase C (PLC)-Ca\(^{2+}\) pathway (104,123). Interaction with G-proteins is suggested by evidence that indicates that guanine nucleotides are capable of decreasing the affinity of the GLP-1 receptor for its ligand in RINm5F cells (126). Indeed, recent evidence suggests that the GLP-1 receptor can interact with different G-protein \(\alpha\)-subunits including \(\text{Go}_s\), \(\text{Go}_{q11}\), and \(\text{Go}_{41,2}\) (127). The majority of studies to date indicate that G-protein activation by the GLP-1 receptor rapidly induces the production of cAMP through the adenylyl cyclase system as demonstrated in both \(\beta\) cells (128) and in heterologous cell lines expressing the GLP-1 receptor (123,126,129-131). However, coupling of the GLP-1 receptor to raised intracellular Ca\(^{2+}\) is also supported by several studies (123,130,132) suggesting that the GLP-1 receptor is similar to the parathyroid hormone receptor in its ability to signal through both the adenylyl cyclase system and intracellular Ca\(^{2+}\) (133). However, this rise in intracellular Ca\(^{2+}\) is rather controversial since there are also reports that Ca\(^{2+}\) levels are not changed in response to GLP-1 stimulation (126). Furthermore, in studies reporting an increase in intracellular Ca\(^{2+}\), the source of this calcium is also controversial and differs depending on the cell line utilized. For example, several studies indicate that extracellular calcium predominantly contributes to the rise in intracellular levels (129,131,134). However, in COS cells, the rise in intracellular Ca\(^{2+}\) is attributed to release
from intracellular stores and is associated with phosphoinositol turnover consistent with the stimulation of PLC (123). A recent study conducted in βTC3 cells links these two sources of Ca^{++} under the notion that a small influx of Ca^{++} from extracellular stores is capable of causing further release from intracellular stores through the PLC system (132). Therefore, the ability of the GLP-1 receptor to stimulate the adenyl cyclase and/or the PLC-Ca^{++} signaling systems is dependent on the cell type utilized. Nonetheless, it appears that the GLP-1 receptor is capable of modulating either or both of these two systems to achieve its biological function in various tissues.

1.3.1.4 Receptor Desensitization

The activation of GPCRs such as the GLP-1 receptor is well known to result in desensitization of the receptor. For example, preinfusion of Hamster Insulin Tumor (HIT) cells with supraphysiological doses of GLP-1 substantially reduces the cAMP and first-phase insulin responses to a second GLP-1 challenge. In contrast, preinfusion of HIT cells with GIP or glucagon does not affect GLP-1-induced insulin secretion, indicating that GLP-1 receptors undergo homologous desensitization (135). This finding was confirmed by a recent study in which GLP-1 pre-exposure caused a decrease in GLP-1-induced cAMP formation (128). In addition, the GLP-1 receptor may also undergo heterologous desensitization, as incubation with phorbol esters reduces the GLP-1-mediated cAMP response. Phosphorylation of the receptor is correlated to both homologous and heterologous desensitization. Interestingly, although the PKA pathway is not implicated in this process (136), residues in the carboxy-terminal 33 amino were found to be phosphorylated by PKC, and therefore, appear to be responsible for the heterologous desensitization. These residues are distinct from those implicated in homologous receptor desensitization induced by GLP-1.
(136). Furthermore, internalization of the GLP-1 receptor may also be involved in mediating homologous, but not heterologous, receptor desensitization (137-139). An alternative mechanism of reducing signaling by GLP-1 receptors may also include ligand degradation at the cell membrane (140) or internalization of the ligand itself (141).

1.3.1.5 Agonists and Antagonists

Recent investigations into the structure-activity relationship of GLP-1 have demonstrated that both the amino and carboxy terminals are important for GLP-1 binding and receptor activation (142). Specifically, the amino terminal histidine (143,144) as well as residues 10, 15, and 17 are essential for insulinotropic activity (143). Furthermore, carboxy terminal residues 28 and 29 are important in receptor binding (144). A careful analysis of the structure-activity relationship in the GLP-1 molecule is necessary in order to discover agonists and receptor antagonists for this important peptide.

Exendin-4 is a 39 amino acid peptide isolated from the venom of the Gila monster (Heloderma suspectum), which is 50% homologous to human GLP-1 and displays similar ligand binding affinities to the human GLP-1 receptor (145). This peptide is an effective agonist at the GLP-1 receptor in β-cell lines, capable of inducing cAMP formation and stimulating insulin secretion (146,147). In contrast, removal of the first eight amino acids of exendin-4 results in a potent antagonist of the GLP-1 receptor, exendin(9-39). Exendin(9-39) is 48% homologous to GLP-1, however, this peptide inhibits the formation of cAMP induced by GLP-1 (147), and prevents the GLP-1-induced rise in insulin in humans (148). These findings indicate that the amino acid residues important for GLP-1 receptor activation are
contained within the first eight amino acids of exendin-4. The inhibitory actions of exendin<sup>[9-39]</sup> will be invaluable in further experiments to delineate specific roles for GLP-1.

A search for proglucagon mRNA transcripts expressed in *Xenopus laevis* has also revealed several GLP-1-like peptides designated xenGLP-1A-C (6). These peptides all bind to the human GLP-1 receptor and stimulate cAMP and insulin secretion from the perfused pancreas of the rat. Interestingly, xenGLP-1B exhibits a higher affinity for the GLP-1 receptor than human GLP-1 (6).

The investigation of GLP-1 metabolism has also promoted the discovery of several analogues that are resistant to degradation by the major enzyme that degrades GLP-1, Dipeptidyl-peptidase IV (DPP-IV) (see section 1.3.7). In particular, alterations at position 2 of the GLP-1 molecule result in peptides that are both resistant to the inactivating properties of DPP-IV and are comparable to GLP-1 in terms of receptor affinity and biological activity (149,150). Several estimates indicate that the majority of circulating GLP-1 exists in the DPP-IV truncated form (151,152). Furthermore, this metabolite of DPP-IV action can function as an antagonist to the GLP-1 receptor (152). This finding introduces a new variable into the extent and physiological action of GLP-1 (153).

1.3.2 Pancreatic Functions

A significant proportion of the importance of GLP-1 action is derived from the fact that GLP-1 regulates several functions of the islets of Langerhans, and thus fuel metabolism. The discovery of the stimulatory effects of GLP-1 on insulin secretion contributed to the overall knowledge of the factors, both nutrient and non-nutrient, that regulate the secretion of insulin. By increasing insulin release in a glucose-dependent manner and inhibiting the
secretion of glucagon, GLP-1 directs the metabolic pathways in an anabolic course. Furthermore, the synthesis of insulin is also increased, therefore preventing depletion of the β cell product. These important regulatory functions of GLP-1 on the endocrine pancreas represent the primary rationale for clinical interest in utilizing GLP-1 as a therapy in type 2 diabetes.

1.3.2.1 Incretin Concept

As early as 1929, it was observed that glucose administered orally stimulated a greater increase in insulin secretion when compared to the same amount of glucose administered intravenously (154). This observation indicated that the gastrointestinal tract released a substance upon oral glucose administration, which was capable of augmenting insulin secretion. The term incretin was used to describe this factor (154,155) and this connection between the intestine and the endocrine pancreas was subsequently termed the enteroinsular axis (156). This axis was found to account for approximately 50% of the insulin released after an oral load of glucose (156). The first substance discovered to possess incretin-like effects was GIP. This hormone, which is produced by K cells in the duodenal mucosa, was first described to possess inhibitory action on gastric acid secretion (157). However when GIP was perfused into the rat pancreas, it was found to potently stimulate the secretion of insulin in a glucose-dependent fashion (158). GIP is secreted into the circulation in response to various luminal nutrients, including glucose (159,160) and fat (161,162), therefore implying that this substance is the factor responsible for the incretin effect. However, immunoneutralization studies demonstrated that a substantial proportion of the incretin effect is preserved when GIP receptor activation is blocked, implying that other factors released from the gastrointestinal tract are also participating in the incretin
phenomenon (163). Several other duodenal hormones, such as secretin and CCK were implicated as incretins, however these were disproved by subsequent experiments (164,165). However, studies involving patients who had undergone ileal resection demonstrated decreased incretin effects, thus raising the possibility that a factor produced by the ileum could also mediate some insulinotropic activities consistent with the presence of another incretin (166).

The identity of the other incretin remained unknown despite the discovery in 1983 of GLP-1, produced from the proglucagon molecule in the ileum and large bowel. Initial experiments to determine bioactivity of GLP-1(1-36NH2) failed to demonstrate any insulinotropic activity of this newly discovered peptide. The insulinotropic potential of GLP-1 was not realized until 1987 when the truncated form of the peptide, GLP-1(7-36NH2/37) was demonstrated to stimulate the glucose-dependent secretion of insulin (167-170). Furthermore, recent studies employing the specific GLP-1 antagonist, exendin(9-39) have demonstrated that GLP-1 is responsible for a significant proportion of the incretin effect (148,171-173). It has now been well established that GLP-1 and GIP stimulate insulin secretion independently; their effects are additive in both the rat (174) and human (175,176). On a molar basis, GLP-1 is the more potent incretin (176). However, an important difference in the insulinotropic activity arises when the two incretins are compared in the diabetic state. Type 2 diabetes is associated with an overall decrease in the incretin effect (177) that is not due to a decrease in the secretion of either GIP (177) or GLP-1 (178) in some patients(179). However, the insulinotropic activity of GIP is lost in some patients with type 2 diabetes whereas that of GLP-1 remains intact (176,180). In this regard, GLP-1 has received an
immense amount of clinical attention since it represents a promising insulinotropic therapy for type 2 diabetes.

1.3.2.2 Regulation of Insulin Secretion

To understand the important role of GLP-1 in the regulation of insulin secretion, a thorough examination of the factors that regulate insulin secretion is necessary (Fig. 1-2). The primary stimulus for insulin secretion is derived from nutrient metabolism within the β cell (181,182). Although metabolism of glucose, fat and amino acids is intimately connected to the elements controlling insulin secretion, glucose is the major stimulator of insulin secretion. Glucose is transported to the interior of the β cell through the actions of glucose transporter-2 (GLUT-2) (183). Glucokinase then phosphorylates the glucose molecule, trapping it within the cell, and glucose-6-phosphate is further processed in the glycolytic pathway, producing adenosine triphosphate (ATP). Increasing intracellular levels of this high-energy compound increase the ratio of ATP to adenosine diphosphate (ADP) and result in closure of the ATP-sensitive K⁺ channel. This causes a depolarization of the cellular membrane potential and activation of the voltage-dependent Ca²⁺ channels, leading to an influx of extracellular Ca²⁺ (181,182,184). This is the trigger for exocytosis of the insulin-containing secretory granules through the interactions of the molecules involved in the soluble NSF attachment protein receptor (SNARE) complex (185). The metabolism of fat within the β cell also produces an increase in the intracellular levels of ATP thus leading to depolarization of the cell via the ATP-sensitive K⁺ channel while amino acids enter the Kreb’s cycle at differing levels and can also increase the levels of ATP. Therefore, nutrients are the primary regulators of insulin secretion.
Figure 1-2 Overview of Insulin Secretion
Extracellular and intracellular mediators of insulin secretion are demonstrated.
Intracellular cAMP plays an important role in modulating nutrient- and, in particular, glucose-induced insulin secretion. It acts in two distinct ways, both of which are dependent on a permissive glucose concentration (186). Firstly, cAMP is a necessary factor in the maintenance of β cell responsiveness to glucose. Isolated β cells do not respond fully to perfusion with high levels of glucose. However, when these β cells are incubated in high glucose along with agents that increase the intracellular levels of cAMP, such as phosphodiesterase inhibitors (187) or glucagon (181), the deficiency in glucose-induced insulin secretion is largely restored. Secondly, cAMP can potentiate the secretion of insulin in the face of a glucose challenge. Humoral and neural factors that modulate the levels of intracellular cAMP are thus capable of influencing the rates of insulin secretion. Endocrine factors that can promote cAMP levels and therefore potentiate glucose-induced insulin secretion include the incretin hormones, GIP (188,189) and GLP-1 (128,130), but also glucagon from nearby α cells, VIP, and pituitary adenylate cyclase-activating polypeptide (190). The latter 2 peptides are released from local nerve fibers (191,192). The classical mediator of the parasympathetic nervous system, acetylcholine, also regulates insulin secretion but this is not through modulation of cAMP levels but, rather, through increased Ca^{++} release (193).

In contrast to these factors, several humoral and neural agents can also impact on the regulation of the β cell at the level of cAMP by decreasing intracellular levels of this important second messenger. Somatostatin, released in a paracrine fashion from nearby δ cells, decreases intracellular levels of cAMP and therefore powerfully inhibits the secretion of insulin (194). Leptin also inhibits insulin secretion from INS-1 cells, but only under
conditions in which intracellular cAMP was previously increased (195). Adrenergic agonists acting via the α2 receptor and the neuropeptide galanin also decrease intracellular levels of cAMP in the β cell (194). Therefore, it is evident that the β cell lies at the intersection of nutrient, neural, and humoral control, with intracellular levels of cAMP being crucial to the competency and level of the β cell response to glucose.

1.3.2.3 Glucose-dependent Insulin Secretion

One important aspect of insulin secretion distinguishes the incretin hormones from the other agents that exert regulatory control over the β cell. This is, significantly, that both GIP and GLP-1 act to increase insulin secretion in a glucose-dependent manner. GLP-1 and GIP bind to GPCRs expressed on the surface of the β cell and activate adenylyl cyclase, which in turn leads to increased levels of cAMP intracellularly. However, the end-result of this intracellular signaling network, namely the secretion of insulin, is dependent on elevated levels of glucose (168-170,174,196). This glucose dependency is a very important aspect in the prospective use of GLP-1 as a therapy in diabetes, as the potentially dangerous effects of insulin-induced hypoglycemia are avoided. In this regard, GLP-1 therapy is superior to the present use of sulfonylureas, which lead to closure of the ATP-dependent K⁺ channel followed by depolarization of the membrane and insulin secretion, regardless of the ambient glucose concentration (197). The complication of hypoglycemia is a prevalent side effect of the use of sulfonylureas.

The mechanism of action of GLP-1 in stimulating glucose-dependent insulin secretion is not totally understood. However, GLP-1 potentiates glucose-induced insulin secretion by acting in a synergistic manner at the level of the ATP-sensitive K⁺ channel.
GLP-1 receptor activation is associated with decreased conductance of the ATP-sensitive K\(^+\) channel and depolarization of the cell only in the presence of glucose (198) indicating that this step is one site of interaction of glucose and GLP-1 leading to the glucose-dependent nature of GLP-1-induced insulin secretion. In this manner, GLP-1 has the ability to induce glucose-competence in cells that were previously insensitive to stimulation by glucose (132,198). Antagonism of cAMP signaling inhibits the depolarizing effect of GLP-1 (198), indicating that the cAMP signaling pathway is necessary for the induction of glucose-competence by GLP-1. However, if the cell is depolarized by an alternative approach, such as incubating the cell in a high K\(^+\) medium, GLP-1 is capable of stimulating insulin secretion even in the absence of glucose (132). Therefore, the effect of GLP-1 to stimulate the secretion of insulin is more precisely dependent on membrane depolarization.

In addition to its effects on the ATP-sensitive K\(^+\) channel, GLP-1 potentiates the secretion of insulin by interacting with several other elements of the insulin secretory pathway. For example, GLP-1 increases the influx of Ca\(^{++}\) through L-type calcium channels and therefore potentiates the Ca\(^{++}\)-induced exocytosis of insulin-containing granules (199,200). Furthermore, as exemplified by experiments utilizing photoreleased cAMP in the presence of clamped Ca\(^{++}\) levels, GLP-1 is capable of potentiating insulin exocytosis at a level distal to the influx of Ca\(^{++}\), likely to be due to a direct effect of the PKA pathway on the secretion machinery itself (201). Furthermore, an effect of raised intracellular cAMP induced by GLP-1 receptor activation has been associated with an increased conductance through non-selective cation channels in rodent \(\beta\) cells (202). However, this finding has not been confirmed in studies of human \(\beta\) cells (201).
1.3.2.4 Insulin Synthesis

In addition to increasing insulin secretion, GLP-1 also up regulates insulin synthesis. In contrast to the sulphonylureas, GLP-1 protects against depletion of the secretory reserve of insulin by promoting the formation of insulin mRNA as well as protein levels (167,203). This is achieved mainly through the activation of insulin gene transcription (167) presumably through the cAMP-dependent activation of the insulin gene CRE (204). However, a recent report indicates that stabilization of insulin mRNA also contributes to the elevation in intracellular insulin mRNA levels (205). Furthermore, an increase in the translation of preproinsulin is also stimulated by exposure to GLP-1 as determined by leucine incorporation studies (203).

GIP acts in a similar manner to GLP-1 in terms of increasing the synthesis of insulin, through stimulation of insulin gene transcription (129,206). This likely occurs in a similar way to GLP-1-induced insulin gene transcription, i.e. through the insulin gene CRE.

1.3.2.5 Glucagon Suppression

Several studies have reported an additional effect of GLP-1 within the endocrine pancreas; this being the ability to suppress the secretion of glucagon both in rodents (207) and humans (170,208). Although the inhibitory effect of GLP-1 on glucagon secretion is also evident in isolated islets (209,210), this effect is not seen in isolated α cells (211) or in a glucagonoma cell line (111). Furthermore, treatment of α cells with concentrations of GLP-1 up to 1 nM does not elicit changes in cellular cAMP (212), indicating that complex paracrine interactions between the cell types of the endocrine pancreas are important in regulating glucagon secretion. GLP-1 receptors have been identified in both β and δ cell types of the pancreas (110) and activation of these receptors leads to increases in both insulin and
somatostatin, which exert negative influences on glucagon secretion (213). Moreover, GLP-1 suppresses glucose and glucagon levels in an insulin-deprived diabetic dog model (214) and in patients with type 1 diabetes (215) indicating an effect of GLP-1 in suppressing glucagon that is independent of the inhibitory action of insulin. In addition, a small subpopulation of islet α cells was found to express the GLP-1 receptor (110). However, activation of the GLP-1 receptors in isolated rat α cells stimulated exocytosis (211) indicating that, in the absence of elevated insulin and somatostatin, GLP-1 actually stimulates the secretion of glucagon. Furthermore, this GLP-1-stimulated exocytosis was inhibited by somatostatin. Thus, important paracrine actions of insulin and somatostatin appear to override the direct action of GLP-1 on the α cell, thus leading to the overall inhibitory effect of GLP-1 on glucagon secretion. Consequently, GLP-1 is of benefit not only to patients with type 2 diabetes (180), but also to those patients with type 1 diabetes (216), as it can inhibit the secretion of glucagon which is a major contributor to hyperglycemia in these conditions.

1.3.3 Gastric Functions

In concert with the pancreatic functions of GLP-1, the gastric effects of this peptide act to regulate nutrient assimilation. GLP-1 infusion into normal humans (217-219) and rodents (220) inhibits gastric acid secretion thereby delaying the eventual absorption of nutrients from the intestinal tract. Both meal- (218,221) and pentagastrin-stimulated (217,222) acid secretion is inhibited by GLP-1. Post-prandial acid secretion was also inhibited by GLP-1 (218). Consistent with these findings, binding of GLP-1 has been reported in gastric glands (223) and GLP-1 receptors have been identified in purified preparations of rat parietal cells (113). However, some evidence indicates that GLP-1 may also affect acid secretion by alteration of gastrin release in perfused rat stomach. Since
gastrin release is also inhibited directly by somatostatin, it is likely that GLP-1 indirectly inhibits the secretion of gastrin through increased gastric somatostatin secretion. Indeed, the inhibition of gastrin secretion is inversely proportional to the increase in somatostatin secretion in this model (224). Analogous to the mechanism of inhibition of the pancreatic α cell, GLP-1 receptors are also found on gastric somatostatin-releasing cells. Activation of these receptors leads to increased secretion of somatostatin (225), which is known to inhibit gastric acid production via a decrease in cAMP mediated by the pertussis toxin sensitive G\textsubscript{i} subunit of the G-protein (226). Therefore, GLP-1 appears to act both directly at the parietal cell and indirectly through the release of somatostatin, leading to a net inhibition of gastric acid secretion. Interestingly, the PGDPs, glucagon and oxyntomodulin, also function in an inhibitory manner with regard to gastric acid production in vivo (84,227). However, these agents stimulate gastric acid secretion when applied directly to a purified population of parietal cells by activation of the GLP-1 receptor (228). Subsequent studies have suggested that these two PGDPs affect the parietal cells indirectly in a similar manner to GLP-1 by acting on the GLP-1 receptor expressed on somatostatin-secreting cells leading to inhibition of the parietal cell mediated by somatostatin (225).

GLP-1 also functions as a prominent enterogastrone by inhibiting the motility of the gastrointestinal tract upon its release from the intestinal L cell. Together with peptide YY (PYY), GLP-1 mediates the ileal brake mechanism, preventing further delivery of nutrients to the ileum in cases when excess nutrients are sensed in the distal gastrointestinal tract (208,229). Consistent with this finding, GLP-1 secretion is intimately correlated to the delivery of nutrients to the absorptive surfaces of the gastrointestinal tract (230). Recent evidence indicates that the GLP-1-induced inhibition of gastric motility occurs by neural
interactions with the vagus nerve (116,220). Furthermore, ablation of vagal afferents by perivagal capsaicin treatment abolished the inhibition of gastric emptying induced by central and peripheral administration of GLP-1 (220). Therefore, GLP-1 is involved in the central regulation of gastric emptying and this process is dependent on an intact vagal afferent network for appropriate function. The inhibition of gastric motility by GLP-1 also contributes to its role as a regulator of nutrient homeostasis by slowing the rate at which ingested nutrients can be absorbed, causing decreased insulin secretion as a result of the decreased level of glycemia (231). As a result, it has been questioned whether GLP-1 is actually a physiological incretin at all, as the function of GLP-1 as an entero gastrone may override its insulinotropic capacity (232).

1.3.4 Satiety Promoting Functions in the Central Nervous System

The involvement of GLP-1 in the complex regulation of nutrient intake is suggested by the fact that proglucagon mRNA, GLP-1, and the GLP-1 receptor are all localized in regions of the brain known to exert regulatory control on food intake and feeding behaviour. Recent experiments have demonstrated that intracerebroventricular (i.c.v.) administration of GLP-1 potently inhibits feeding in fasted rats (233). The specificity of this effect for GLP-1 was demonstrated by the infusion of the GLP-1 receptor antagonist, exendin (9-39), which abolished the inhibitory effect of GLP-1 on feeding. In satiated, but not fasted animals, exendin (9-39) stimulated an increase in food intake (233), consistent with the possibility that GLP-1 may act as an indicator of satiety. Furthermore, GLP-1 inhibits food intake induced by Neuropeptide Y (NPY), indicating that GLP-1 interacts with elements of the network regulating food intake (233). Additionally, i.c.v. infusions of GLP-1 stimulate the expression of c-fos, a marker of neuronal activity, in several areas of the hypothalamus centrally.
involved in feeding behaviour, including the paraventricular nucleus (233,234). In a large proportion of the neurons expressing c-fos as a result of GLP-1 administration, corticotropin releasing hormone (CRH) immunoreactivity was coexpressed, indicating that GLP-1 activates the hypothalamo-pituatary-adrenocortical axis through stimulation of CRH neurons (234). CRH is itself a potent inhibitor of NPY-stimulated food intake (235). Therefore, this activation of CRH-containing neurons may mediate some of the inhibitory effects on food intake induced by GLP-1 (234). Furthermore, GLP-1-containing neurons in the nucleus of the solitary tract were found to express the signaling form of the leptin receptor indicating that leptin may mediate some of its inhibitory effects on food intake and body weight through the release of GLP-1 (206). In this regard, exendin (9-39) inhibited the reduction in food intake induced by leptin (236). Interestingly, GLP-1 receptor mRNA is located in hypothalamic neurons that also express glucokinase and GLUT-2. Thus, GLP-1 activation of these neurons may regulate subsequent neuronal activity in these glucose-sensing neurons (237). GLP-1 receptors are also located in regions of the brain that are positioned outside of the blood-brain barrier such as the area postrema and the subfornical organ (117). Whether circulating GLP-1 influences food intake through these receptors remains controversial at the present time. Peripheral GLP-1 was previously shown not to affect food intake (237), however, a recent study in humans demonstrated that i.v. infusion of GLP-1 reduced food intake and fluid ingestion at physiological plasma levels (238). In addition to its regulatory role in food intake, GLP-1 also inhibits water intake (237,238) and stimulates the secretion of vasopressin and corticosterone (234). Therefore, GLP-1 is emerging as an important regulator in the homeostasis of food and water intake.
1.3.5 Peripheral Functions

In addition to the effects of GLP-1 on the endocrine pancreas and stomach, there is accumulating evidence that GLP-1 can affect peripheral tissues in its role as a regulator of nutrient fluxes. Initial GLP-1 infusion studies performed in human volunteers confirmed the insulinotropic and glucagonostatic effects of this hormone. However, when the meal-related insulin requirement was measured in patients with type 2 diabetes, it was found to be decreased during the GLP-1 infusion as compared to the saline infusion. This was postulated to be due not only to the insulinotropic effects of GLP-1, but also to hepatic and/or peripheral effects (239). This finding is supported by the fact that GLP-1 reduces the meal-related insulin requirement in type 1 diabetic patients maintained on a normoglycemic clamp (239). Further studies performed in healthy human volunteers undergoing i.v. glucose tolerance tests demonstrated that infusions of GLP-1 improve glucose tolerance through insulinotropic actions as well as through enhancement of the effectiveness of glucose in stimulating its own disposal (240). Furthermore, when calculated according to the minimal model of glucose kinetics, GLP-1 infusion did not alter insulin sensitivity in this study (240). However, since the measure of glucose effectiveness incorporates both insulin-independent glucose disposal as well as the ability of glucose itself to suppress hepatic glucose uptake, it is not clear whether GLP-1 is acting at a peripheral location or at the level of the liver or both (240). Similar effects on glucose effectiveness and glucose clearance rates were observed in a follow-up study in which endogenous GLP-1 levels were stimulated by a meal rather than being elevated by i.v. infusion (241). GLP-1 treatment also led to increases in the glucose clearance rates in fed and fasted rats (242). This effect is specific for GLP-1 since the increases in glucose infusion rates induced by GLP-1 in hyperglycemic clamp studies are
reduced by concomitant infusion of the GLP-1 receptor antagonist, exendin\(^{(9-39)}\) (172). Conversely, infusions of exendin-4, the GLP-1 receptor agonist, cause an increase in the glucose requirements necessary to maintain the hyperinsulinemic-euglycemic clamp (243). In contrast to these studies is the finding that GLP-1 does not acutely influence insulin sensitivity in healthy humans (244). Moreover, several other studies report that GLP-1 can influence glucose disappearance rates only in the presence of elevated insulin levels, suggesting that the increase in glucose disappearance rate is a function of the increased secretion of insulin induced by GLP-1 (245). However, somatostatin was used to inhibit the endocrine pancreas in these studies and this may have an inhibitory effect on a putative GLP-1-mediated effect on glucose disposal. Additional evidence implicating insulin in the peripheral effects of GLP-1 is obtained from experiments performed in depancreatized dogs in which somatostatin was used to clamp gastric glucagon levels. GLP-1 administration increased the glucose infusion rate necessary to maintain the hyperinsulinemic clamp, due to an increased glucose utilization rate. The effect of GLP-1 was abolished when the GLP-1 infusion was accompanied by a low dose insulin infusion indicating that GLP-1 enhances insulin-induced glucose utilization (122). These studies were performed with an eye to whole body glucose homeostasis and, thus, are not helpful in properly resolving the tissues and physiological processes which are responsible for the altered glucose utilization rates achieved by GLP-1. Many sites have been postulated for mediating this effect of GLP-1 including muscle, adipose and liver.

1.3.5.1 Muscle

Muscle is a major site of insulin-mediated glucose disposal, however, whether the \textit{in vivo} effects of GLP-1 on glucose utilization and insulin sensitivity are manifested in muscle
is unclear at the present time. Recent studies have demonstrated specific GLP-1 binding to the membranes of rat skeletal muscle. This binding was not associated with an increase in intracellular cAMP, indicating a GLP-1 receptor that is somehow different from the pancreatic β-cell receptor (246). Furthermore, this binding is associated with increased incorporation of glucose into glycogen as well as increased glucose oxidation and lactate formation. The GLP-1 agonist, exendin-4, also stimulated glycogenesis and glucose utilization in a subsequent study whereas the antagonist, exendin(9-39) inhibited these parameters (247). Thus, these actions in muscle may account for the peripheral effect of GLP-1 in lowering plasma glucose concentrations (248). In support of this finding, application of GLP-1 to L6 myocytes increased glycolysis, glucose oxidation and glycogen synthesis, in an additive fashion to that of insulin (249). However, another study in rat skeletal muscle failed to detect a role for GLP-1 in stimulating glycogenesis (250). Interestingly, however, experiments performed in L6 myocytes revealed a possible alternate mode of action for GLP-1. GLP-1 led to an increase in glycogen synthesis in parental cells, but in cells transfected with the pancreatic form of GLP-1 receptor, application of GLP-1 led to a decrease in glycogen formation. These results therefore suggest that a separate GLP-1 receptor may exist in muscle, which acts in a cAMP-independent manner (249). Therefore, the effects of GLP-1 in muscle tissue are extremely controversial, but may explain the in vivo findings of increased glucose effectiveness and glucose utilization with GLP-1 infusion. Clearly, further investigation is required to resolve this issue.

1.3.5.2 Adipose Tissue

Within adipose tissue, GLP-1 acts to increase glucose uptake both in an insulin-independent (251) and independent manner (252). The enhanced glucose uptake was reflected
in increased levels of fatty acid synthesis (251-253). Infusions of GLP-1 in depancreatized dogs also increased glucose utilization and led to a decrease in circulating free fatty acids (FFA) and glycerol (122) consistent with the observed lipogenic effects of GLP-1 in vitro. Therefore, GLP-1 has interesting effects in adipose tissue that are consistent with a role for this peptide in the direct regulation of glucose utilization.

1.3.5.3 Liver

GLP-1 receptor localization in this tissue is controversial. However, several reports of biological function exist. Binding to rat hepatic membranes (254) and isolated rat hepatocytes (255) has been reported. In this tissue, GLP-1 did not increase intracellular cAMP levels, and receptor binding was equally displaced by GLP-1(7-36NH2) (254) in an analogous manner to GLP-1 binding in L6 myocytes (249). This finding suggests that the GLP-1 receptor in liver is similar to the muscle form and both of these are different from the pancreatic form of the receptor since the pancreatic receptor does not bind GLP-1(1-36NH2) (104). An alternative form of the receptor may also explain the controversial results achieved with receptor localization studies. Within the liver, GLP-1 leads to elevation in the rates of glycogen synthesis. This is abolished by glucagon and is accompanied by an increase in glycogen synthase A activity (255).

1.3.5.4 Lung

GLP-1 receptors have been identified in vascular smooth muscle of pulmonary arteries and mucosal glands, and GLP-1 stimulates macromolecule secretion from the trachea (256). Furthermore, GLP-1 relaxes preconstricted pulmonary arteries; this effect is dependent on an intact epithelium (256). The physiological significance of these findings are unresolved at the present time.
1.3.6 **GLP-1 Receptor Knock-out Mouse**

The inactivation of the GLP-1 receptor in mice, accomplished by homologous recombination, created a useful model with which to examine the physiologic functions of GLP-1 and its role in the processes of fuel homeostasis (257). These mice exhibit impaired glucose tolerance upon oral challenge with glucose due to decreased insulin secretion. Furthermore, male mice exhibit elevations in fasting glycemia compared to normal littermates as a result of the requirement for basal cAMP levels in maintaining β cell competence. Importantly, exogenous administration of GLP-1 did not affect glucose levels in GLP-1 receptor knock-out animals after an oral glucose tolerance test, indicating that a second form of the GLP-1 receptor is not present, at least in mice. The fact that GLP-1 receptor knock-out animals are not obese and exhibit normal feeding behaviour indicates that the mechanisms controlling food intake are extremely complex and subject to adaptation in the event of chronic loss of GLP-1 function. Genetic analysis of GLP-1 receptor function in the context of humans indicated that inherited defects of the GLP-1 receptor in two racial groups are not major risk factors for type 2 diabetes (258). Nevertheless, the findings demonstrated in the GLP-1 receptor knock-out animal indicate that GLP-1 plays a prominent role in the regulation of nutrient metabolism.

1.3.7 **Therapeutic Potential for GLP-1 in the Treatment of Diabetes**

With the discovery of the various functions of GLP-1, interest in its therapeutic use in diabetes dramatically increased. Type 2 diabetes is characterized by two major defects, insulin resistance and relative insulin insufficiency, which predispose to a major disturbance in nutrient homeostasis (259). Insulin resistance is defined as a decreased ability of insulin to stimulate glucose uptake in peripheral tissues as well as a decreased effectiveness of insulin
in the suppression of hepatic glucose production. Environmental factors, which can lead to obesity, such as diet and physical inactivity, compound the insulin resistant phenotype. In the early stages of insulin resistance, normal glycemia is maintained by a compensatory increase in the secretion of insulin from the β cell. However, as the prediabetic phase of the disease continues, the capability of the β cell to sustain an increased secretory rate begins to wane. Eventually, the β cell fails to respond to further glucose loads and the clinical features of type 2 diabetes, namely, polyuria, polydipsia, and hyperglycemia, predominate. The failure of the β cell is thought to be mediated by processes that promote β cell insensitivity to glucose-stimulation (260).

Recent clinical studies examining the effects of exogenous GLP-1 infusion demonstrate the effectiveness of this peptide in normalizing blood glucose levels in patients with type 2 diabetes (261-264). Consistent with these findings, exendin-4 also exerts glucose-lowering actions in several models of type 2 diabetes (243). Clearly, the various effects of GLP-1 offer benefit to the treatment of both the insulin resistance and relative insulin deficiency that characterize type 2 diabetes. By increasing the synthesis and secretion of insulin from the β cell, GLP-1 effectively overcomes the relative deficiency in insulin without the side effects of conventional sulfonylurea treatment. Glucagon secretion is also inhibited by GLP-1 thereby decreasing the degree of hepatic glucose output and reducing the requirement for insulin. Furthermore, through its actions as an enterogastrone, GLP-1 effectively delays nutrient absorption, thus diminishing the acute nutrient load on the β cell, while the insulin sensitizing actions of GLP-1 in muscle and adipose tissue may prevent
peripheral insulin resistance. These functions of GLP-1 therefore offer great promise for the treatment of type 2 diabetes.

The ability of GLP-1 to suppress the secretion of glucagon may also be an important aspect of the treatment of type 1 diabetes. The lack of insulin in this setting leads to unregulated secretion of glucagon causing increased hepatic glucose output and severe hyperglycemia. In humans with type 1 diabetes, GLP-1 infusion produced a pronounced decrease in plasma glucagon levels and partially decreased fasting levels of glucose. Although the reduction in glucagon level was not different in type 1 and type 2 diabetes, the reduction in glycemia in patients with type 1 diabetes did not reach the near-normalization achieved in those with type 2 diabetes, indicating that an effect on β cell secretion is also necessary (216).

Despite the demonstrated benefits of GLP-1 in the deficient insulin secretion and insulin resistance of diabetes, it appears that the incretin effect is reduced in type 2 diabetes (177). However, the secretion rates of both GIP (265) and GLP-1 (178,179) are normal or elevated in patients with type 2 diabetes, indicating that a reduced sensitivity of the β cell to the incretin hormones, and to GIP in particular (180), may be the cause of the reduced incretin effect in type 2 diabetes. Hyposecretion of the incretin hormones in type 2 diabetes has also been reported (180), indicating that the responses of GIP and GLP-1 are quite variable in type 2 diabetes and may correlate with the extent of the disease. However, at supraphysiological levels, GLP-1 infusion was capable of stimulating insulin secretion in type 2 diabetic individuals (266). Importantly, the insulin secretory action of GLP-1 is preserved in type 2 diabetic patients when compared with normal controls (180), albeit at
higher therapeutic concentrations, indicating that homologous desensitization of the GLP-1 receptor may have an impact on insulin secretion in type 2 diabetes (239,261). The ability of GIP to stimulate insulin secretion decreases in aging (267) as well as in type 2 diabetes (176,180). Therefore, the effectiveness of GLP-1 may be reduced in diabetes whereas the insulinotropic function of GIP is lost to a greater extent. This may be the result of GIP receptor desensitization at the level of the β cell which has recently been demonstrated (268). Thus, GLP-1 treatment in diabetes offers a viable and effective approach to therapy.

1.3.8 Metabolism and Other Obstacles to Therapy

Like insulin, GLP-1 is a peptide hormone and is therefore subject to proteolysis and degradation in the gastrointestinal tract. For this reason, therapeutic interventions to date have been limited to parenteral administration of the peptide. Such studies have revealed that the effects of exogenously administered GLP-1 are extremely short-lived (269) with the immunoreactive half-life of GLP-1 reported to be in the range of 4-17 min (78,217). The kidney has been determined to play a role in the degradation and clearance of circulating GLP-1 with a half-life of approximately 4 min (270,271). However, more recent studies of GLP-1 metabolism have identified a major conversion of GLP-1 from GLP-1\(^{7-36\text{NH}_2}\) to GLP-1\(^{9-36\text{NH}_2}\) that occurs with a half-life of approximately 0.9 min (150,151,272). The enzyme responsible for this degradation has been identified as DPP-IV, as inhibitors to this enzyme decrease the extent of metabolism of GLP-1 in vitro (151,272). This enzyme is ubiquitously located in mammalian tissues with highest activity in renal and intestinal brush-border cells (273) but is also found in the capillaries abutting the intestinal L cell (274) and in the circulation (151). DPP-IV preferentially cleaves peptides at the N-terminus, specifically those with proline or alanine as the penultimate amino acid residue (275). This enzyme has
also been implicated in the degradation of the other major incretin hormone, GIP (272). The major metabolite of DPP-IV activity is GLP-1(9-36NH2) which is found to comprise up to 80% of the GLP-1 immunoreactivity in circulation (269). This metabolite of GLP-1 has been demonstrated to act as an antagonist at the GLP-1 receptor, however, its affinity to the receptor is only 1% of that of GLP-1(7-36NH2) (152). Furthermore, GLP-1 is also degraded at the C-terminal end of the peptide by neutral endopeptidase-24.11 (276) although this appears to be less important than the actions of DPP-IV.

Attempts to increase the half-life of GLP-1 have been made in order to maximize the therapeutic potential of GLP-1 in diabetes. Since GLP-1 is predominantly inactivated by DPP-IV, the development of DPP-IV resistant analogues or the inhibition of DPP-IV activity are viable options. A modification of GLP-1 at position two renders the peptide resistant to the activities of DPP-IV (149,150) while inhibition of DPP-IV activity potentiates the insulinotropic effect of GLP-1 (277). However, these strategies cannot increase the biological half-life of GLP-1 beyond that normally conferred by renal clearance of the peptide (270,271).

Currently, several approaches of delivering therapeutic doses of GLP-1 are being developed. Subcutaneous injections of GLP-1 have been reported to increase insulin and decrease glucagon secretion. Repeated s.c. injections of GLP-1 also normalized plasma glucose (278). Therapeutic levels of GLP-1 can also be achieved through the use of a buccal tablet. Increased insulin and decreased glucagon secretion were witnessed following meal ingestion in conjunction with a GLP-1 buccal tablet (279). However, given the drawbacks and inconvenience of the above therapeutic maneuvers, alternative strategies also need to be
considered. One such alternative is to optimize the endogenous secretion of GLP-1 in an attempt to bypass the issues of bioavailability and patient compliance, and to efficiently match the benefits of GLP-1 action with nutrient ingestion.

1.4 Secretion of Glucagon-like Peptide-1

GLP-1 is secreted from the L cell, an enteroendocrine cell located predominately in the ileum and colon. Both in vitro and in vivo model systems have been utilized to study the mechanisms involved in GLP-1 secretion. Several primary cultures systems such as the FRIC cultures and isolated canine enteroendocrine cells have been utilized, as well as cell line models such as the GLUTag and STC-1 cells, to determine the intracellular and extracellular factors which regulate GLP-1 secretion. These models have the advantage of isolating the L cell to a certain degree, which allows for cell-specific analyses. The cell lines are homogeneous populations of cells and are useful for investigations of intracellular signaling studies. However, as the L cell is isolated in these cell culture systems, physiological interactions between the L cell and governing endocrine/neuroendocrine systems is disengaged which may confound results of GLP-1 secretion when applied to the intact organism. On the other hand, various in vivo models such as the isolated vascularly perfused ileum/colon as well as in situ models in various species, have the advantage of providing physiological information about the mechanisms that govern the secretion of GLP-1. The vascular, paracrine and nervous inputs to the L cell remain intact in these model systems and thus provide a more accurate picture of the physiological regulation of the L cell. However, it is difficult to distinguish between direct and indirect regulatory mechanisms within these models. Therefore, information about the mechanisms that govern
GLP-1 secretion from the L cell is best ascertained through analysis of a combination of the *in vitro* and *in vivo* models.

The L cell is under complex regulation from luminal nutrients, as well as endocrine and neural factors, involving the activation of several intracellular signaling systems. Physiological stimulation of GLP-1 secretion occurs promptly after nutrient ingestion, with levels of GLP-1 peaking in the blood within 30 min. The distal location of the GLP-1-secreting L cell therefore introduces a significant paradox when this profile of nutrient-induced GLP-1 secretion is considered and suggests that the regulation of the L cell is far more complex than previously anticipated.

The L cell produces and secretes several products as a result of post-translational processing of the proglucagon molecule. In the past, secretion from the L cell has been quantified by measuring several of these released peptides including GLP-1, oxyntomodulin, glicentin and GLP-2. Since all of these peptides are cleaved from a single precursor molecule, it follows that they are released in a one-to-one ratio (179,280,281). Therefore, when one considers the factors and mechanisms that lead to the secretion of GLP-1, these can also be applied to the other members of the PGDP family.

1.4.1 L cell

The enteroendocrine L cell is a member of the multitude of gastrointestinal enteroendocrine cells. It is an open-type cell, which samples the lumen and secretes substances into the circulation via the basolateral membranes. The frequency of L cell distribution increases in an aboral manner such that the ileum and colon possess the greatest density of L cells (41).
Several intracellular signaling pathways including the PKA, PKC, and calcium pathways govern GLP-1 secretion from the L cell. The study of these intracellular signaling systems has been accomplished mainly by the use of in vitro model systems, including the primary FRIC cultures (282), and the GLUTag cell line (36). Activation of the PKA and PKC pathways leads to increases in the secretion of GLP-1 in both models (34,38,283). Inhibition of calcium channel activity also interferes with basal GLP-1 secretion indicating that calcium signaling is fundamental in L cell secretion (283).

As the enteroendocrine cells are derived from a common precursor stem cell, many of these are capable of secreting more than one product. In the case of the L cell, GLP-1 is co-secreted with PYY (284). In the rabbit colon, PYY and GLP-1 are colocalized in 100% of the L cells (285). However, in the canine colon, glicentin/oxyntomodulin and PYY are colocalized in only 15% of the L cells (284). The physiological reasons for this are unclear. Several investigations of the factors controlling PYY secretion from the distal gastrointestinal tract have been performed and potentially offer some insight into the mechanisms that govern secretion from the L cell. Circulating levels of PYY increase within 30 min after nutrient ingestion in both humans (286) and rats (287) in a similar temporal pattern to nutrient-induced GLP-1 secretion. Stimulators of PKA and PKC lead to increased secretion of PYY from the FRIC cultures (288). Nutrients are potent stimulators of PYY secretion. Intraduodenal oleic acid and mixed nutrients potently enhance both PYY and GLP-1 secretion (289). The neuropeptide GRP significantly elevated PYY secretion in FRIC cultures (288) and in the isolated perfused pig ileum (290). Vagal stimulation (290,291) and the cholinergic agonist betahaneol (288,291) induce PYY secretion as well. In agreement with these findings is the result in rat, that hexamethonium, a nicotinic cholinergic
antagonist, inhibited the secretion of PYY stimulated by a mixed meal (291) or intraduodenal oleic acid (292). Interestingly, neurally-induced secretion of PYY and GLP-1 is differentially regulated in the rat, as an inhibitor of nitric oxide synthase blocked the meal-induced secretion of PYY but had no effect on the secretion of GLP-1 (289). Therefore, the secretion of PYY from the L cell is controlled at several levels, including nutrient, humoral, and neural regulation. These mediators may also regulate GLP-1 secretion from the L cell in a similar fashion.

1.4.2 Regulation by Nutrients

GLP-1 is secreted into the circulation upon the ingestion of nutrients (293-295). However, the peak of GLP-1 secretion occurs within 30 min of nutrient ingestion (293,294) at which time, digested nutrients have not reached the ileum in order to directly stimulate the secretion of GLP-1 (296). Therefore, indirect mechanisms exist that link the ingestion of nutrients to GLP-1 secretion from the distally located L cells. This is supported by the demonstration that meal-induced GLP-1 secretion is maintained in patients in which ileostomies divert the bulk of nutrient flow away from the ileum (294).

GLP-1 is released from the L cell in a pulsatile fashion with peaks of secretion occurring 5-7 times per hour. Upon nutrient stimulation, the pulses of GLP-1 increase in amplitude but not frequency. This pulsatile nature of GLP-1 secretion is similar to that of pancreatic hormones and of insulin, in particular (297).

1.4.2.1 Carbohydrates

Glucose is the major carbohydrate that exerts a stimulatory effect on the L cell (170,293). Galactose (295,298), but not fructose or lactose (298), can also lead to the
stimulation of GLP-1 secretion. The majority of glucose entering the gastrointestinal tract is absorbed rapidly within the upper segment (299,300); very little glucose is present in the distal segments, which contain the majority of the GLP-1-secreting cells. Although supraphysiological concentrations of glucose in the ileum can stimulate GLP-1 secretion (162,282), glucose does not reach the luminal surfaces of the L cell to stimulate GLP-1 secretion under physiological conditions. However, it is well established that GLP-1 secretion stimulated by glucose occurs within a short time period after ingestion (293,294) indicating that indirect mechanisms must be in operation to elicit a prompt secretory response within the L cell. This will be disclosed in more detail in section 1.4.3.

The mechanisms by which glucose directly acts on the L cell are relatively unknown; however, it appears that glucose must be absorbed in order to stimulate secretion of GLP-1. Glucose-induced GLP-1 secretion occurs in a sodium-dependent manner, implicating the involvement of the Na-glucose co-transporter molecule found on the brush border of the intestine (295,301). Hyperglycemia per se does not stimulate GLP-1 secretion thus demonstrating the importance of luminal absorption (162,302). Interestingly, α-glucosidase inhibitors cause increased secretion of GLP-1 presumably due to increased shunting of carbohydrates to the lower segments of the intestine, thereby enabling a direct effect of glucose on the L cells (303,304).

The direct effect of glucose on the L cell also appears to be dose-dependent. Lower total levels of glucose (3.6% w/v) perfused into an isolated segment of rat ileum were incapable of stimulating GLP-1 secretion (305) but, when higher amounts of glucose (18% w/v) were perfused, a significant GLP-1 response was achieved (162). Not surprisingly,
therefore, GLP-1 secretion is increased in states associated with accelerated rates of gastric emptying such as the dumping syndrome (170) and post-esophageal resection (306).

1.4.2.2 Fat

In a similar manner to carbohydrates, the ingestion of fats produces a prompt increase in the secretion of GLP-1 (293-295). In contrast to glucose, the temporal profile of GLP-1 secretion induced by a fat meal in humans contains two major peaks, an early peak at 20-40 minutes and a later peak arising 60-90 minutes after nutrient ingestion (294,307). It follows that the early peak represents an indirect mechanism of fat-induced GLP-1 secretion analogous to the glucose situation since nutrients do not reach the ileum within this time frame. However, in contrast to glucose, which is largely absorbed in the proximal gut (299), significant proportions of fat reach the distal gut (308). The later peak of fat-induced GLP-1 secretion occurs at a time that is consistent with normal intestinal transit times in humans (309). These findings are consistent with a direct effect of fat on the luminal surface of the L cell, and are likely involved in the late rise in GLP-1 observed after fat ingestion. In support of this claim, fat perfusion of ileum and colon significantly elevated the level of secretion in a number of studies (162,295). Furthermore, exposure of L cell cultures to fats has resulted in increased secretion of GLP-1 (310,311).

Studies on the effect of the structural characteristics of individual fatty acids on the release of other gastrointestinal hormones have been performed and demonstrate that long chain fatty acids are necessary to elicit elevated neurotensin secretion (310). On the other hand, the secretion of PYY is not dependent on fatty acid chain length (312). Whether fatty acid chain length is an important determining factor in the activation of the L cell was
unknown at the time the present studies were initiated, however, fatty acid saturation reportedly did not influence the secretion of GLP-1 in healthy human volunteers (313).

1.4.2.3 Protein

The role of protein in the secretion of GLP-1 is controversial. Contrasting studies indicate that protein ingestion has very little effect on GLP-1 secretion as evidenced by administering protein meals to healthy human volunteers (293,294). However, mixtures of amino acids are reported to be stimulatory in vivo (295) but not ex vivo in the isolated perfused rat intestine (42). Peptones specifically derived from egg albumin and meat have also been reported to stimulate the secretion of GLP-1 in vitro and ex vivo (42).

1.4.3 Regulation by Endocrine Factors

1.4.3.1 Stimulators

The fact that GLP-1 secretion is elevated shortly after a meal raises the possibility that mediators released from the proximal gastrointestinal tract after meal ingestion act in an indirect manner to stimulate GLP-1 secretion. However, the exact nature of this indirect mechanism is relatively unknown. Experiments performed in the dog examining the factors that control PYY secretion from the L cell in response to nutrient ingestion have demonstrated regulatory control by the upper gastrointestinal hormone cholecystokinin (CCK) (314). However, CCK does not have a direct effect on PYY secretion from the rat L cell in vitro (288), nor does it have a direct (311) or indirect effect on GLP-1 secretion in vivo (162). Other proximal hormones such as secretin, gastrin, and motilin are also without effect on the secretion of GLP-1 in the rat (162). The indirect mechanism linking proximal nutrient ingestion with secretion from the distal L cell must therefore involve other hormones released upon nutrient ingestion from the upper gastrointestinal tract. One such candidate
has been found to be GIP, which is released from the upper gastrointestinal K cell upon nutrient ingestion (159,315,316). GIP is stimulated by the presence of fat in the duodenum (162,317) and can stimulate the secretion of GLP-1 in vitro (38,311,318) as well as in vivo in rats (162). Interestingly, plasma GIP levels are elevated in GLP-1 knock-out mice further emphasizing the relationship between these two incretin hormones (319). However, GIP does not seem to be the mediator of the glucose signal from the proximal gut in humans, as GIP infusions into patients with type 2 diabetes did not elicit significant GLP-1 responses (180).

1.4.3.2 Inhibitors

Somatostatin-28 is produced from endocrine D cells found predominantly in the intestinal tract (320,321). This peptide has been demonstrated to potently inhibit a variety of gastrointestinal endocrine cells, including the L cell, through a Gi-specific pathway (311).

Indirect evidence for an inhibitory role for insulin in the regulation of the L cell exists from studies performed in diabetic states. Secretion of GLP-1 is elevated in both type 1 and type 2 diabetes, which are characterized by a total or relative lack of insulin (180,239,302). Furthermore, insulin-treatment of streptozotocin-induced diabetic rats reverses the elevation in GLP-1 secretion, implying a negative feedback function of insulin on the L cell (322). Consistent with these in vivo findings, insulin treatment of FRIC cultures causes an inhibition of secretion (282). Finally, GLP-1 and GLP-2 are themselves incapable of inhibiting the L cell in an autocrine feedback manner when tested in the FRIC culture model of the L cell (311).
1.4.4 Regulation by Neural Factors

The influence of the nervous system on various endocrine cells, including the β cell (323,324) and the α cell (324,325) is well established. Furthermore, the nervous system and the vagus nerve in particular, play large roles in the cephalic phase of digestion in which neural stimulation of cells of the digestive system aids in preparing for the ingestion, digestion and absorption of nutrients. Whether the nervous system is involved in the regulation of the GLP-1-secreting L cell remains to be established. However, several key points of evidence point toward a role for the nervous system in this regard.

1.4.4.1 Neuropeptides

GRP is a potent stimulator of GLP-1 release in vitro (311). Furthermore, GLP-1 secretion is stimulated by infusions of GRP in a variety of species (326) including man (327). GRP is the human analogue of bombesin and stimulates a variety of enteroendocrine cells by enhancing the activity of the PKC pathway (328). GRP is a component of both the enteric nervous system (329) as well as the nonadrenergic/noncholinergic branch of the vagus nerve (330). Therefore, GRP may be a neural mediator of nutrient-induced GLP-1 secretion. Interestingly, the infusion of a GRP-receptor antagonist, BW-10, completely abrogated the nutrient-induced response of GLP-1 (326), strongly implicating a prominent role for GRP in the regulation of the L cell. Paradoxically, the inhibitory action of BW-10 on the L cell occurred despite the presence of elevated levels of GIP. Thus, the mechanism of indirect stimulation of the distal L cell mediated by the duodenal hormone GIP was thrown into doubt in light of the importance of GRP.

Another neuropeptide with stimulatory action at the level of the L cell is calcitonin gene related peptide (CGRP). Two separate genes encode for CGRP-like peptides, termed
CGRP-I and CGRP-II (331). Both CGRP-I and CGRP-II are stimulatory to the L cell \textit{in vitro} (311,332), consistent with the fact that these two peptides differ by only two amino acids. Furthermore, infusion of CGRP-I into isolated segments of rat ileum (333) and colon (334) produces dramatic increases in the amount of GLP-1 secreted. CGRP-I is mainly localized to the extrinsic nervous system whereas CGRP-II is found in neurons of the intrinsic enteric nervous system (335). CGRP mediates its stimulatory effect on the L cell through the PKA pathway (336). However, when CGRP is infused into humans, GLP-1 secretion rates are decreased, suggesting that an indirect mechanism mediates the inhibition of the L cell induced by CGRP. In fact, CGRP causes an increased secretion of somatostatin (332), which, likely, contributes to the appearance of its negative effect on the L cell.

Substance P is a member of the tachykinin family of enteric neuropeptides that is found in all layers of the porcine ileum (337). Substance P was found to stimulate GLP-1 secretion from the perfused porcine ileum (338). This effect was mediated by neurokinin-1 receptors as a specific antagonist inhibited Substance P-induced secretion.

In contrast to the enteroendocrine hormone, somatostatin-28, the related peptide somatostatin-14 is associated with the enteric nervous system (339) and has been found to inhibit the L cell both \textit{in vitro} (311,340) and \textit{in vivo} (341,342). However, somatostatin-28 is much more potent at inhibiting the L cell than is somatostatin-14 (311).

\textbf{1.4.4.2 Neurotransmitters}

The major parasympathetic neurotransmitter, acetylcholine, appears to act in a stimulatory manner on the L cell. Agonists of acetylcholine directly stimulate the secretion of GLP-1 in FRIC cultures (311) and in the GLUTag cell line (38), as well as in the secretin
turnour cell (STC-1) line (343). Evidence for a stimulatory role for acetylcholine comes also from several in vivo studies. Cholinergic agonists lead to increased secretion of GLP-1 in vascu larly perfused, isolated segments of ileum (333) and colon (334). Furthermore, infusion of atropine, an acetylcholine receptor antagonist, diminished the response of GLP-1 to an oral glucose load in healthy human volunteers (297). In the mouse STC-1 cell line, acetylcholine acts through the muscarinic M3 receptor subtype (343), however, preliminary data from FRIC cultures suggests that the rat L cell expresses an M1 receptor (P.L. Brubaker, unpublished observations).

Agonists of the adrenergic receptors are largely ineffective at stimulating GLP-1 secretion when applied directly to models of the L cell (38,311). However, when these agents are administered into perfused segments of the gastrointestinal tract, stimulation of GLP-1 secretion is evoked (334,344) indicating that this effect may be dependent on the release of an intermediate stimulatory factor. Nonetheless, β2-adrenergic agonists are stimulatory to the L cell. This is differentially modulated by α-adrenergic stimulation; α1 receptors appear to synergistically modulate GLP-1 secretion induced by β-agonists whereas agonists of α2-adrenergic receptors negatively modulate the effect of β-adrenergic stimulation (344). Therefore, the adrenergic control of the L cell is extremely complex.

1.5 Role of Fatty Acid Structure on GLP-1 Secretion

Clinical evidence in humans has demonstrated that both carbohydrates and fats (293-295) potently stimulate GLP-1 secretion. The mechanisms that are responsible for these phenomena are only partially understood, but likely include both direct and indirect mechanisms.
Although the structural requirements necessary for carbohydrate-induced GLP-1 secretion have been well-defined (298), those for fat-induced stimulation of the L cell were unknown upon initiation of the present studies. However, fatty acid structure is an important determinant in the secretion of another gastrointestinal hormone, neurotensin. Unsaturated long chain fatty acids specifically stimulate secretion of neurotensin from primary cultures of canine enteroendocrine cells. This effect is dependent on mobilization of cellular calcium and an inhibitor of PKC reduces fatty acid-induced neurotensin secretion (310). These findings indicate that fatty acids may be influencing classical intracellular signaling mechanisms in order to elicit secretion of neurotensin. For example, fatty acids have been demonstrated to activate PKC (345-347). As this intracellular signaling pathway has been implicated in the secretion of GLP-1 (283), it is reasonable to question whether fatty acids can stimulate GLP-1 secretion by modulating this signaling system.

1.5.1 Benefits of Monounsaturated Fatty Acid Diets on Glycemic Control

Recently, monounsaturated fat diets have been advocated for patients with type 2 diabetes (348,349). In the past, it was suggested that high carbohydrate diets were beneficial in type 2 diabetes, since they were thought to reduce the risk of coronary heart disease through decreases in low-density lipoprotein levels (350). Furthermore, it was believed that diets high in carbohydrates improve glycemic control and enhance insulin sensitivity (349). However, with further examination it was noted that high carbohydrate diets tended to accentuate hyperglycemia and elevate plasma glucagon levels. Furthermore, diets high in carbohydrate actually deteriorated lipoprotein status in patients with type 2 diabetes (351). By contrast, diets in which a portion of the carbohydrate was replaced with monounsaturated fatty acids (MUFA) were found to result in lower mean glucose levels and reduced insulin
requirements, as well as lower very low-density lipoprotein levels in patients with type 2 diabetes (352-354). Thus, diets rich in MUFA have been recommended for patients with type 2 diabetes (349,355,356). The mechanisms responsible for these improvements were not ascertained in these preliminary studies. Given the fact that GLP-1 possesses important functions that ameliorate glucose homeostasis, coupled with the knowledge that fats are an important stimulus for GLP-1 secretion, it can be hypothesized that the benefits in glycemic control achieved by MUFA diets in type 2 diabetes are mediated, in part, by increases in the secretion of GLP-1.
2 RATIONALE FOR STUDIES OF GLP-1 SECRETION

2.1 Global Hypothesis

Secretion of the antidiabetic hormone, GLP-1, by ingested fat activates a complex network of luminal, humoral and neural elements, leading to stimulation of the L cell by both direct and indirect pathways.

2.2 Rationale for Study 1

Fat meals are potent stimulators of GLP-1 secretion. Furthermore, fat ingestion stimulates two distinct peaks of GLP-1 secretion in the human; the first peak is an immediate response whereas the second peak occurs approximately 1 hour after fat ingestion. Given the distal location of the GLP-1-secreting L cell in the intestine, this suggests that the effects of fat on the L cell are mediated through both indirect and direct pathways. The potential direct effects of fatty acids on the L cell have not been examined previously. In addition, different structural classes of fatty acids exhibit different effects on the release of other gut hormones such as neurotensin.

2.2.1 Hypothesis and Aims for Study 1

The hypothesis of this study was that fatty acids exert direct effects on the L cell to stimulate the secretion of GLP-1; this effect is dependent on the structural class of the fatty acid. The aim of this study was therefore to characterize the effects of fatty acids with varying chain length and degrees of unsaturation on GLP-1 secretion. A secondary aim was to examine the potential intracellular signaling cascades involved in fat-induced secretion of GLP-1.
2.3 **Rationale for Study 2**

It has been previously suggested that diets rich in MUFA are beneficial to patients with type 2 diabetes, through improvements in both glycemic tolerance and lipid status. Given the role of GLP-1 in glucose homeostasis and the finding in study 1 that MUFAs specifically stimulate the secretion of this antidiabetic hormone, it is plausible that the benefits exhibited by MUFA feeding in vivo are mediated, in part, by increased GLP-1 secretion.

2.3.1 **Hypothesis and Aims for Study 2**

The hypothesis for this study was that the glycemic benefits observed with MUFA diets are attributable to increases in secretion of the antidiabetic hormone, GLP-1. The specific aims were therefore to establish that the improvements in glycemic tolerance induced by MUFA diets are due to increases in plasma levels of GLP-1 secretion.

2.4 **Rationale for Study 3**

Previous findings suggest that there is an indirect mechanism that links proximal nutrients to GLP-1 secretion from distal sections of the gastrointestinal tract. Previous studies have demonstrated that the endocrine peptide, GIP, is the proximal mediator of fat-induced GLP-1 secretion. However, subsequent studies have shown that infusions of an antagonist to the GRP receptor suppress fat-induced GLP-1 secretion, despite the fact that GIP levels remain elevated. This finding indicates that GIP is not solely responsible for mediating the indirect signal to the distal L cells and that there must be other mediators involved in the indirect regulation of GLP-1 secretion. Consistent with this notion, the secretion of other gastrointestinal hormones, such as PYY, has been shown to be similarly regulated by an indirect mechanism involving the vagus nerve.
2.4.1 Hypothesis and Aims for Study 3

The hypothesis for the third study was that the early phase of fat-induced GLP-1 secretion is mediated by an indirect mechanism that involves vagal innervation. The specific aims of this study were to demonstrate that the vagus nerve participates in the indirect mechanism governing GLP-1 secretion. A secondary aim was to demonstrate that the endocrine mediator of the indirect pathway, GIP, is capable of interacting with the neural elements of the indirect pathway regulating GLP-1 secretion.
3 STUDY 1

DIRECT EFFECTS OF MONOUNSATURATED FATTY ACIDS ON PROGLUCAGON-DERIVED PEPTIDE SECRETION IN FETAL RAT INTESTINAL CULTURES

Portions of this study have been previously published in Endocrinology 136:5593-5599 (Rocca AS and Brubaker PL). Reproduced with permission from the publisher.
3.1 Abstract

The ingestion of fats is a potent stimulus for the secretion of the PGDPs including the insulinotropic peptide GLP-1 from the intestinal L cell. The aim of the study was to characterize the structural requirements for fatty acid-induced secretion of the PGDPs, and investigate the cellular mechanisms through which fatty acids mediate PGDP secretion. FRIC cultures were incubated with 10-150 μM fatty acids differing in chain length (14-18) and degree of unsaturation (0-2). Inhibitors of PKC and of fatty acid esterification and oxidation were also incubated with the cells in the presence of stimulatory fatty acids. The cultures were assayed for glucagon-like immunoreactivity (GLI) and GLP-1(9-36NH2) secretion. MUFAs of chain length greater than 14 carbons stimulated PGDP secretion by 1.8 to 3.4-fold, in a dose-dependent fashion (P<0.05-P<0.001). This is consistent with the immunohistochemical demonstration of the Intestinal fatty acid binding protein within the L cell, as this molecule is capable of binding only long chain fatty acids. Enhanced PGDP secretion was lost upon full saturation of the stimulatory fatty acids. Furthermore, while blockade of fatty acid esterification with a carboxyl methyl ester group prevented fatty acid-induced PGDP secretion, inhibition of fatty acid oxidation with methyl palmoxirate did not prevent PGDP secretion. Finally, the use of various inhibitors of classical and novel isoforms of PKC (staurosporine, H7, 24h down regulation) also did not alter fatty acid-induced PGDP secretion. The presence of an atypical isoform of PKC, PKC-ζ, was demonstrated in the L cell by immunohistochemistry. In conclusion, MUFAs possessing a free carboxyl group stimulate intestinal PGDP secretion. Neither fatty acid oxidation nor classical isoforms of PKC appear to be directly involved in this response. Therefore, the structure of the fatty acid plays a central role in inducing intestinal PGDP secretion. These
findings suggest that dietary fat composition may significantly affect the magnitude of the GLP-1 response to ingested nutrients.
3.2 Introduction

Ingestion of nutrients stimulates the secretion of various hormones including the intestinal PGDPs, glicentin, oxyntomodulin, GLP-1 and GLP-2 (162,293-295,305,357). Fats are one of the three major nutrients in the diet that are absorbed from the intestine and are important components of biological membranes and internal energy stores. Fat ingestion or placement of mixed fats directly into the intestine increases plasma levels of the intestinal PGDPs by 2-10 fold (162,293-295,305,357). Furthermore, the fatty acid sodium oleate (at $10^{-4}$ M) has been shown to increase glicentin/oxyntomodulin secretion by 3-fold from both FRIC (311) and canine enteroendocrine cell (310) cultures. GLP-1 and oxyntomodulin act in the enterogastric axis to decrease gastric acid secretion and gastric motility (84,217,358), and thus, nutrient fluxes into the intestine. Another important role of GLP-1, is in the disposal of ingested nutrients through its stimulatory effects on glucose-dependent insulin secretion (168,175,261,266,359). This incretin activity of GLP-1 has led to interest in its potential use for the treatment of type 2 diabetes. Given the importance of fats in the regulation of intestinal PGDP secretion, the present study was designed to characterize the effects of long-chain fatty acids and their mechanism of action on the secretion of PGDPs, including GLP-1, from the intestinal L cell.

3.3 Methods and Materials

3.3.1 FRIC Cultures

The in vitro studies were performed using previously described techniques (282,283,311). Briefly, intestines from a 20-21 day litter of fetal Wistar rats were pooled and minced in Hanks Balanced Salt Solution. The cells were dispersed by two digestions with Collagenase (SigmaBlend Type H; 40 mg/dl), Hyaluronidase (Type I-S; 50 mg/dl) and
DNase-I (5 mg/dl) (Sigma Chemical Co., St. Louis, Mo). The cells were washed with culture medium [Dulbecco’s Minimal Essential Medium (DMEM) containing 5% (vol/vol) fetal bovine serum, 4.5 g/l glucose, 50 IU/ml penicillin and 50 μg/ml streptomycin], and 60 x 15mm dishes containing 0.6 fetal rat intestines/2ml were incubated overnight at 37°C with 7% (vol/vol) CO₂ and constant humidity.

3.3.2 Experimental Protocols

Stock solutions (150 mM) of oleic acid [18:1, (n-9)], methyl ester of oleic acid, linoleic acid [18:2, (n-6)], stearic acid (18:0), palmitoleic acid [16:1, (n-9)], palmitic acid (16:0), myristoleic acid [14:1, n-9]] and myristic acid (14:0) (Sigma Chemical Co., St. Louis, Mo) were made in dimethyl sulfoxide (DMSO). The stock solutions were diluted in experimental culture medium [DMEM containing 0.5% (vol/vol) fetal bovine serum, 1 g/l glucose, 50 IU/ml penicillin, 50 μg/ml streptomycin and 20 μU/ml insulin] to the desired final concentration and applied to the cells for an incubation time of 2 h. Neither the pH of the medium nor the cell viability was affected by addition of the fatty acids. Controls included experimental culture medium alone, 0.1% (vol/vol) DMSO (vehicle) and 1 μM phorbol 12-myristate 13-acetate (PMA; positive control). This concentration of DMSO represents the maximum that was added to the cell cultures with any of the test agents. Methyl paloxirate (150 μM; McNeill Pharmaceuticals, Springhouse, Pa.) was preincubated with cells for 30 min prior to the 2 h incubation with fatty acids in order to investigate the role of fatty acid oxidation in PGDP secretion (360). In experiments examining the role of PKC in fatty acid-induced PGDP secretion, cells were preincubated with staurosporine (1 μM; Upstate Biotechnology Inc., Lake Placid, NY), H7 (6 μM; LC Services Corp., Woburn,
Ma.) or HA-1004 (40 µM; LC Services Corp., Woburn, Ma.) for 30 min or with PMA (1 µM) for 24 h, prior to the 2 h incubation period.

3.3.3 Extraction of Peptides

Media was collected and made to 0.1% (vol/vol) trifluoroacetic acid (TFA) and cells were homogenized in extraction medium [1 N HCl containing 5% (vol/vol) HCOOH, 1% (vol/vol) TFA and 1% (wt/vol) NaCl] at 4°C. Peptides were collected separately from media and cells by reversed-phase adsorption to C18 silica (C18 SepPak, Waters Associates, Milford, Ma.) as previously described (282,283,311). Recoveries of the PGDPs exceed 88% using this methodology (282,283). The extracts were stored at -20°C until analysis by RIA.

3.3.4 Radioimmunoassay

The presence of GLI was detected using an antiserum (K4023; BiosPacific, Emeryville, Ca.) that recognizes the mid-sequence of glucagon. This antiserum crossreacts with all glucagon-containing peptides and provides an accurate measure of the glicentin and oxyntomodulin content of the FRIC cultures (282,283,311). The assay sensitivity is 4-400 pg/tube and the inter- and intra-assay variations are 18.5% and 7.6%, respectively. GLP-1 levels were determined using an antiserum (Affiniti Research Products, Nottingham, UK) that recognizes the C-terminal amide group of GLP-1 (1-36NH2) and GLP-1 (7-36NH2) (361). Over 60% of the immunoreactive GLP-1(1-36NH2) in FRIC cultures consists of GLP-1(7-36NH2) (34). The assay sensitivity is 3 to 800 pg/tube and the inter- and intra-assay variations are 15.9% and 4.9%, respectively. For each experiment, differences in GLI or GLP-1 levels between treatment groups and controls were determined within the same assay.
3.3.5 Tissue Staining

Ileal tissue was obtained from fasting male Wistar rats (Charles River Canada, Inc., St. Constant, QC) and rinsed with cold saline solution. Tissue was fixed in 10% formalin for 24 hours and then embedded in paraffin blocks from which 5 μm sections were obtained. On the day of staining, tissue slides were deparaffinized through a xylene:alcohol gradient and stored in phosphate buffered saline (PBS) prior to the staining. Tissue slices were pretreated with a blocking solution made up of 4% normal goat serum for a period of 30 min. Following this pre-incubation, slides were incubated overnight with primary antibody (mouse monoclonal anti-GLP-1 [1:100; a gift from Dr. D.D'Allessio, Seattle, WA]; rabbit anti-intestinal-Fatty acid binding protein (I-FABP) [1:500; a gift from Dr. J.Gordon, St. Louis, MS]; or rabbit anti-PKC-ζ [1:500; a gift from Dr. C.Whiteside, Toronto, ON]) in blocking solution in order to decrease non-specific binding. Addition of the primary antibody was withheld in control slides, which were otherwise treated as the experimental slides.Slides were rinsed with PBS and treated with the blocking solution once more for 30 min prior to the addition of the secondary antibody. Anti-mouse immunoglobulin G (IgG) labeled with Cy2 (1:100) and anti-rabbit IgG labeled with Cy3 (1:500) (Jackson ImmunoResearch Laboratories, West Grove, PA) were incubated with the tissue sections for 3 hours. Tissue sections were then rinsed with PBS and mounted in a glycerol:PBS solution as a mounting solution. Tissue sections were then visualized under a fluorescence microscope (courtesy of Dr. U. DeBon, Toronto, ON). Some experiments involved double immunostaining for GLP-1 and I-FABP. For these experiments, the same staining protocols described above were performed in sequence. Double immunofluorescent images were captured using a confocal microscope (Electron Microscopy Unit, University of Toronto, Toronto, ON).
3.3.6 Cell Staining

GLUTag and STC-1 cells were plated on individual glass coverslips and allowed to grow to approximately 75% confluence. Cells were then fixed in 10% formalin for 6 hours, rinsed and stained for I-FABP, PKC-ζ or GLP-1 according to the above methodology. Similar dilutions of both primary and secondary antibodies as used in the tissue staining procedure were utilized.

3.3.7 Data Analysis

Media content of GLI or GLP-1 was divided by the total culture content of GLI or GLP-1 (media + cell content), respectively, to determine the percentage of total immunoreactive-peptide secreted during the incubation period. In some experiments, the GLI and GLP-1 responses were combined to determine changes in intestinal PGDP secretion. Synthesis of intestinal PGDPs was determined as a function of the total GLI or GLP-1 content of media and cells and did not change during the 2 hour incubation period (data not shown). Statistical significance was assessed by analysis of variance (ANOVA) or paired Student’s t test using Statistical Analysis System Software (SAS Institute, Cary, NC.) for IBM computers. Some data were converted to percent of control and log10 transformed prior to analysis. Pearson’s product moment analysis was used to assess the relationship between changes in GLI secretion and those of GLP-1. All data are expressed as mean ± standard error of the mean (SEM). For all experiments, n = 4 - 7.

3.4 Results

3.4.1 Effect of Fatty Acid Structure on PGDP Secretion

The effect of varying fatty acid chain length on intestinal PGDP secretion was examined in the first series of experiments (Fig. 3-1). In this, and all experiments, DMSO did
Figure 3-1  Effects of Varying Chain Length on Fatty Acid-Induced PGDP Secretion.

PGDP secretion was measured as the percent of total GLI (medium and cell content) or GLP-1 (medium and cell content) that was secreted into the medium. Fatty acids were incubated with the cells for 2 h at concentrations of 10 - 150 μM. Con = normal culture medium; PMA = 1 μM PMA (positive control). (A) Oleic acid (18:1) (B) Palmitoleic Acid (16:1) (C) Myristoleic Acid (14:1). * = P<0.05, ** = P<0.01, *** = P<0.001 vs. control.
not alter PGDP secretion from control values (data not shown), while the positive control, PMA, stimulated PGDP secretion to 179 ± 9% (P<0.001) (n=35). Oleic acid (18:1) induced a dose-dependent increase in secretion of GLI to a maximum value of 254 ± 23% of controls at a concentration of 150 μM (P<0.001 vs control, P<0.01 vs 50 μM). Similarly, GLP-1 release was also stimulated by oleic acid to 175 ± 36% of controls (P<0.05). The GLI data is consistent with previous findings using oleic acid with this intestinal culture system (311). Palmitoleic acid (16:1) also stimulated a dose-dependent increase in GLI and GLP-1 secretion, reaching 340 ± 85% (P<0.001 vs control, P<0.01 vs 50 μM) and 284 ± 84% (P<0.05 vs control, P<0.01 vs 50 μM) of controls, respectively at 150 μM. In contrast, the 14:1 fatty acid, myristoleic acid, failed to significantly increase either GLI or GLP-1 secretion at any concentration tested (Fig. 3-1).

The next series of experiments focused on the effect of the degree of unsaturation of the fatty acid on PGDP secretion. Doubling the degree of unsaturation of the 18 carbon chain (linoleic acid; 18:2), or complete saturation of the chain (stearic acid; 18:0) did not increase GLI secretion above control values (Fig. 3-2). Similar results were found when the saturated 16 and 14 carbon fatty acids were tested.

As GLI and GLP-1 are produced in quantitatively identical amounts by the post-translational processing of proglucagon in the L cell (34,47), the relationship between the secretion of these peptides in FRIC cultures was determined. Correlation analysis of the data presented in Figs. 3-1 and 3-2 demonstrated a highly significant relationship (r = 0.5, P<0.0001). Therefore, as GLP-1 secretion paralleled that of GLI in these experiments, only GLI secretion was determined in subsequent experiments.
Figure 3-2  Effects of Degree of Unsaturation on Fatty Acid-Induced PGDP Secretion.

PGDP secretion was measured as the percent of total GLI (medium and cell content) or GLP-1 (medium and cell content) that was secreted into the medium. Fatty acids were incubated with the cells for 2 h at concentrations of 10 - 150 μM. Con = normal culture medium; PMA = 1 μM PMA (positive control). (A) Linoleic acid (18:2) (B) Stearic acid (18:0) (C) Palmitic acid (16:0) (D) Myristic acid (14:0). * = P<0.05, ** = P<0.01 vs. control.
In an attempt to determine whether the long chain fatty acids also modulate intestinal PGDP synthesis, monounsaturated and saturated fatty acids of 16 to 18 carbons in chain length were incubated with the cells for 24 h. None of these fatty acids (at 100 to 150 μM) significantly changed the synthesis of the PGDPs from that seen in control cells (data not shown).

3.4.2 Cellular Mechanisms of Fatty Acid-Induced PGDP Secretion

The cellular mechanisms through which fatty acids may stimulate PGDP secretion were investigated in the next three series of experiments (Figs. 3-3 to 3-5). Prevention of oleic acid esterification using the methyl ester of oleic acid completely abrogated the stimulatory effects of oleic acid alone on GLI secretion throughout the concentration range tested (Fig. 3-3). In contrast, 30 min of preincubation with methyl palmoxirate (150 μM), an inhibitor of fatty acid oxidation (360), did not prevent GLI secretion in response to stimulation with PMA, oleic acid or palmitoleic acid, and stimulated GLI secretion in the control cells (P<0.05) (Fig. 3-4).

Since fatty acids are known to operate through the PKC pathway in some cell types (345,362), intestinal cultures were pre-incubated with several PKC inhibitors for 30 min prior to the addition of PMA or stimulatory concentrations (100 μM) of fatty acids (Fig. 3-5). The non-specific PKC inhibitor, staurosporine (1 μM) (363-365), decreased basal GLI secretion to 73 ± 5% of control levels (P<0.001), and reduced PMA-mediated GLI secretion by a similar amount, although this did not reach significance. Furthermore, pretreatment with staurosporine did not decrease GLI secretion induced by either oleic acid or palmitoleic acid. Similarly, H7 (6 μM), another non-specific inhibitor of PKC
Figure 3-3  Role of Fatty Acid Esterification in Fatty Acid-Induced PGDP Secretion.

GLI secretion was measured as the percent of total GLI (medium and cell content) that was secreted into the medium. The methyl ester of oleic acid was incubated with the cells for 2 h at concentrations of 10 - 150 μM. Con = normal culture medium; PMA = 1 μM PMA (positive control). *= P<0.05 vs. control.
Figure 3-4  Role of Fatty Acid Oxidation in Fatty Acid-Induced PGDP Secretion

GLI secretion was measured as the percent of total GLI (medium and cell content) that was secreted into the medium. Con = normal culture medium; PMA = 1 μM PMA (positive control); 18:1 = 100 μM oleic acid; 16:1 = 100 μM palmitoleic acid. Methyl palmoxirate (MPX, 150 μM) was preincubated with the cultures for 30 min prior to the addition of test agents. * = P<0.05, ** = P<0.01, *** = P<0.001 vs. control. §§§ = P<0.001 vs. the same treatment without methyl palmoxirate.
Figure 3-5  Role of PKC in Fatty Acid-Induced PGDP Secretion.

GLI secretion was measured as the percent of total GLI (medium and cell content) that was secreted into the medium. Con = normal culture medium; PMA = 1 μM PMA (positive control); 18:1 = 100 μM oleic acid; 16:1 = 100 μM palmitoleic acid. Staurosporine (1 μM), H7 (6 μM), HA-1004 (40 μM) were preincubated with the cultures for 30 min prior to the addition of PMA and fatty acids. The cultures were also treated for 24 h with 1 μM PMA in order to downregulate PKC prior to the addition of PMA and fatty acids. * = P<0.05, ** = P<0.01, *** = P<0.001 vs. control. § = P<0.05, §§ = P<0.01, §§§ = P<0.001 vs. the same treatment without protein kinase inhibition.
also failed to inhibit the phorbol ester- and fatty acid-induced GLI secretion. Interestingly, HA-1004 (40 μM), a control for the non-specific effects of H7 on PKA (365-367) inhibited basal GLI secretion to 85 ± 3% of control levels (P<0.01), but had no effect on PMA- or fatty acid-stimulated GLI secretion. Finally, in an attempt to down regulate PKC activity, FRIC cultures were pretreated for 24 h with PMA (366,368). Surprisingly, this actually increased GLI secretion by 48 ± 33% (P<0.05) in the control group, but did not decrease the effect of the phorbol ester on GLI secretion (Fig. 3-5 C). Likewise, 24 h pretreatment with PMA did not significantly alter the GLI secretion induced by either the 18:1 or 16:1 fatty acids.

3.4.3 Tissue Staining

Tissues sections of ileum incubated without the addition of the primary anti-GLP-1 antibody did not exhibit any fluorescence due to the secondary Cy2-linked anti-rabbit IgG antibody (not shown). Low frequency positive staining was observed in cells located in the crypt regions of ileum (Fig. 3-6 Upper Panel). Green fluorescent staining was confined to the cytoplasm and extended from the lumen to the basolateral membrane of the cells. Thus, in terms of both localization and staining, these cells appear to resemble L cells (41,369).

Ileal sections incubated without the addition of the primary anti-I-FABP antibody did not accumulate red fluorescent staining. However, positive I-FABP staining was evident in most cells along the villus tips (data not shown), consistent with its role in the absorption and metabolism of fatty acids (370). Sporadic I-FABP staining was also evident in the crypt region of the ileum. Staining was confined to the cytoplasm of the cell (Fig. 3-6 Lower Panel).
Figure 3-6  Anti-GLP-1 and Anti-I-FABP Double Staining of Rat Ileal L Cells

Upper Panel: Section of rat ileum labeled with mouse monoclonal anti-GLP-1 antibody. Secondary antibody consisted of anti-mouse IgG conjugated to the green fluorescent Cy2 molecule.

Lower Panel: Same section of rat ileum labeled with rabbit anti-I-FABP antibody. Secondary antibody consisted of anti-rabbit IgG conjugated to the red fluorescent Cy3 molecule.
Double immunofluorescence experiments conducted with the two primary antibodies directed against I-FABP and GLP-1 demonstrated that I-FABP staining in the crypt regions colocalized with specific staining for GLP-1 (Fig. 3-7).

### 3.4.4 GLUTag Cell Staining

GLUTag cells stained positively for GLP-1, as immunofluorescence was readily detectable in a large subset of the cells (Fig. 3-8 Upper Panel). This was seen as a punctate pattern in the cytoplasm of the stained cells, however, some cells did not express GLP-1 immunofluorescence. This finding is of unknown significance but is consistent with previous accounts of GLP-1 immunostaining in GLUTag cells (36).

Positive staining within GLUTag cells was evident with the incubation of the anti-I-FABP primary antibody. Intense cytoplasmic staining was evident in all cells (Fig. 3-8 Lower Panel).

Positive cytoplasmic staining within GLUTag cells was present within all cells upon the exposure to the anti-PKC-ζ antibody (Fig. 3-9 Upper Panel). Staining was specific as removal of the primary antibody abolished fluorescent staining by the Cy3-conjugated secondary antibody (not shown).

### 3.4.5 STC-1 Cell Staining

STC-1 cells possessed positive staining for GLP-1, and indeed, most cells stained positively for GLP-1 (Fig. 3-10 Upper Panel) in contrast to the GLUTag cells, which exhibited sporadic staining for GLP-1. STC-1 cells also stained positively for both I-FABP and PKC-ζ (Figs. 3-9 Lower Panel, 3-10 Lower Panel).
Figure 3-7  Rat Ileal L Cells Express Both GLP-1 and I-FABP

Confocal image of a section of rat ileum double labeled with rabbit anti-I-FABP and mouse anti-GLP-1. Secondary antibodies consisted of anti-rabbit IgG conjugated to the red fluorescent Cy3 molecule and anti-mouse IgG conjugated to the green fluorescent Cy2 molecule.
Figure 3-8  Immunohistochemical Studies of GLUTag Cultures

Upper Panel: GLUTag cells labeled with anti-GLP-1 antibody. The fluorescent marker Cy2 was linked to anti-mouse IgG. Lower Panel: GLUTag cells labeled with rabbit anti-I-FABP antibody. The fluorescent marker Cy3 was linked to anti-rabbit IgG.
Figure 3-9  Anti-PKC-ζ Labeling of GLUTag and STC-1 Cell Lines

Upper Panel: GLUTag cells labeled with rabbit anti-PKC-ζ antibody. Cy3 conjugated to anti-rabbit IgG was the fluorescent marker. Lower Panel: STC-1 cells labeled with anti-PKC-ζ antibody. Cy3 conjugated to anti-rabbit IgG was the fluorescent marker.
Figure 3-10 Immunohistochemical Studies in STC-1 Cells

Upper Panel: STC-1 cells labeled with anti-GLP-1 antibody. Cy2 conjugated to anti-rabbit IgG served as the fluorescent marker. Lower Panel: STC-1 cells labeled with anti-I-FABP antibody. The secondary antibody was Cy3 conjugated to anti-rabbit IgG.
3.5 Discussion

Ingestion of fats or administration of corn oil into the intestine stimulates a 2-10 fold increase in the release of the intestinal PGDPs, including GLP-1 from the intestinal L cell (162,293-295,305,357). The effects of a variety of fatty acids on PGDP secretion were investigated in the present study in order to determine the structural requirements for PGDP release. Consistent with the in vivo responses to mixed fat, MUFAs of 18 and 16 carbon chain length both stimulated PGDP secretion in FRIC cultures by 2-3-fold. However, as the chain length was shortened below 16 carbons the ability of the fatty acid to stimulate PGDP secretion was abolished. Indeed, short chain fatty acids such as butyric acid, have also been found to have no effect on PGDP release in FRIC cultures (283). Furthermore, as the degree of unsaturation was reduced to zero in each of the stimulatory fatty acids, PGDP secretion was also attenuated. Surprisingly, doubling the degree of unsaturation of the stimulatory oleic acid also prevented elevated PGDP secretion. Therefore, it appears that fatty acids must be monounsaturated and of chain length greater than 14 carbons in order to significantly stimulate intestinal PGDP secretion. Interestingly, the effective doses used in the present study appear to be an order of magnitude lower than physiological fat concentrations, which are estimated to be in the range of 6 mM, as observed in the effluent collected from ileostomy patients (371). These findings suggest that the L cell is exquisitely sensitive to the presence of luminal fatty acids.

The present study also demonstrates that GLP-1 secretion in response to fatty acid treatment is highly correlated with that of GLI in the FRIC culture system utilized. This result is not surprising as proglucagon has been shown to be processed to glicentin, oxyntomodulin and GLP-1 within the FRIC cultures (34) as well as in the adult intestinal L
cell (47,372). When taken with the finding that fatty acids stimulate GLP-1 secretion to a similar extent in both FRIC cultures and in vivo, the results of this study further demonstrate the utility of the FRIC culture system as a model for the study of GLP-1 synthesis and secretion. It must be noted, however, that the FRIC cultures are comprised of a heterogeneous cell population, including cells that produce cholecystokinin, PYY and somatostatin (35,288,373). Neither cholecystokinin nor PYY modulate intestinal PGDP release when administered to FRIC cultures (311). However, somatostatin inhibits GLI secretion by FRIC cultures (311). Although oleic acid is known to stimulate intestinal somatostatin release (373), somatostatin levels in FRIC cultures are approximately 100 times lower than those required to affect GLI secretion (311,373).

Oleic acid, in addition to stimulating PGDP release, is known to be effective in augmenting secretion of two other gut peptides, namely PYY (288), an inhibitor of pancreatic secretion and intestinal motility (312), and neurotensin (310), an inhibitor of gastric acid secretion (374). PYY and neurotensin are distributed in a similar pattern to that of the PGDPs in the intestinal tract and interestingly, PYY is colocalized with the PGDPs within the L cell (284,285,375,376). Consistent with this distribution, PYY secretion from FRIC cultures is increased 2-fold by treatment with 100 μM oleic acid (288). Secretion of glicentin/oxyntomodulin from canine enteroendocrine cells in culture is also increased 3-fold upon treatment with 100 μM oleic acid (310). Furthermore, unsaturated fatty acids from chain length 14 to 20 increased neurotensin release by 3-fold in the same preparation (310). In contrast, PGDP secretion in the present study was not significantly elevated upon incubation with myristoleic acid (14:1). Consistent with the canine neurotensin data (310) however, neither saturated fatty acids nor the methyl ester of oleic acid altered PGDP
secretion in FRIC cultures. These findings are in contrast to in vivo data from pigs in which palmitic acid (16:0) has been reported to increase glicentin release (357). The subtle differences between fatty acid structural requirements for stimulation of rat and porcine PGDP and canine neurotensin release may be explained by species- and cell-specific differences, as well as by differences between in vivo and in vitro models.

In addition to the effect of chain length and saturation on PGDP secretion, functional blockade of the free carboxyl group of oleic acid with a methyl ester group prevented oleate-induced PGDP secretion. This is consistent with recently published data demonstrating that triglycerides, which also lack free carboxyl groups, cannot stimulate GLP-1 secretion in the perfused rat ileum model (295). The inability of the medium-chain fatty acids and the methyl ester of oleic acid to stimulate PGDP secretion may be explained by the presence of a fatty acid binding protein (FABP) in the intestinal L cell. FABPs within intestinal mucosal cells mediate the transfer of fatty acids from the apical membrane to the smooth endoplasmic reticulum for reconstitution into triglycerides (370,377). It is known that long-chain fatty acids with a free carboxyl end are preferred by FABP over medium-chain fatty acids (eg. 14:1) (378). Therefore, it appears that FABP may be responsible for some of the effects of fatty acids on PGDP secretion. These FABPs are structurally related to the retinoic acid receptors, which modulate transcription of various genes by interacting in heterodimers with other steroid nuclear receptors (379). Therefore, fatty acids may impart regulation on cellular activities via direct interaction of FABPs with regulatory transcriptional elements. Some isoforms of FABPs are also capable of undergoing phosphorylation, which can further modulate intracellular trafficking of fatty acids and therefore regulate downstream signaling (380). Although FABP is known to be present within the rat intestinal mucosa (378), the
present study has established that the intestinal subtype of FABP is present within the L cell itself. Nonetheless, FABP alone cannot account for the observed selectivity of PGDP secretion in terms of the degree of unsaturation, since it can bind both saturated and unsaturated fatty acids equally (378). Furthermore, these findings do not necessarily preclude the other members of the FABP family, such as liver-FABP (L-FABP), from being involved in fatty acid-induced PGDP secretion.

Methyl esterification of oleic acid, in addition to its effects on FABP binding, also inhibits re-esterification into triglycerides. Thus, the inability of the methyl ester to induce PGDP secretion may also indicate that the process involves re-esterification. However, this seems unlikely since re-esterification was possible in the experiments involving the polyunsaturated and saturated fatty acids, which did not alter PGDP secretion from control levels. Furthermore, the inhibitor of fatty acid oxidation, methyl palmoxirate, also did not alter fatty acid-induced PGDP secretion. Therefore, it appears that neither fatty acid oxidation nor re-esterification are directly involved in the stimulation of PGDP secretion from the intestinal L cell.

In addition to the role of fatty acids in providing a source of energy for cellular function, fatty acids may also modulate the internal signaling pathways of the cell. Fatty acids are known to increase the mobilization of calcium within certain cell types (310,347,362) and can also enhance the activation of the atypical isoform ζ of PKC (366,381). Thus, the effects of the stimulatory fatty acids on PGDP secretion were investigated in the presence of a number of agents known to inhibit PKC activity. The non-specific PKC inhibitor, staurosporine, decreased basal PGDP secretion, suggesting the
presence of a classical PKC isoform in the L cell. Unexpectedly, however, staurosporine did not significantly decrease PMA-induced PGDP secretion. It is possible that the concentration of staurosporine utilized in these experiments was insufficient to overcome the stimulatory effects of PMA, however, higher concentrations were not tested as they induced marked morphological changes in the FRIC cultures. Finally, staurosporine also had no effect on fatty acid-induced PGDP secretion. Similarly, PMA- and fatty acid-induced PGDP secretion were not inhibited by H7, an inhibitor that competes for the ATP binding site on PKC (365,366). HA-1004 was used as a control against cross inhibition of the PKA pathway by H7 since H7 is known to inhibit a variety of serine/threonine kinases (365-367). Consistent with the known effects of PKA in stimulating PGDP secretion from the L cell (283,311), HA-1004 significantly inhibited basal PGDP secretion but had no effect on PMA- or fatty acid-induced PGDP secretion, suggesting that neither PMA nor fatty acids stimulate PGDP secretion through a PKA-dependent mechanism. Interestingly, neither saturated nor unsaturated long-chain fatty acids altered the synthesis of the PGDPs, again indicating a lack of involvement of the PKA pathway, as this pathway also increases PGDP synthesis (35,283).

In an alternative attempt to inhibit the actions of PKC, a 24 h period of PMA-induced PKC down regulation preceded incubation of the cells with the stimulatory fatty acids. Such PMA treatment induced a significant rise in PGDP secretion in the control group indicating that the PMA response was still active over the extended time period. The inability of PKC down regulation to inhibit basal and fatty acid-induced PGDP secretion indicates that a PMA-insensitive isotype of PKC may also be involved in PGDP secretion. Further support for this theory is provided by the fact that treatment of FRIC cultures with oleoyl acetyl
glycerol, an analog of diacylglycerol, also does not increase secretion of intestinal GLI (P.L. Brubaker, unpublished data). PKC-ζ is a member of the atypical class of the PKC family that is insensitive to diacylglycerol stimulation as well as phorbol ester-induced stimulation and down regulation (381). Interestingly, PKC-ζ is also preferentially stimulated by unsaturated fatty acids over saturated fatty acids. Therefore, it appears that this atypical PKC isoform may be responsible for the stereospecific requirement of one degree of unsaturation versus complete saturation in terms of the fatty acid-induced rise in PGDP secretion. The presence of more than one PKC isoform within the intestinal L cell may explain the apparent contradiction presented in these studies by an elevated PGDP response upon PMA stimulation and the possible involvement of a fatty acid-sensitive, phorbol ester-independent isoform of PKC. Several PKC isoforms, including the atypical ζ isoform, have been identified in rabbit ileal enterocytes (382) and, through the use of immunohistochemical staining techniques, the presence of PKC-ζ within L cells was demonstrated. These findings, therefore, support the postulation that this molecule may be involved in the signaling cascade of fatty acid-induced GLP-1 secretion. However, PKC-ζ cannot be solely responsible for the fatty acid specificity observed in FRIC cultures as it is also preferentially stimulated by linoleic acid, which did not stimulate the secretion of GLP-1 in the above study. This indicates that another molecule, perhaps I-FABP, is necessary in the discrimination of fatty acids in the signaling pathway involved with GLP-1 secretion.

Fatty acids, especially long chain MUFAs, stimulate secretion of GLP-1 from the intestinal L cell. However, apart from the possible effects of fatty acids on PKC ζ and I-FABP, the intracellular mechanisms responsible for the MUFA effect on the L cell have not
been delineated. Although originally thought of as a simple fuel source, fatty acids are now considered as sophisticated intracellular signaling mediators. Fatty acids can be liberated from the phospholipid bylayer by the action of phospholipases as the result of receptor activation. Arachidonic acid is one example of such a fatty acid that can function as a powerful intracellular signal (383). Moreover, fatty acids can modify the activity of intracellular signaling systems through the process of acylation. In the case of PKC, this is associated with increased translocation to and affinity of the enzyme for the plasma membrane, which modulates its phosphorylating activity (383,384). In addition, G proteins are also known to be acylated by fatty acids leading to alterations in their activity (383,385).

Interestingly, fatty acids have also been reported to affect ion channels (386) and thus may regulate secretion by altering membrane potential. Furthermore, fatty acids have been demonstrated to increase the transcription of cellular enzymes involved in fatty acid metabolism through interactions with the nuclear peroxisome proliferator-activated receptor (PPAR) family of transcription factors (387). Therefore, fatty acids including the long-chain MUFAs may utilize a number of novel intracellular mechanisms in order to stimulate the secretion of GLP-1.

In summary, the present study has focused on the direct effects of fatty acids on the L cell, and has demonstrated that MUFAs possessing a free carboxyl group potently stimulate secretion of the intestinal PGDPs, including GLP-1. FABP and the atypical PKC-ζ isoform may be implicated in this fatty-acid induced secretory response. Therefore, the current study establishes that fatty acid structure is an important characteristic in stimulating the secretion of the insulinotropic peptide GLP-1. These findings suggest that fat composition may significantly affect the magnitude of the GLP-1 response to ingested nutrients.
4 STUDY 2

Monounsaturated Fatty Acid Diets Improve Glycemic Tolerance Through Increased Secretion of Glucagon-Like Peptide-1.

Two undergraduate physiology project students contributed to the results presented in this section. Jonathon LaGreca contributed to a portion of the Oral and Duodenal Glucose Tolerance experiments as well as the analysis of tissue GLP-1 levels. Juliana Kalitsky contributed to the analysis of essential fatty acid levels. The data contained in this chapter has been submitted for publication in Endocrinology.

(Rocca AS, LaGreca J, Kalitsky J, Brubaker PL).
4.1 Abstract

Diets enriched in MUFAs have been shown to benefit glycemic control. Furthermore, MUFAs specifically stimulate secretion of the antidiabetic hormone, GLP-1 in vitro. To determine whether the MUFA-induced benefit in glycemic tolerance in vivo is due to increased GLP-1 release, lean Zucker rats were pair-fed a synthetic diet containing 5% fat derived from either Olive Oil (OO; 74% MUFA) or Coconut Oil (CO; 87% saturated fatty acids; SFA) for 2 wk. Food intake and body weight gain were similar for both groups over the feeding period. The OO group had improved glycemic tolerance as compared to the CO group in both oral and duodenal glucose tolerance tests (AUC 121±61 vs 290±24 mM·120min, P<0.05; and 112±28 vs 266±65 mM·120min, P<0.05, respectively). This was accompanied by increased secretion of gut Glucagon-Like Immunoreactivity (gGLI; an index of GLP-1 levels) in the OO rats as compared to the CO rats (402±96 vs 229±33 pg/ml at t=10min, P<0.05). Tissue levels of GLP-1 and plasma insulin and glucagon levels were not different between the two groups. To determine the total contribution of GLP-1 to the enhanced glycemic tolerance in OO rats, the GLP-1 receptor antagonist exendin9-39 (Ex9-39) was infused 3 min prior to a duodenal glucose tolerance test. Ex9-39 abolished the benefit in glycemic tolerance conferred by OO feeding (OO+Ex9-39 vs CO+Ex9-39, P=NS), and resulted in a deterioration of glycemic tolerance in the OO+Ex9-39 group when compared to the OO controls (AUC 331±21 vs 112±28 mM·120min, P<0.05). To probe the mechanism by which the OO diet enhanced GLP-1 secretion, a GLP-1-secreting L cell line was incubated for 24 h with either 100μM oleic acid (MUFA) or 100μM palmitic acid (SFA) and subsequently challenged with GIP, a known stimulator of the L cell. Pre-exposure to oleic acid but not to palmitic acid significantly increased GIP-induced GLP-1 secretion when compared to
controls (55±12 % vs 34±9 %, P<0.01). These results demonstrate that the benefit in glycemic tolerance obtained with MUFA diets occurs in association with increased GLP-1 secretion, through a mechanism of enhanced L cell sensitivity. These results suggest that diet therapy with MUFAs may be useful for the treatment of patients with impaired glucose tolerance and/or type 2 diabetes through increased GLP-1 secretion.
4.2 Introduction

The intestinal hormone GLP-1 represents a potential therapeutic agent in the treatment of the insulin resistance and relative insulin deficiency that characterize type 2 diabetes. GLP-1 is one of two major incretin hormones that are secreted from the intestinal tract upon nutrient ingestion which act to increase insulin secretion. Produced from the proglucagon molecule by tissue-specific post-translational processing within the ileal L cell (62,67), GLP-1 is secreted promptly after ingestion of carbohydrate and fat (293,294). GLP-1 receptor activation within the pancreatic islets results in an increase in glucose-dependent insulin secretion, as well as inhibition of glucagon release (167-170,180,207). This ability to decrease glucagon secretion suggests that GLP-1 therapy may also be applicable to patients with type 1 diabetes (216). GLP-1 action in the stomach also reduces gastric acid secretion and gastric motility (217,230), thereby decreasing the rate at which ingested nutrients are absorbed, while in peripheral tissues, GLP-1 may increase sensitivity to insulin (122,240,241). Recent evidence also indicates a role for GLP-1 in the central mechanisms that contribute to satiety (233). Therefore, these actions suggest a potential role for GLP-1 in the treatment of patients with diabetes.

One approach to the therapeutic use of GLP-1 is to enhance its endogenous secretion, in an effort to avoid the compliance issues related to the necessary injections of peptide hormones. A number of studies have been performed that have examined the factors that regulate the secretion of GLP-1. These have indicated that GLP-1 secretion from the ileal L cell is governed by humoral, neural, and nutrient factors. GIP, a hormone released from the K cells of the duodenum, has been demonstrated to increase the secretion of GLP-1 in several experimental models (38,311,318), although not in humans (180), while somatostatin (311)
and insulin (322) are inhibitory to the L cell. Agents of the nervous system such as adrenergic (333) and cholinergic (297,311,388) agonists, and the neuropeptide, GRP (311,326,333), also stimulate GLP-1 secretion. However, as the L cells of the ileum are also exposed to luminal contents, perhaps the most important regulation of GLP-1 secretion is derived from digested nutrients, particularly carbohydrate and fat. Both carbohydrates and fat potently stimulate the secretion of GLP-1 (293,294) and appear to act in an indirect manner since peak GLP-1 levels occur within 30 min of nutrient ingestion, a time frame that is not consistent with the delivery of these nutrients to the ileal L cell (299). However, certain fatty acids have also been shown to directly stimulate the L cell. Specifically, it has been determined that MUFAs are stimulatory to GLP-1 secretion, whereas SFAs are not (refer to Section 3.4.1). Furthermore, the chain length of the fatty acid is an important factor in determining GLP-1 secretion, as only long chain MUFA (≥C16) were found to stimulate the L cell in vitro.

MUFA diets are being increasingly advocated for use in the treatment of patients with type 2 diabetes. Previously, diets high in carbohydrate content were recommended to patients with diabetes, primarily to decrease the cardiovascular risks associated with high levels of saturated fat (350). However, diets high in carbohydrate may be detrimental to glycemic control (351). In contrast, several studies have demonstrated that diets with increased proportions of MUFAs, compared with high carbohydrate diets, produce improvements in glycemic control and also provide benefit to lipid profiles [eg. decreased triglycerides and very low-density lipoprotein (349,351-356)]. Glucagon levels are also elevated in patients who are fed diets high in carbohydrates compared to high MUFA diets (351). Given the benefits to glucose homeostasis produced by the many actions of GLP-1,
and the finding that MUFAs potently stimulate the secretion of GLP-1 \textit{in vitro}, the present study was undertaken to explore the potential link between increased GLP-1 secretion and the benefits in glycemia induced by diets containing MUFAs as compared to SFAs.

4.3 Materials and Methods

4.3.1 Diets and Feeding

Lean Zucker male rats (Fa/?, Charles River Canada Inc., St. Constant, Canada) were used at 7-8 weeks of age in all experimental procedures. The animals were acclimatized in individual cages with free access to food and water for one week. Chow was provided in separate canisters, which were used to determine daily food intake. Subsequent to the acclimatization period, groups of animals were pair-fed on synthetic diets enriched with either MUFAs or SFAs. The dietary composition and energy content of each diet are listed in Table 4-1. In brief, each diet was composed of 75% carbohydrates, 20% protein and 5% fat (Harlan Teklad, Madison, WI). The fat component of the MUFA diet was derived entirely from olive oil (74% MUFA), whereas the fat component of the SFA diet was derived entirely from coconut oil (87% SFA). All other constituents of the synthetic diets were identical between the two groups. Rats were maintained on the synthetic diets for a period of two weeks during which food intake and body weight were monitored daily. Following the two week feeding period, rats were fasted overnight and underwent experiments the following day.

4.3.2 Glucose Tolerance Tests

Oral glucose tolerance tests (OGTT) were performed on conscious animals. In brief, a bolus of 10% glucose was administered by gastric gavage at a dose of 1g/kg body weight. Tails were anesthetized with Xylocaine (Astra Pharma, Mississauga, ON) and blood samples
Table 4-1  Composition and Energy Content of Synthetic Rat Diets

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Source</th>
<th>% of Total Calories</th>
<th>Grams/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>Cornstarch (90%)</td>
<td>75</td>
<td>626.4</td>
</tr>
<tr>
<td></td>
<td>Sucrose (10%)</td>
<td></td>
<td>69.6</td>
</tr>
<tr>
<td>Protein</td>
<td>Casein</td>
<td>20</td>
<td>188</td>
</tr>
<tr>
<td>Fat*</td>
<td>Olive Oil or Coconut Oil</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>Fibre</td>
<td>Cellulose</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>Supplements</td>
<td>DL-Methionine</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Vitamins</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Minerals</td>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

* The two diets differed only in the use of OO or CO to provide the fat component.
were obtained at $t = 0, 10, 20, 30, 60, 90$ and $120$ min for determination of blood glucose utilizing a One Touch Basic blood glucose monitoring system (Lifescan Canada Ltd., Burnaby, BC, Canada).

Duodenal glucose tolerance tests (DGGT) were also performed to avoid any potential effects of GLP-1 on gastric emptying (230). Rats were anesthetized with $60$ mg/kg pentobarbital and the portal vein was cannulated for blood sampling. The portal vein was exposed and was cannulated by placing a free-floating cannula in the vein. Portal blood flow was not disrupted and the cannula was secured to the body wall by loose clips. A bolus of $10\%$ glucose ($1$g/kg body weight) was injected into the duodenum and blood samples were obtained from the tail vein as for the OGTT. In addition, $1$ ml blood samples were also collected from the portal vein at $0, 10, 30, 60, 120$ min into $10\%$ (vol/vol) Trasylol ($5000$ Kalikrein Inactivating Units/ml; Bayer, Inc., Etobicoke, ON, Canada)-EDTA ($12$ mg/ml)-diprotin A ($34$ μg/ml; Calbiochem, La Jolla, CA), and plasma was stored at $-20^\circ$C until time of RIA. In some experiments, Ex$^{(9-39)}$ (Bachem California Inc., Torrance, CA), a GLP-1 receptor antagonist (172), or vehicle control was administered as a bolus dose through a jugular cannula at a dose of $18.3$ nmol/kg ($61.7$ μg/kg), $3$ minutes prior to the administration of duodenal glucose. This protocol was derived from a similar study in which Ex$^{(9-39)}$ infusion antagonized the effect of GLP-1 on glycemic profiles in rats (389). Table 4-2 lists the mean basal plasma glucose levels obtained in the various glucose tolerance tests.
Table 4-2  Basal Plasma Glucose Levels for Glucose Tolerance Tests

Means and SEM are expressed in units of mM for both OGTTs and DGTTs.

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal Plasma Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGGT- Olive Oil</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>OGGT- Coconut Oil</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>DGTT- Olive Oil</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>DGTT-Coconut Oil</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>DGTT- Olive Oil + Exendin(9-39)</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>DGTT-Coconut Oil + Exendin(9-39)</td>
<td>4.7 ± 0.1</td>
</tr>
</tbody>
</table>
4.3.3 GLUTag Cell Cultures

The GLUTag cell line is an L cell model derived from intestinal tumours induced in transgenic mice by expression of the SV40 large T antigen under the control of the proglucagon promoter (36). GLUTag cultures were maintained in DMEM with 10% fetal bovine serum. At the time of experiment, GLUTag cells were trypsinized and plated into 24 well culture plates and allowed to grow to 60-80% confluence. The cells were then rinsed with Hank’s Balanced Salt Solution and exposed to either normal experimental media (DMEM with 1% fetal bovine serum; control), or experimental media containing either 100μM oleic or palmitic acid (Sigma Chemicals; St. Louis, MO) for 24 h. Media was then removed and replaced with either normal experimental media or media containing 100nM human GIP (Bachem California, Torrance, CA) for 2 h. Following the incubation period, media was collected in TFA to a final concentration of 0.1% and small peptides and proteins were extracted by reversed-phase adsorption on a C₁₈ silica cartridge (C₁₈ SepPak, Waters Associates, Milford, MA). The recovery of intact PGDPs with this protocol is greater than 88% (283).

4.3.4 Assays

Plasma samples were analyzed by RIA for gGLI, which correlates directly with GLP-1 levels in the rat in vivo (refer to Section 5.4.1). Briefly, gGLI is derived by subtracting immunoreactive glucagon (IRG), determined in 0.2 ml plasma using antiserum 04A (Dr. R.H. Unger, Dallas, TX), from total GLI, determined using 0.1 ml plasma with antiserum K4023 (Biospacific, Emeryville, CA). Plasma insulin levels were determined using an immunoreactive insulin kit using 0.1 ml of plasma (Linco Research Inc., St. Charles, MO).
Five cm segments of ileum were homogenized in 1 N HCl containing 5% (vol/vol) HCOOH, 1% (vol/vol) TFA and 1% (vol/vol) NaCl. Extraction of small peptides and proteins was carried out by reversed-phase adsorption, as above. Ileal and cell culture media extracts were analysed by RIA for GLP-1 using a GLP-1 antiserum (Affinititi Research Products, Mammead, UK) directed against the carboxy-terminus of the peptide. This antiserum has been shown to recognize predominantly GLP-1\(^{(7-36NH_{2})}\) in extracts of ileum and GLUTag cells (36,59). Protein levels in ileal extracts were assayed by the Lowry Protein method (390).

Fatty acid composition of the plasma was determined by gas chromatography in the laboratory of Dr. S. Cunnane (University of Toronto, Toronto, ON, Canada), as previously described (391).

4.3.5 Data Analysis

Area-under-the-curve (AUC) of glycemic profiles was determined according to the trapezoidal rule. All data are expressed as mean ± SEM. For all experiments \(n = 4-7\). Statistical analysis was assessed by Student’s t-test or ANOVA followed by \(n-1\) post-hoc custom hypothesis tests, as appropriate, on Statistical Analysis System Software (SAS Institute, Inc., Cary, NC). Significance was established to be at the \(P<0.05\) level.

4.4 Results

4.4.1 Food Intake and Body Weight

Food intake during the paired feeding protocol was monitored daily and did not differ significantly between the OO group and the CO group for the duration of the feeding period (Fig. 4-1 A). As a result, the two groups of rats gained weight at similar rates over the two-
Lean Zucker rats were fed diets containing either 5% Olive Oil (solid line) or 5% Coconut Oil (dashed line) for two weeks. A) Food intake and B) subsequent changes in body weight are expressed. C) Levels of essential fatty acids obtained from animals at the end of the two week feeding protocol are expressed as a percentage of total fatty acids.
week course of feeding (Fig. 4-1 B). As deficiencies in the levels of essential fatty acids can cause impaired glucose tolerance (392), circulating essential fatty acids were determined in rats from each group. Levels of essential fatty acids were not significantly different between the two groups of animals (Fig. 4-1 C), consistent with previous studies demonstrating that a two week feeding protocol preserves the levels of essential fatty acids (393,394).

4.4.2 Oral Glucose Tolerance Tests

Following the two week feeding period, rats in both the OO and CO groups were challenged with an oral load of glucose and blood glucose was monitored over the following two hour period. Overall, glycemic responses were reduced in rats that received the OO diet as compared to those rats fed the CO diet (Fig. 4-2 A). OO-fed rats displayed a lower peak in glycemia when compared to the CO group (P<0.05). Furthermore, the OO group demonstrated better glycemic tolerance as evidenced by the return to baseline in blood glucose at t = 120 min in the OO but not the CO group (P<0.05). When the glycemic response was quantified over the entire 120 min period, OO-fed rats were found to have a significantly reduced glycemic AUC as compared to CO-fed rats (121±61 vs 290±24 mM·120min, respectively P<0.05) (Fig. 4-2 B).

4.4.3 Duodenal Glucose Tolerance Tests

In a separate group of animals, DGTTs were performed in order to avoid the inhibitory effects of GLP-1 on gastric emptying (230). Similar results to those achieved with the OGTTs were obtained in this set of experiments (Fig. 4-3A), such that peak glycemia was again lower and blood glucose returned to baseline in OO-fed rats in contrast to the rats fed the CO diet (Fig. 4-3A). The glycemic AUC was also significantly reduced in the OO
Figure 4-2  Effect of Two Week Fat Feeding on Oral Glucose Tolerance Test

A) Oral Glucose Tolerance Test in lean Zucker rats fed diets containing either 5% Olive Oil (solid line) or 5% Coconut Oil (dashed line) for two weeks. The change in blood glucose above basal is depicted. B) The area under the glycemic response curves is also plotted. (*=P<0.05)
Figure 4-3  Effects of Two Week Fat Feeding on Duodenal Glucose Tolerance Tests
A) Duodenal Glucose Tolerance Test in lean Zucker rats fed diets containing either 5% Olive Oil (solid line) or 5% Coconut Oil (dashed line) for two weeks. The change in blood glycemia above basal is expressed. B) The area under the glycemic response curves is also plotted. (*=P<0.05)
as compared to the CO group (112±28 vs 266±65 mM, 120 min, respectively, P<0.05) (Fig. 4-3B). During the DGTTs, blood was also sampled from the portal vein for the determination of hormone levels. Although insulin and glucagon levels were not different between the two groups of animals (Fig. 4-4), plasma gGLI levels were significantly elevated at the 10 min time-point in the OO-fed rats as compared with the CO group (402±96 pg/ml vs 229±33 pg/ml respectively, P<0.05) (Fig. 4-5 A). In contrast, the ileal content of GLP-1 was not significantly different between the OO- and CO-fed rats (Fig. 4-5 B).

4.4.4 Duodenal Glucose Tolerance Tests + Ex(9-39) Infusions

To establish whether different GLP-1 levels between the OO- and CO-fed groups were responsible for the observed differences in glucose tolerance, DGTTs were carried out with concomitant administration of the GLP-1 receptor antagonist, Ex(9-39). Pre-infusion of Ex(9-39) completely abolished the observed benefit in glucose tolerance produced by the OO diet (Fig. 4-6). Peak glucose levels were higher, glycemia remained elevated above baseline for the entire experimental period, and the glycemic AUC was elevated in OO-fed rats that received Ex(9-39) as compared to OO rats receiving a saline (control) infusion (Fig. 4-6 C). In contrast, Ex(9-39) treatment did not alter the glycemic response to an oral glucose challenge in CO-fed rats. Plasma insulin levels were not significantly different between the OO- and the CO-fed rats throughout the experimental period (data not shown), however, after infusion with Ex(9-39), glucagon levels rose dramatically and to a similar extent in both the OO- and CO-fed animals (Fig. 4-7).
Figure 4-4  Insulin and Glucagon Levels During Duodenal Glucose Tolerance Tests

Plasma levels of A) Insulin and B) Immunoreactive Glucagon (IRG) in lean Zucker rats fed diets containing either 5% Olive Oil (diamonds) or 5% Coconut Oil (squares) for two weeks.
Figure 4-5  Plasma Levels of gGLI in Animals Undergoing Two Week Feeding Study
A) Plasma levels of Gut Glucagon-Like Immunoreactivity (gGLI) and B) tissue levels of Glucagon-like peptide-1 (GLP-1) in lean Zucker rats fed diets containing either 5% Olive Oil (diamonds) or 5% Coconut Oil (squares) for two weeks. (*=P<0.05)
Figure 4-6  Effect of Exendin\(^{(9-39)}\) on Duodenal Glucose Tolerance Tests

Duodenal Glucose Tolerance Tests in lean Zucker rats fed diets containing either 5% Olive Oil (diamonds) or 5% Coconut Oil (squares) for two weeks. (A) Olive Oil and Coconut Oil groups pretreated with exendin\(^{(9-39)}\) (18nmol/kg). (B) Olive Oil group (diamonds; data from Fig. 4-3 A) compared to olive oil + exendin\(^{(9-39)}\) (squares; data from panel A). C) Area under the glycemic excursion curve for olive oil group compared with olive oil rats receiving exendin\(^{(9-39)}\). * = P<0.05.
Figure 4-7  Immunoreactive Glucagon Levels in Rats Receiving Exendin\(^{(9-39)}\) Infusions Prior To A Duodenal Glucose Tolerance Test

Immunoreactive Glucagon (IRG) levels during a Duodenal Glucose Tolerance Test. Both Olive Oil- (open diamonds) and Coconut Oil-fed rats (open squares) received a bolus injection of exendin\(^{(9-39)}\) 3 min prior to the duodenal glucose tolerance test.
4.4.5 Effects of MUFA and SFA on GIP-Induced GLP-1 Secretion In Vitro

To determine the effects of chronic exposure to MUFA and SFA on the L cell response to secretagogues, GLUTag cells were pre-incubated for 24 h in media alone (control) or media containing either oleic acid (MUFA) or palmitic acid (SFA). The 24 h pre-treatment was then followed by a 2 h challenge with GIP, a known stimulator of GLP-1 secretion in the L cell (38,311,318). Pre-treatment with either oleic acid or palmitic acid did not change basal secretion of GLP-1 from that observed in cells treated with control media alone (Fig. 4-8). In cells pre-incubated with control media, treatment with GIP resulted in a 34 ± 9 % (P<0.05) increase in GLP-1 secretion. In contrast, in cells pre-incubated with oleic acid, GIP not only induced a significant increase in GLP-1 secretion (by 55 ± 12%; P<0.01), but this increase was significantly greater than that observed in cells that were pre-incubated with control media alone (P<0.01). Pre-incubation of GLUTag cells with media containing 100μM palmitic acid diminished the subsequent GLP-1 response to the same challenge with GIP.

4.5 Discussion

The benefits of diets rich in MUFA have been reported to include improvements in both lipid status and glycemic control (348-356). However, few studies have attempted to investigate the mechanisms by which dietary MUFA mediate these benefits. The demonstration of increased secretion of the antidiabetic hormone GLP-1 in rats following 2 weeks of feeding with an OO diet provides one mechanism for the observed benefit of MUFA to glycemic tolerance. OO feeding, in contrast to feeding with CO, was associated with improvements in glycemic tolerance in rats, as evidenced by lower glycemic AUCs in response to both oral and duodenal glucose tolerance tests. In association with the observed
Cells were preincubated with control media or media supplemented with 100uM Palmitic or Oleic Acid. The cells were then challenged for 2 hours with either media alone (solid bars) or 100nM Glucose Dependent Insulinotropic Peptide (hatched bars). *=P<0.05 vs Control, **=P<0.01 vs Control.

Figure 4-8  24 Hour Preexposure of GLUTag Cells with Monounsaturated or Saturated Fatty Acids
benefit in glycemic tolerance achieved in this study was the concomitant increase in the secretion of gGLI, a molecule released in parallel with the antidiabetic hormone GLP-1 (refer to Section 5.4.1). An alternative perspective on this finding is that chronic CO feeding acted to depress GLP-1 secretion in this setting. However, this seems unlikely since oleic acid, the main fatty acid constituent of OO, has been demonstrated to significantly increase GLP-1 secretion in intestinal cultures in vitro (refer to Section 3.4.1). Consistent with this finding, OO, but not butter, also stimulates GLP-1 secretion acutely in humans (395).

Curiously, the benefit in glycemic tolerance observed in the MUFA-fed rats persisted for the entire experimental time period (120 min) even though GLP-1 secretion was elevated only at the early time point of the experiment (10 min). This pattern of GLP-1 secretion is consistent with stimulation of the L cell through indirect mechanisms since the glucose bolus is rapidly absorbed in the proximal regions of the gastrointestinal tract. Thus, glucose does not progress to the distal location of the majority of the L cells under physiological conditions and is therefore incapable of eliciting later peaks in GLP-1 secretion. Therefore, in order to clearly define the contribution of increased GLP-1 secretion to the benefit in glycemic tolerance induced by MUFA feeding, experiments utilizing a specific inhibitor of the GLP-1 receptor were undertaken. Ex(9-39) is a peptide homologue of GLP-1 isolated from the venom of the Gila monster and has been demonstrated to be a potent antagonist at the GLP-1 receptor (172,389). Ex(9-39) administration prior to a duodenal glucose tolerance test in rats fed OO completely abolished the benefit in glycemic tolerance obtained with this diet, therefore demonstrating that the beneficial effects of MUFA feeding on glycemic tolerance are specific to activation of the GLP-1 receptor. A similar experiment conducted in the CO group did not significantly alter the glycemic response to the duodenally administered
glucose challenge, consistent with the observation that GLP-1 secretion was not elevated in this group. It may also be postulated that the CO diet partly decreased glucose tolerance in the present study, as SFA have been associated with decreases in insulin sensitivity (396,397). However, the results of the Ex^{9-39} study clearly implicate GLP-1 as a causative factor in the enhanced glycemic tolerance conferred by MUFA feeding in the present study.

The fact that the benefit in glycemic tolerance induced by OO feeding in OGTIs was maintained in DGTTs indicates that differences in the rates of gastric emptying were not responsible for the MUFA-induced improvement in glycemic tolerance. This is an important finding, as GLP-1 is known to inhibit gastric emptying as part of its ability to improve glycemic handling (217,230). Also, as tissue levels of GLP-1 were not significantly different between the two groups of rats, altered synthesis of GLP-1 cannot account for the increased secretion of GLP-1 observed in the OO-fed group. This is also consistent with the fact that fatty acids do not affect total cell content of GLP-1 in vitro (refer to Section 3.4.1). Furthermore, the increase in GLP-1 secretion in rats fed OO was not associated with alterations in the plasma levels of either insulin or glucagon. In support of this finding is a recent report that demonstrated that elevated secretion of GLP-1 did not alter insulin levels in normal humans fed a meal supplemented with OO (395). Similarly, GLP-1 administration during an OGTT in rats improves glycemic tolerance in the absence of any effect on insulin secretion (398). Given that the primary stimulator of insulin secretion is the level of glycemia, the fact that glucose levels were diminished in the OO rats despite unaltered plasma levels of insulin demonstrates that the amount of insulin secreted per unit of glycemia must have increased as a result. Therefore, the enhanced secretion of GLP-1 observed in the
present study may have benefited glycemic tolerance through its stimulatory effects on the \( \beta \) cell despite the fact that plasma levels of insulin were not different.

However, in this model it is not possible to accurately define the site of the benefit to glycemic tolerance induced by the increased secretion of GLP-1. Thus, the decreased levels of glycemia accompanied by unchanged insulin levels observed in OO-fed rats may also be explained by extrapancreatic-extragastric functions of GLP-1 acting to enhance insulin sensitivity. In support of this concept, GLP-1 has been reported to stimulate glycogen formation in liver cells (255), and GLP-1 receptors have been identified in both muscle and adipose tissue (122-124). GLP-1 also enhances insulin-dependent and insulin-independent glucose disposal in dogs and humans, respectively (122,240,241), although this remains controversial (244). In addition, high-MUFA diets can improve glycemic control when compared to diets high in carbohydrate (348,399) and have been shown to enhance insulin sensitivity (352). Interestingly, the effects of the qualitative features of dietary fat on insulin sensitivity were examined recently, demonstrating that chronic feeding of diets high in MUFAs improved insulin sensitivity compared to a high SFA diet in healthy human volunteers (355). It is not known whether the dietary manipulation altered the secretion of GLP-1 in these experiments. Additionally, the experiments utilizing the GLP-1 receptor antagonist, Ex\(^{9-39}\), demonstrated that the deterioration of glycemic tolerance was partly attributable to the elevated secretion of glucagon, consistent with a role for GLP-1 in the inhibition of secretion from the \( \alpha \) cell. Therefore, the improvements in glycemic tolerance mediated by enhanced secretion of GLP-1 witnessed in the present study can be attributed to effects on the endocrine pancreas and/or peripheral insulin-sensitive tissues.
Of some interest was the finding that GLP-1 secretion in the O0-fed rats was elevated above basal levels very early during the glucose challenge. Similar rapid increments in GLP-1 secretion have also been observed in humans (293,294). These findings are inconsistent with the fact that nutrients do not reach the ileal L cell within this time frame (299), and have led to the hypothesis that the early phase of nutrient-induced GLP-1 secretion may be mediated indirectly (162,305). In the rat, this indirect regulation of GLP-1 secretion is mediated through complex interactions between the endocrine and nervous systems, and involves the enteric hormone GIP (162) and the vagus nerve (refer to Section 5.4). However, GIP can also directly stimulate the L cell (38,311,318), and for this reason, was used to investigate the potential mechanism of action of MUFAs on the L cell. Exposure to oleic acid for 24 hr prior to a 2 hr challenge with GIP led to a greater stimulation of GLP-1 secretion as compared to the secretion elicited by the same dose of GIP in cells pre-exposed to control media only. Furthermore, pre-exposure of the L cells to palmitic acid, the predominant SFA in CO caused a blunting in the GLP-1 secretion induced by GIP. Therefore, it appears that chronic exposure to MUFAs can enhance the sensitivity of the L cell to subsequent stimulation, resulting in greater levels of GLP-1 secretion, whereas SFAs depress the secretory response to the same stimulus. The cellular mechanism(s) underlying these responses is not known, however, several factors may be speculated to play a role. For example, PKC-ζ has been reported to be preferentially activated by unsaturated as opposed to saturated fatty acids (366,381) and therefore may be implicated in this process. PKC is a known intracellular mediator of GLP-1 secretion (283), and the intestinal L cell expresses the ζ isoform of this enzyme (refer to Section 3.4.4). Alternatively, the increased L cell sensitivity to GIP induced by oleic acid may occur consequent to altered membrane fluidity.
(400), possibly through changes in the activity of the I-FABP. I-FABP is present in the intestinal L cell (refer to Section 3.4.3) and this molecule is known to influence cell membrane structure and function (401,402). Further experimentation is clearly required in order to identify the intracellular mechanism(s) that is responsible for the MUFA-induced increase in L cell sensitivity to GIP stimulation.

In conclusion, the benefits in glycemic control that have been ascribed to diets enriched with MUFAs can be explained, at least in part, by the fact that such diets increase secretion of the antidiabetic hormone GLP-1. Manipulation of dietary fatty acid composition to increase the content of MUFAs may therefore be a useful approach with which to increase the secretion of GLP-1 in patients with impaired glucose tolerance or type 2 diabetes.
ROLE OF THE VAGUS NERVE IN MEDIATING PROXIMAL NUTRIENT-INDUCED GLUCAGON-LIKE PEPTIDE-1 SECRETION

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5.1 ABSTRACT

Plasma levels of GLP-1 rise rapidly after nutrient ingestion, suggesting the existence of a proximal gut signal regulating GLP-1 release from the L cells of the distal small intestine. GIP has been shown to be one such proximal signal, however, the dependence of GIP on GRP, a neuromodulator, suggested a possible role for the nervous system in this proximal-distal loop. Investigations into the nature of this proximal signal were therefore conducted in an in situ model of the rat gastrointestinal system. Infusions of corn oil into a 10 cm segment of duodenum that was isolated by loose ligation (to ensure that luminal contents did not progress to the ileal L cell) increased the secretion of GLP-1 in parallel with that of gGLI (r=0.85, P<0.05). Infusion of fat into a transected segment of duodenum also significantly raised gGLI secretion compared to saline infusion, reaching a peak value of 132 ± 37 pg/ml. However, peak secretion was significantly delayed when the gut was transected as compared to ligation alone (19 ± 4 vs 6 ± 1 min, respectively; P<0.05). Furthermore, bilateral subdiaphragmatic vagotomy in conjunction with gut transection completely abolished the fat-induced rise in gGLI secretion (P<0.001). Consistent with a role for the vagus in the regulation of the L cell, stimulation of the distal end of the celiac branch of the subdiaphragmatic vagus nerve significantly stimulated the secretion of gGLI to a level of 71 ± 14 pg/ml above basal (P<0.05). As found previously, supraphysiological infusion of GIP significantly increased gGLI secretion in control animals by 123 ± 32 pg/ml (P<0.05); this was not prevented by hepatic branch vagotomy [96 ± 25 pg/ml (P<0.05)]. In contrast, although infusion of GIP at physiological levels into sham vagotomized animals also increased gGLI secretion, by 40 ± 6 pg/ml (P<0.05), selective hepatic branch vagotomy abolished the GIP-induced gGLI secretion (P<0.05). The results of these experiments
therefore demonstrate that the secretion of GLP-1 and gGLI from the ileal L cell in response to fat is regulated by a complex neuroendocrine loop, involving the myenteric plexus, afferent and efferent vagus nerves, as well as the duodenal hormone GIP.
5.2 INTRODUCTION

Proglucagon is cleaved by tissue-specific prohormone convertases to the PGDPs (67). The major pancreatic product of proglucagon processing is glucagon, while the intestinal products include glicentin, oxyntomodulin, GLP-1 and GLP-2. While GLP-1 possesses inhibitory action in terms of gastric emptying (208,217,230), its main effect is its role as an incretin, stimulating the secretion of insulin in a glucose-dependent manner (168,170,208). Other metabolic effects of GLP-1 include suppression of glucagon secretion (208), enhancement of glucose disposal (241), and inhibition of central feeding behaviour (233). These pleiotropic actions of GLP-1 therefore offer great potential for the treatment of the insulin resistance and relative insulin insufficiency that define type 2 diabetes mellitus (259).

The release of GLP-1 and the other PGDPs from ileal L cells is under complex regulation. Agents that can directly stimulate the secretion of GLP-1 have been analyzed in various models of the intestinal L cell including the primary FRIC cultures (311,403), a murine intestinal endocrine cell line (343) and a perfused model of the rat ileum (333,404). The major secretagogues identified using these models include GIP, GRP, CGRP and agonists of acetylcholine. Fatty acids, when applied directly to the FRIC cultures also stimulate GLP-1 secretion (refer to Section 3.4.1).

In the in vivo setting, GLP-1 is rapidly released from ileal L cells upon the ingestion of a mixed meal (293,294) or by the infusion of fat directly into the duodenum (162,305). Indeed, plasma levels of GLP-1 peak within 30 min of nutrient administration, despite the fact that nutrients do not reach the ileum within this time frame (296). Furthermore, in patients with ileostomies to divert nutrient flow from the ileum, GLP-1 levels after ingestion
of a mixed meal are similar to those observed in individuals with an intact gastrointestinal tract (294). These observations are not consistent with the notion that nutrients act directly on ileal L cells to stimulate the release of GLP-1, at least during the early phase of secretion. Therefore, it has been postulated that the secretion of GLP-1 is under the regulation of a proximal-distal loop, relaying information about nutrient, and in particular, fat ingestion from the proximal duodenum to the distal site of GLP-1-containing L cells (162,305). Consistent with this concept, we have demonstrated that the duodenal peptide GIP, which is secreted in response to fat ingestion (162,293), is an endocrine mediator of this proximal-distal loop in rats (162). However, recent data have demonstrated that the secretion of GLP-1 induced by duodenal fat can be completely inhibited by infusion of an antagonist for the neuropeptide GRP (326). These findings suggest that physiological doses of GIP act through the nervous system (either vagal or myenteric) to indirectly stimulate GLP-1 secretion, rather than acting directly at the level of the L cell.

The present investigation further examined the possible existence of a neural component of the proximal-distal loop regulating secretion of GLP-1 in the rat. Specifically, the intrinsic and extrinsic nervous systems were analyzed with respect to their ability to affect duodenal fat-induced GLP-1 secretion. In addition, the humoral mechanism by which nutrients in the proximal duodenum signal the distal L cell was reevaluated by examining the potential interaction of GIP with the vagus nerve.

5.3 MATERIALS AND METHODS

5.3.1 Surgery

After fasting for 18-24 h, male Wistar rats weighing 337 ± 6 g (Charles River Canada Inc., St. Constant, Quebec, Canada) were lightly anesthetized with halothane and given an
intra
eritoneal injection of sodium pentobarbital (60 mg/kg) to achieve a surgical plane of
anesthesia. The carotid artery was cannulated to allow for arterial blood sampling. A
maximum of 9 samples were collected from each experimental animal. Blood was collected
into 10% (v/v) Trasylol:EDTA:Diprotin A [5,000 Kalikrein Inactivating Units/ml (Bayer
Inc., Etobicoke, Canada): 12 mg/ml: 34 μg/ml (CalBiochem, La Jolla, CA)]. Plasma was
collected and stored at -20°C until time of assay, and the red blood cells were resuspended in
an equal volume of heparinized saline and re-infused via a cannula in the jugular vein. To
ensure that the experimental procedures were not stimulating a counter-regulatory response,
corticosterone levels (Diagnostic Products Corp., Los Angeles, CA) were determined in 50
μl of plasma from all animals (n=42) upon initiation (585 ± 25 ng/ml) and completion (552
± 18 ng/ml) of blood sampling, and were not found to be significantly different.

5.3.2 Model of the Proximal Distal Loop
In order to separate indirect from direct effects of nutrients on the ileal L cell, the
proximal-distal loop governing GLP-1 secretion was modeled by infusing nutrients into a 10
cm segment of proximal duodenum (162,305,326). The duodenum was cannulated above the
common bile duct, and isolated from the remaining gut by loose ligation 10 cm distal to the
site of the duodenal catheter. This ligation ensures that nutrients do not progress to the distal
portions of the small intestine to directly stimulate GLP-1 secretion from the ileal L cells, but
permits retention of all neurovascular connections. Blood was collected at t=-10 and 0 min
to ascertain basal levels of secretion, and manipulation of the gastrointestinal tract was
performed in the interval between these two time points. At t=0 min, 3-4 ml of fat in the
form of corn oil (56% polyunsaturated fatty acids, 32% MUFAs, 12% saturated fatty acids;
Sunfresh Ltd., Toronto, ON) was infused into the proximal duodenal segment, and blood samples were collected at every five-ten minutes thereafter to \( t=60 \) min.

### 5.3.3 Vagotomy

To ascertain the role of vagal innervation in the regulation of GLP-1 secretion, a series of vagotomies were performed prior to the start of experimental sampling, in the interval between the first and second basal blood sample. The procedures included: a) left cervical vagotomy, b) bilateral subdiaphragmatic vagotomy, and c) selective hepatic branch vagotomy. The bilateral subdiaphragmatic vagotomy was performed by transection of the esophagus including the accompanying vagal trunks.

### 5.3.4 Vagal Stimulation

The vagal stimulation protocol was based on parameters used in a similar study of vagally-induced bombesin-like immunoreactivity release (405). Briefly, the celiac branch of the subdiaphragmatic vagus nerve, which innervates the distal portions of the gastrointestinal tract containing the majority of GLP-1-secreting L cells, was transected 30 min prior to the commencement of the experimental procedure. During this interval the distal portion of the celiac branch was placed over a bipolar stimulating electrode connected to a Grass Stimulator. This preparation was then immersed in paraffin oil throughout the duration of the experiment. The electrical stimulation was begun at \( t=0 \) and lasted for 15 min at a voltage of 10 V and a frequency of 20 Hz (preliminary studies with lower frequencies did not significantly alter gGLI secretion). Blood was collected at \( t=5, 10 \) and 15 min during the stimulation period and then every 10 min throughout the remainder of the experimental time course.
5.3.5 GIP Infusions

Human GIP (Bachem California, Torrance, CA) was administered as a bolus via the jugular vein followed by a maintenance infusion through the femoral vein for 40 min and then a 20 min recovery period. Blood samples were collected every 10 min. GIP was infused at either a high (supraphysiological; 715 ng/kg bolus + 1070 ng/kg•h infusion) or low (physiological; 143 ng/kg bolus + 214 ng/kg•h infusion) dose. The physiological dose of GIP was based on that used in a previous study which was found to mimic levels of GIP produced by the ingestion of a fat meal (162). The supraphysiological dose of GIP was 5 times greater than the physiological dose.

5.3.6 Assays

In correlation experiments, RIA for GLI (glicentin/oxyntomodulin/glucagon) was carried out using 0.1 ml of plasma with antiserum K4023 (Biospacific, Emeryville, CA), while RIA for IRG (glucagon) utilized 0.2 ml of plasma, with antiserum 04A (Dr. R.H. Unger, Dallas, TX). The plasma levels of gGLI were obtained by subtraction of IRG from GLI (162,305,326). Immunoreactive GLP-1(7-36NH2) was detected by RIA using an antiserum directed against the carboxy terminus of GLP-1(7-36NH2) (Affinity Research Products, UK). Prior to RIA for GLP-1(7-36NH2), 1 ml of plasma was diluted with 2 ml of 1% TFA (pH 2.5 with diethylamine) and purified by passage through a C18 SepPak cartridge (Waters, Milford, MA). Peptides were eluted with 3 ml of 80% isopropanol/0.1%TFA, and the eluate was dried in vacuo to make a single sample for RIA (406). This procedure resulted in a recovery rate of 53.9 ± 6% when spiked plasma samples were analyzed. Immunoreactive GIP levels were determined using 0.1 ml of plasma in a human GIP RIA kit (Peninsula Laboratories Inc., Belmont, CA).
5.3.7 Data Analysis

Peptide secretion is expressed as the change from basal values. All data are expressed as mean ± SEM. For all experiments, n = 4-7. Statistical significance between experimental groups was assessed by ANOVA using n-1 post hoc custom hypothesis tests on the Statistical Analysis System Software (SAS Institute, Cary, NC). Comparisons of plasma levels of hormones between basal levels and subsequent time points within an experimental group were made using repeated measures ANOVA. Significance was determined at the P<0.05 level in these comparisons. Where single determinations were made, a paired Student’s t test was performed. Some data were log_{10} transformed prior to analysis.

5.4 Results

5.4.1 Correlation of GLP-1 and gGLI Secretion

The large amount of plasma required for assay of GLP-1 (one ml per determination) limited repeated sampling over a prolonged period of time. Since the proglucagon molecule is cleaved in the intestinal L cell to produce glicentin/oxyntomodulin and GLP-1 in a 1:1 relationship, the secretion of gGLI has been used to monitor the secretion of GLP-1 indirectly, at least in vitro (refer to Section 3.4). To determine the relationship between gGLI and GLP-1 levels in the rat in vivo, plasma levels of gGLI and GLP-1 were determined in the same animals under basal conditions and in response to administration of fat into the duodenum (Fig. 5-1). gGLI levels were highly correlated with those of GLP-1 (r=0.85, P<0.05, n=3). As the gGLI assay requires only 300 µl of plasma, as compared to 1 ml for the GLP-1 RIA, gGLI was therefore used as a measure of GLP-1 secretion in all subsequent studies.
The change in GLP-1 levels obtained from corn oil challenge of the proximal-distal loop model (n=3) were corrected for GLP-1 assay recovery rate and plotted as a function of the change in gGLI levels obtained from the same plasma samples. The open circle indicates an outlier in the triplicate for GLP-1 at that level of gGLI. This outlier was not included in the regression analysis.
5.4.2 Effect of Left Cervical Vagotomy on Basal Levels of gGLI

Basal levels of gGLI were 193 ± 12 pg/ml (n=36) in anesthetized rats. To assess whether the vagus nerve has a global effect on basal levels of gGLI, a left cervical vagotomy was performed in unstimulated animals. The right cervical vagus nerve was left intact to maintain cardiorespiratory function. Left cervical vagotomy reduced the mean basal level of gGLI from 205 ± 11 pg/ml in control animals to 113 ± 9 pg/ml (P<0.001, n=6). This finding therefore suggested that the vagus nerve plays a role in modulating basal gGLI secretion. Levels of plasma glucose and glucagon are shown in tables 5-1 and 5-2 and were not affected by left cervical vagotomy.

5.4.3 Corn Oil Infusion in the Model of the Proximal-Distal Loop

To confirm that the presence of fat in the proximal duodenum can signal the GLP-1-containing L cells in the ileum, the model of the proximal-distal loop was employed (162,305). Infusion of 3-4 ml of saline into a ligated segment of duodenum did not significantly alter the secretion of gGLI from basal levels (Fig. 5-2). In contrast, infusion of 3-4 ml of fat in the form of corn oil induced a rapid rise in gGLI secretion (P<0.05 at 5 min), which was maintained throughout the remainder of the experimental time course (P<0.001 vs saline infusion, n=5). The role of the enteric nervous system in mediating this proximal signal to the ileal L cells was investigated by transection of the gastrointestinal tract at a site immediately distal to the isolated segment of duodenum prior to the infusion of corn oil. This procedure did not reduce the gGLI secretion induced by duodenal fat, as the infusion of corn oil elicited a significant rise in gGLI levels, when compared to saline controls (P<0.05, n=6) (Fig. 5-2). The peak change in gGLI from basal was 132 ± 37 pg/ml (P<0.05, n=6),
Table 5-1  Changes in Plasma Glucose over Time with Left Cervical Vagotomy and Celiac Vagal Stimulation.

Means and SEM are expressed in units of mg/dl and represent changes from basal levels. No significant differences were observed from control animals.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>L. Cervical Vagotony</th>
<th>Time (min)</th>
<th>Celiac Vagal Stim.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>86 ± 2</td>
<td>92 ± 5</td>
<td>Basal</td>
<td>111 ± 4</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>7 ± 4</td>
<td>11 ± 7</td>
<td>5</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>20</td>
<td>11 ± 5</td>
<td>14 ± 8</td>
<td>10</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>29 ± 8</td>
<td>22 ± 9</td>
<td>15</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>40</td>
<td>26 ± 8</td>
<td>24 ± 10</td>
<td>25</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>50</td>
<td>29 ± 9</td>
<td>21 ± 11</td>
<td>35</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>60</td>
<td>28 ± 9</td>
<td>19 ± 12</td>
<td>45</td>
<td>35 ± 6</td>
</tr>
</tbody>
</table>
Table 5-2  Changes in Plasma Immunoreactive Glucagon over Time with Left Cervical Vagotomy and Celiac Vagal Stimulation.

Means and SEM are expressed in units of pg/ml and represent changes from basal levels. No significant differences were observed from control animals.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>L. Cervical Vagotom</th>
<th>Time (min)</th>
<th>Celiac Vagal Stim.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>105 ± 14</td>
<td>106 ± 13</td>
<td>Basal</td>
<td>135 ± 23</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>-11 ± 20</td>
<td>11 ± 14</td>
<td>5</td>
<td>7 ± 18</td>
</tr>
<tr>
<td>20</td>
<td>-16 ± 15</td>
<td>23 ± 13</td>
<td>10</td>
<td>2 ± 5</td>
</tr>
<tr>
<td>30</td>
<td>22 ± 17</td>
<td>41 ± 20</td>
<td>15</td>
<td>40 ± 16</td>
</tr>
<tr>
<td>40</td>
<td>22 ± 16</td>
<td>59 ± 38</td>
<td>25</td>
<td>15 ± 20</td>
</tr>
<tr>
<td>50</td>
<td>72 ± 42</td>
<td>120 ± 68</td>
<td>35</td>
<td>24 ± 22</td>
</tr>
<tr>
<td>60</td>
<td>111 ± 72</td>
<td>211 ± 145</td>
<td>45</td>
<td>40 ± 21</td>
</tr>
</tbody>
</table>
Figure 5-2  Indirect Stimulation of GLP-1 Secretion by Duodenal Fat in the Proximal-Distal Model of GLP-1 Secretion

Change in plasma levels of gGLI in response to 3-4 ml of corn oil infused into the proximal segment of the duodenum, isolated by loose ligation (closed triangles, n=5) or gut transection distal to the proximal segment (open circles, n=6). Changes in plasma levels of gGLI in response to 3-4 ml of saline are shown by the closed squares (n=5). Differences between groups are represented by the P values above the figure. Differences from basal levels within each individual experimental group are indicated by the asterisk. * = P<0.05 vs basal.
and this was not significantly different from that achieved by infusion of corn oil into the ligated duodenal segment. However, a significant difference was observed in the timing of the corn oil-induced response of gGLI secretion in the two protocols, such that the fat-induced rise in gGLI levels was significantly delayed by transection of the gut as compared to the animals undergoing gut ligation (P<0.05 for corn oil/ligation vs corn oil/transection at t=5 min). Indeed, the peak change in gGLI secretion occurred at 19 ± 4 min in the transected enteric nervous system group compared to 6 ± 1 min in the ligated group (P<0.05).

To investigate the possibility that extrinsic vagal fibers mediate the proximal signal to the ileal L cells, bilateral subdiaphragmatic vagotomies were performed in addition to gut transection, prior to the administration of fat (Fig. 5-3). In contrast to the response found in rats with an intact vagus, corn oil infusion into the proximal duodenum of vagotomized rats completely failed to stimulate gGLI secretion (P<0.001 vs intact vagus, n=5). Changes from basal levels of plasma glucose and glucagon are shown in tables 5-3, 5-4.

5.4.4 Effect of Vagal Stimulation on gGLI Secretion

Electrical stimulation of the celiac branch of the vagus nerve, which innervates the distal portions of the gastrointestinal tract containing GLP-1-secreting L cells, was performed in unstimulated animals to determine whether the efferent vagus nerve mediates the signal from the proximal duodenum to the distal L cells (Fig. 5-4). The celiac branch of the vagus nerve was transected and the distal end was stimulated for a 15 min period. Electrical stimulation using 10 V and a frequency of 20 Hz resulted in a significant rise in gGLI levels which reached a peak of 71 ± 14 pg/ml above basal at the 10 min time point (P<0.05, n=5). gGLI levels returned to basal during the final phase of the stimulation period, but surprisingly, increased again to significance during the recovery period. No significant effect
Figure 5-3  Effect of Subdiaphragmatic Vagotomy on Changes in Plasma Level of gGLI in Response to Corn Oil.

Changes of gGLI induced by corn oil infusion in gut transected control animals (open circles; n=6) and subdiaphragmatic vagotomized animals (closed circles, n=5) are represented. Differences between groups are represented by the P values above the figure. Differences from basal levels within each individual experimental group are indicated by the asterisk. *=P<0.05 vs basal.
Table 5-3  Changes in Plasma Glucose over Time with Duodenal Saline or Corn Oil Administration.

Means and SEM are expressed in units of mg/dl and represent changes from basal levels. * = P<0.05 vs duodenal saline control group.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Duodenal Saline</th>
<th>Duodenal Corn oil Infusions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gut Transection</td>
<td>Gut Ligation *</td>
</tr>
<tr>
<td>Basal</td>
<td>127 ± 16</td>
<td>121 ± 6</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>12 ± 4</td>
<td>29 ± 19</td>
</tr>
<tr>
<td>20</td>
<td>13 ± 6</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>30</td>
<td>12 ± 9</td>
<td>25 ± 13</td>
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<td>40</td>
<td>12 ± 20</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>50</td>
<td>35 ± 20</td>
<td>54 ± 16</td>
</tr>
<tr>
<td>60</td>
<td>55 ± 29</td>
<td>52 ± 17</td>
</tr>
</tbody>
</table>
Table 5-4  Changes in Plasma Immunoreactive Glucagon over Time with Duodenal Saline or Corn Oil Administration.

Means and SEM are expressed in units of pg/ml and represent changes from basal levels.  

*** = P<0.001 vs Gut ligation group.  # = P<0.05 vs basal level within subdiaphragmatic vagotomy group.  Subdiaphragmatic vagotomy increased basal levels of IRG by 41 ± 9 pg/ml.  This value was set to zero and taken as the reference for subsequent changes in this group.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Duodenal Saline</th>
<th>Duodenal Corn oil Infusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>96 ± 19</td>
<td>111 ± 10</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>-3 ± 13</td>
<td>-24 ± 8</td>
</tr>
<tr>
<td>20</td>
<td>-2 ± 18</td>
<td>-17 ± 6</td>
</tr>
<tr>
<td>30</td>
<td>-1 ± 20</td>
<td>-18 ± 10</td>
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<td>40</td>
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<tr>
<td>50</td>
<td>27 ± 5</td>
<td>23 ± 20</td>
</tr>
<tr>
<td>60</td>
<td>20 ± 28</td>
<td>54 ± 27</td>
</tr>
</tbody>
</table>

Subdiaphragmatic Vagotomy
Figure 5-4  Responses of Plasma gGLI to Electrical Stimulation of the Distal End of the Celiac Branch of the Vagus Nerve.

Electrical stimulation consisted of 10 V at 20 Hz for a stimulation period of 15 min. Differences from basal are indicated by the asterisk. * = P<0.05 vs basal.
of celiac branch stimulation on plasma glucose and glucagon levels was detected when compared with the control group (table 5-1).

5.4.5 Effect of High GIP Infusion with or without Hepatic Branch Vagotomy

In order to determine the relative contribution of the vagus nerve to the proximal-distal loop, with respect to that of the humoral component mediated by GIP (162), primed infusions of GIP at varying doses were performed in animals with or without a hepatic branch vagotomy (Fig. 5-5). The hepatic branch of the vagus nerve innervates the duodenum, which contains the GIP-secreting K cells. Primed infusion of GIP at a supraphysiologic dose resulted in rapidly elevated GIP levels, which were not significantly different between the control and hepatic branch vagotomized animals. Peak immunoreactive GIP levels reached 1030 ± 121 pg/ml above basal (P<0.05, n=7) in the control group at 13 ± 2 min, and returned towards basal during the subsequent infusion and recovery periods. These elevations in GIP levels resulted in significant increases in gGLI in control as well as vagotomized animals. The peak of gGLI above basal induced by the supraphysiological GIP infusion in the control group was 123 ± 32 pg/ml (P<0.05, n=7), and this was not significantly different from the gGLI response achieved by supraphysiological GIP infusion in the hepatic branch vagotomized animals (96 ± 25 pg/ml, P<0.05, n=6). Interestingly, GIP-induced gGLI secretion rapidly returned to baseline within 20 min of reaching peak levels, despite elevated concentrations of GIP in both control and vagotomized groups.
Figure 5-5 Supraphysiological Levels of GIP Exert Direct Effects on the L Cell.

Changes in plasma GIP (A) and gGLI (B) levels in response to primed supraphysiological infusions of GIP into control (sham hepatic branch vagotomized animals; closed circles, n=7) or hepatic branch vagotomized animals (open circles, n=6). The 40 min infusion period (solid bar) was followed by a 20 min recovery period. Differences between groups are represented by the P values above the figure. Differences from basal levels within each individual experimental group are indicated by the asterisk. * = P<0.05 vs basal.
5.4.6 Effect of Low GIP Infusion with or without Hepatic Branch Vagotomy

The effects of physiological increases in GIP levels on gGLI secretion were also examined in the presence or absence of intact vagal innervation. The infusion of the physiological dose of GIP into control animals resulted in a peak change of 223 ± 43 pg/ml above basal at 13 ± 3 min (P<0.05, n=4). The level of GIP achieved by the physiological infusion of GIP in the vagotomized group was not significantly different from that in the control group, reaching a maximum of 169 ± 30 pg/ml above basal at 12 ± 2 min (P<0.05, n=5) (Fig. 5-6). Furthermore, the GIP levels achieved by the infusion were significantly decreased compared to the supraphysiological dose of GIP (P<0.001). In control animals, the physiological dose of GIP led to a small but significant elevation in gGLI secretion, reaching a peak of 40 ± 7 pg/ml above basal levels (P<0.01, n=4), and rapidly returning to basal levels by the 20 min time point. In contrast, hepatic branch vagotomy completely abolished the rise in gGLI secretion induced by the physiological infusion of GIP (P<0.05 vs controls). Changes in plasma glucose and glucagon produced by the infusions of GIP are demonstrated in tables 5-5 and 5-6.

5.5 Discussion

Due to its numerous actions on metabolic processes, GLP-1 holds great promise as a treatment for type 2 diabetes mellitus. Therefore, it is imperative that the factors that modulate the secretion of GLP-1 are understood. The results of the present paper further this knowledge by demonstrating that a neuroendocrine loop exists which acts to stimulate the ileal L cells when nutrients are present in the proximal duodenum. The neural component of this proximal-distal loop has been demonstrated to involve the vagus nerve, while the
Figure 5-6  Physiological Levels of GIP Act Indirectly via the Vagus Nerve on the GLP-1-Secreting L Cell.

Changes in plasma GIP (A) and gGLI (B) levels in response to primed physiological infusions of GIP into control (sham hepatic branch vagotomized animals; closed circles, n=4) or hepatic branch vagotomized animals (open circles, n=6). The 40 min infusion period (solid bar) was followed by a 20 min recovery period. Differences between groups are represented by the P values above the figure. Differences from basal levels within each individual experimental group are indicated by the asterisk. * = P<0.05 vs basal.
Table 5-5 Changes in Plasma Glucose over Time with Supraphysiological or Physiological Administration of GIP in Sham or Hepatic Branch Vagotomized Animals.

Means and SEM for plasma glucose are expressed in units of mg/dl and represent changes from basal levels.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Supraphysiological GIP Infusion</th>
<th>Physiological GIP Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham Vagotomy</td>
<td>Hepatic Vagotomy</td>
</tr>
<tr>
<td>Basal</td>
<td>94 ± 6</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>17 ± 7</td>
<td>11 ± 8</td>
</tr>
<tr>
<td>20</td>
<td>-8 ± 14</td>
<td>2 ± 6</td>
</tr>
<tr>
<td>30</td>
<td>-0 ± 7</td>
<td>-1 ± 5</td>
</tr>
<tr>
<td>40</td>
<td>-3 ± 3</td>
<td>-4 ± 4</td>
</tr>
<tr>
<td>50</td>
<td>-2 ± 5</td>
<td>0 ± 9</td>
</tr>
<tr>
<td>60</td>
<td>7 ± 7</td>
<td>10 ± 10</td>
</tr>
</tbody>
</table>
Table 5-6 Changes in Plasma Immunoreactive Glucagon over Time with Supraphysiological or Physiological Administration of GIP in Sham or Hepatic Branch Vagotomized Animals.

Means and SEM are expressed in units of pg/ml and represent changes from basal levels. * = P<0.05 vs sham vagotomy with supraphysiological GIP infusion. ## = P<0.01 vs sham vagotomy with physiological GIP infusion.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Supraphysiological GIP Infusion</th>
<th>Physiological GIP Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham Vagotomy</td>
<td>Hepatic Vagotomy</td>
</tr>
<tr>
<td>Basal</td>
<td>67 ± 9</td>
<td>102 ± 21</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>54 ± 10</td>
<td>38 ± 14</td>
</tr>
<tr>
<td>20</td>
<td>23 ± 7</td>
<td>25 ± 17</td>
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<td>23 ± 10</td>
</tr>
<tr>
<td>60</td>
<td>23 ± 8</td>
<td>57 ± 22</td>
</tr>
</tbody>
</table>
humoral component has previously been described to be mediated by the duodenal hormone GIP (162). Furthermore, these two limbs of the proximal-distal loop have now been shown to interact within the physiological range to induce a rapid secretory response from the ileal L cell.

In order to investigate the secretion of GLP-1 in vivo, a method to evaluate GLP-1 levels over a prolonged period of time is necessary. The major limitation inherent to the RIA of GLP-1 is the large plasma volume that is necessary to accurately measure GLP-1 levels in rats. The results of the present study demonstrate that changes in the plasma levels of GLP-1 in rats in vivo are highly correlated with those of gGLI, a finding that is not surprising, since glicentin/oxyntomodulin and GLP-1 are cleaved from proglucagon in a one to one relationship (67). Furthermore, plasma levels of GLP-2, which is also cleaved from proglucagon in a one to one relationship with glicentin/oxyntomodulin, has recently been shown to be highly correlated with gGLI levels in the rat (322). Similar findings have also been made for gGLI, GLP-1 and GLP-2 using in vitro models of the intestinal L cell [(34,403) and P.L.B., unpublished data]. The results obtained for any one of these peptides may therefore be extrapolated to include all of the other intestinal PGDPs, at least in the rat.

To investigate the hypothesis that a neural component of the proximal-distal loop operates in conjunction with the previously described endocrine-based loop (162,305), the vagus nerve was examined for global effects on basal GLP-1 secretion. This nerve represents the major parasympathetic innervation of the proximal portion of the gastrointestinal tract and is known to influence the secretion of other gut-derived hormones, including GRP (407) and secretin (408). Left cervical vagotomy significantly depressed the basal secretion of
gGLI, to approximately 56% of basal levels, in control rats. This finding demonstrates a tonic stimulatory role for the left cervical vagus nerve in regulating secretion of GLP-1 from the L cell.

Nutrients such as fat can exert effects on GLP-1 secretion directly by stimulating the L cells at their luminal surface (311) or can have indirect effects on the L cell by exerting their influence through an intermediate messenger such as GIP. This duodenal hormone has previously been shown to stimulate secretion of GLP-1 in response to fat ingestion (162). The direct and indirect effects of fat can be separated in vivo by ensuring that the progress of nutrients is impeded before they can reach the site of the majority of the GLP-1-containing L cells within the ileum. This was accomplished in our model by applying a ligature loosely around the gastrointestinal wall at a position approximately 10 cm downstream from the duodenal site of infusion. Saline infusion into the proximal isolated segment did not induce secretion of GLP-1 from the L cells indicating that the effects of fat on GLP-1 secretion are specific to this nutrient and are not due to distension of the duodenal wall or the mere presence of a fluid in the lumen of the duodenum. Consistent with the results of previous studies (162,305), infusion of fat into the duodenum induced a rapid and prolonged rise in gGLI secretion, despite the fact that this fat did not reach the ileum at any time during the experimental time course. Furthermore, the secretion of GLP-1 induced by duodenal fat cannot be explained by the presence of a few hypersensitive L cells in this region, since the response to fat is completely abolished when all sections of the intestine distal to the duodenum are removed (326). However, the rapid response of the L cell to duodenal fat (approximately 5 min), as well as the finding that this response is dependent on the neuropeptide GRP (326), led to the current hypothesis that, in addition to GIP, a component
of the nervous system may also be involved in mediating the proximal nutrient signal to the distal L cell (Fig. 5-7).

To explore the possibility that fibers within the submucosal or myenteric nervous plexus may play a role in the rapid L cell response to fat ingestion, possible contributions of the enteric nervous system were abolished by transection of the gastrointestinal tract prior to infusion of corn oil. This procedure did not prevent gGLI secretion as compared to the control group, thereby indicating that the enteric nervous system is not responsible for relaying information about nutrient ingestion to the ileal L cells. However, an element contained within the walls of the gastrointestinal tract may be necessary in maintaining the appropriate timing of the response, since transection of the gut wall caused a marked delay in the peak of gGLI secretion. One possible explanation for this finding is that local afferents of vagal fibers may have been transected in this procedure. Local afferents are extensions of vagal afferents that divide from the main afferent nerve and supply a target organ directly without synapsing with higher centers (409). These have been shown to contain CGRP, which is a potent stimulator of gGLI secretion in vitro (311). In the present scenario, such afferents could possibly branch from vagal afferents that innervate the duodenal region and travel to the ileum within the walls of gastrointestinal tract to exert effects on gGLI secretion in a reflex manner. As the main vagal nerves are not affected by the gut wall transection, this might explain why the gGLI response was only delayed rather than being totally abolished in these studies.
Fat in the lumen of the duodenum stimulates the release of GIP from the K cells which, in turn, activates the L cell indirectly through vagal afferent pathways. The proximal signal is then mediated by vagal efferent pathways present in the celiac branch of the subdiaphragmatic vagus nerve which are thought to synapse with GRP-ergic neurons present in the myenteric plexus. At supraphysiologic concentrations, GIP may also act directly through receptors on the L cell (dotted line). Mechanisms which stimulate early secretion may also be mediated through the enteric nervous system in the walls of the gastrointestinal tract (dashed line).
Consistent with a major role for the vagus nerve in mediating the proximal-distal loop, bilateral subdiaphragmatic vagotomy performed in addition to gut transection completely abolished the fat-induced rise in gGLI secretion. Two scenarios or a combination of the two may provide the reason that bilateral subdiaphragmatic vagotomy abolished the fat-induced rise in gGLI secretion. The first deals with the possibility that transection of the subdiaphragmatic vagus nerve has rendered higher brain centers insensitive to sensory messages from the duodenum. The vagus nerve is known to contain many afferent fibers arising from the proximal gastrointestinal tract, and has been previously shown to mediate both nutrient and hormonal signals from the gut in response to nutrient administration (408,410,411). In fact, the vagus nerve has been shown to express receptors that are able to differentiate between distinct classes of ingested lipid (412). This is an important finding considering that both fat and GLP-1 are potent inhibitors of gastric motility (208,217,230,413). Thus, activation of vagal afferents by ingested lipid may induce secretion of GLP-1, thereby augmenting the enterogastrone effects of fat. Another possibility that may explain the reduced secretion of gGLI in response to bilateral subdiaphragmatic vagotomy is that the efferent mechanisms that act to stimulate GLP-1 secretion were disrupted by the vagotomy. It has been previously suggested that the parasympathetic nervous system is involved in the modulation of L cell secretion (297), and indeed, muscarinic agonists stimulate the L cell in vitro (311). Thus, disruption of afferent and/or efferent vagal fibres in the rat results in a loss of the rapid L cell response to ingested nutrients. Consistent with this finding, it has recently been reported that administration of atropine abolishes the GLP-1 response to an oral glucose tolerance test in humans, although
the effects of cholinergic blockade to inhibit gastric emptying were not taken into account in this study (297).

In order to differentiate between the afferent and efferent signals mediating GLP-1 secretion, the celiac branch of the vagus nerve was directly stimulated, resulting in significant stimulation of gGLI secretion at the 10 minute time point. The delay observed in secretion of gGLI induced by electrical stimulation was very similar to that observed in the experiments involving fat-induced secretion in gut-transected animals. These findings lend further support to the notion of a role for the enteric nervous system in mediating a component of the proximal-distal loop, as this pathway was not affected by the electrical stimulation. The delay may also occur due to involvement of a peptidergic mediator in the efferent signaling pathway, as the effects of these neuromodulators are known to be preceded by long latent periods (414). Such a possibility is also supported by recent experiments showing that infusion of an antagonist to the GRP receptor completely abolishes proximal fat-induced gGLI secretion, despite the fact that GIP levels remained elevated (326). A role for GRP in the signaling process at a level downstream of GIP was therefore indicated. GRP is localized extensively in the myenteric plexus (415) and potently stimulates the secretion of GLP-1 (311,333,404). Therefore, GRP is a likely candidate neuropeptide that may be released upon electrical stimulation of the celiac branch of the vagus nerve. It should be noted that the delayed secretion of gGLI in response to electrical stimulation is not dependent upon release of GIP from the duodenum, since vagal stimulation has previously been shown to have no effect on the GIP-containing K cell (416). The mechanism underlying the rebound secretion of gGLI during the recovery period remains to be elucidated. However, GLP-1 secretion is known to be pulsatile with a frequency of approximately one pulse every
10-15 min. This pattern of secretion has been hypothesized to be regulated by the parasympathetic nervous system (297). Thus, it is possible that the initial vagal stimulation stimulating release of gGLI in the present study coordinated the dispersed L cells in the distal small intestine, leading to an integrated rebound pulse of gGLI secretion during the recovery period.

The finding that a GRP receptor antagonist completely inhibits gGLI secretion in the presence of elevated GIP levels suggested that the neural and humoral arms of the proximal-distal loop may be integrated in the regulation of the L cell. This hypothesis was examined by experiments in which GIP was infused at different doses into animals that possessed intact or disrupted vagal innervation at the level of the hepatic afferent. This branch of the subdiaphragmatic vagus nerve innervates the proximal segments of the gastrointestinal tract, from which GIP is secreted upon ingestion of fat, and may serve to sense information regarding fat ingestion in the regulation of fat-induced GLP-1 secretion. When infused at supraphysiological doses, GIP stimulated gGLI secretion significantly in both control and vagotomized animals; however, a vagal dependence of GIP became evident when GIP was infused at physiological doses. This finding indicates that, in the normal rat, the hepatic branch of the subdiaphragmatic vagus nerve mediates the stimulatory effect of GIP on the L cell. Consistent with this finding, both CCK (411) and secretin (408) have been reported to act on the exocrine pancreas through vagal afferents when administered at physiological doses, exerting direct effects only when infused at supraphysiological concentrations. Vagal sensory endings in the duodenum do not make contact with epithelial cells but terminate in close association with the lamina propria (417). Thus, GIP-containing cells may function as
‘taste cells’, by sampling the duodenal contents and in turn, activating vagal afferents to stimulate GLP-1 secretion (418,419).

In conclusion, the secretion of GLP-1 and gGLI from the rat ileal L cell is regulated by a complex proximal-distal loop that involves both endocrine and neural factors (Fig. 5-7). Fat is sensed in the duodenum by luminal K cells which secrete GIP in response to fat and glucose. GIP exerts its effects on the ileal L cell in two ways, depending on the concentration achieved. Physiologic levels of GIP act through vagal afferent pathways contained in the hepatic branch of the vagus nerve to stimulate the L cell indirectly. This stimulation is carried to the L cell by efferent pathways located in the celiac branch of the vagus nerve and is thought to involve GRP. GIP can also stimulate the L cell directly at higher levels. Finally, a component of the enteric nervous system also appears to be responsible for the early stimulation of GLP-1 secretion within this loop. The secretion of GLP-1 from the distal L cell is, therefore, intimately connected with the presence of nutrients in the proximal duodenum through an interaction of neural and endocrine pathways. As knowledge is gained concerning the functioning of the neuroendocrine loop, examination of the function of this axis can be made in disease states, such as type 2 diabetes mellitus.
6 DISCUSSION

6.1 Summary of Results

The examination of the effects of fat on GLP-1 secretion in the above work has demonstrated that fat-induced GLP-1 secretion is the result of both direct and indirect effects of fat on the L cell (Fig. 6-1). Long-chain MUFAs specifically stimulate secretion of GLP-1 when exposed directly to the L cell. Shorter-chain fatty acids and saturated fatty acids are incapable of inducing GLP-1 secretion. An investigation into the mechanisms governing the direct effects of fat has led to the implication of both the atypical isoform of PKC, PKC-ζ, as well as I-FABP. The presence of these two molecules within the L cell has been confirmed by a series of immunohistochemical experiments. Furthermore, the benefits of MUFAs on glycemic control, which have been previously observed in patients with type 2 diabetes, have been positively correlated to increases in the secretion of the antidiabetic hormone, GLP-1. The beneficial effect of MUFAs, such as olive oil, on glycemic tolerance is specific to increased GLP-1 secretion, as the infusion of the GLP-1 receptor antagonist, exendin\(^{9-39}\), completely abolished the beneficial effects of the olive oil diet. With regard to the indirect effects of fat on the secretion of GLP-1, it has now been determined that this mechanism operates through the vagus nerve as demonstrated by the finding that subdiaphragmatic vagotony completely abolished the fat-induced rise in GLP-1 secretion. Electrical stimulation of the celiac branch of the vagus nerve was also stimulatory to GLP-1 secretion, thus supporting a role for the vagus nerve in the regulation of GLP-1 secretion. Additionally, an interaction with the endocrine peptide, GIP, occurs in the indirect pathway governing GLP-1 secretion. Therefore, these studies provide the groundwork for future studies aimed
Figure 6-1  Overview of the Mechanisms Regulating Fat-Induced GLP-1 Secretion
at increasing the secretion of GLP-1 through manipulation of these regulatory mechanisms governing the secretion of GLP-1.

Many issues remain to be resolved in the field of GLP-1 secretion. The exact intracellular signaling cascade involved in fat-induced GLP-1 secretion remains elusive. Furthermore, knowledge of the regulation of GLP-1 secretion in certain disease conditions and populations susceptible to diabetes continues to remain obscure. The indirect mechanism governing the secretion of GLP-1 has been relatively well studied in the rodent, however, knowledge concerning this mechanism in humans is limited especially since GIP, the proximal mediator of the indirect loop governing GLP-1 secretion has been demonstrated to be ineffective in humans. In short, the complex nature of nutrient homeostasis and the role that GLP-1 plays requires further investigation in order to achieve the full therapeutic potential of this promising antidiabetic hormone.

6.2 Intracellular Mediators of Fat-Induced L Cell Stimulation

Fatty acids are increasingly being considered as important signaling molecules and the potential interactions of dietary fatty acids with plasma and intracellular membranes introduces additional complexities into the regulatory mechanisms governing GLP-1 secretion. In addition to the potential involvement of a FABP and PKC-ζ, other intracellular mechanisms may also be participating in fat-induced secretion of GLP-1 from the L cell. Interestingly, dietary fatty acids have recently been demonstrated to modify the process of gene transcription. Several transcriptional factors, including the PPAR family, are known to be specifically regulated by dietary fatty acids. The PPAR family of nuclear receptors is related in structure to the steroid family of nuclear receptors (420). Three separate genes
encode three different subtypes of the PPAR family. PPARα, δ and γ are all expressed in the enterocytes of the colon. However, their affinity for fatty acids varies, and other molecules can also activate these nuclear receptors including fibrates and thiazolidinediones. All three isoforms have been demonstrated to bind polyunsaturated fatty acids. PPARα and γ are capable of binding the MUFA, oleic acid, whereas PPARδ is thought to preferentially bind fatty acids rather than fibrates or the thiazolidinediones (421). Interestingly, the fibrate class of compounds is beneficial in improving lipid metabolism (421) and the thiazolidinediones are also being used clinically to enhance the sensitivity of tissues to insulin and reduce the hepatic glucose output that are characteristic of impaired glucose tolerance and type 2 diabetes (422). PPARs are also involved in the induction of several enzymes involved in regulating lipid metabolism, including the FABPs. Interestingly, the PPARα knock out mouse is incapable of undergoing induction of the L-FABP (421). Thus, a role for the PPARs in fat-induced GLP-1 may be possible, as the FABPs have been implicated in this process (refer to Section 3.4). Whether the L cell expresses any isoforms of the PPAR family is unknown at the present time. A role for this nuclear receptor family in the synthesis of the PGDPs is unlikely as 24 hour fatty acid treatment of the FRIC cultures did not stimulate total cell content in these experiments (refer to Section 3.4). However, chronic exposure of the GLUTag L cell line led to enhanced sensitivity of the cells to GLP-1 secretagogues (refer to Section 4.4.5). PPAR stimulation of gene transcription within the L cell may be responsible for this phenomenon given the speculation that FABP may be involved in this and that FABP expression is regulated by the PPARs. Alternatively, other transcription factors that are responsive to fatty acids such as HNF-4α or the sterol response element binding protein 1C
may be implicated in this process. The nature and function of the intracellular molecules that interact with these fatty acids requires further investigation.

6.3 Role of I-FABP in the Regulation of GLP-1 Secretion

Interestingly, recent reports concerning the Pima Indians, a group of native Americans from Arizona with the highest reported incidence of Type 2 Diabetes (423,424), have indicated that a subpopulation possess a polymorphic version of I-FABP (425). This polymorphism occurs at codon 54 of the fabp2 gene, which results in an alanine to threonine substitution at a frequency of 0.29 (425). Furthermore, the fabp2 gene locus encoding for I-FABP has been positively linked with in vivo insulin action and fasting insulin levels in the Pimas (426), and also with 2 hour insulin responses after oral glucose challenge in a related population of Mexican-Americans (427). The threonine-encoding allele results in higher 2 hour oral glucose-induced insulin responses, increased rates of fat oxidation, and higher fasting insulin levels (425). However, a clear physiological mechanism has not been postulated to explain the link between the insulin responses after oral glucose challenge and the I-FABP gene locus (426). In vitro studies on the function of this polymorphic I-FABP have also been completed and reveal that the major conformational adjustment of the I-FABP induced by fatty acid binding occurs at the tight turn containing residues 54 and 55 (425). The polymorphic I-FABP (ala54thr) possesses a higher affinity for long chain fatty acids than the normal version of the fat carrier protein (425,428). Therefore, it can be speculated that this polymorphism in I-FABP may lead to altered levels of post-prandial GLP-1, since I-FABP has been implicated in fat-induced GLP-1 secretion (refer to Section 3.4). This hypothesis may therefore provide a potential physiological mechanism explaining the observed insulin responses within these populations.
Expression of the *fabp2* gene is induced by fatty acids acting through a PPAR and a PPAR response element located in the *fabp2* gene promoter (429). These facts taken together with the postulation that I-FABP is implicated in the direct stimulation of GLP-1 secretion lead to the interesting hypothesis that the beneficial effects of high MUFA diets on GLP-1 secretion may be mediated by a FABP. Bezafibrates are known to induce the expression of FABP (430, 431) and may be useful in the investigation of the potential relationship between FABP and the L cell.

The potential involvement of I-FABP in fat-induced GLP-1 secretion is interesting to contemplate. However, the other members of the FABP family also need to be considered as potential signaling proteins involved in this process. Intestinal epithelial cells contain L-FABP in addition to I-FABP. L-FABP is structurally different from I-FABP in that it can accommodate other molecules such as bilirubin and prostaglandins in addition to fatty acids (370). The L-FABP is reported to bind MUFA with a higher affinity when compared to SFA whereas I-FABP has similar affinity for MUFA and SFA (432). It has been postulated that the two FABPs present within the enterocytes carry out separate functions and potentially direct fatty acids to different compartments within the cell. The L-FABP is capable of binding more than one molecule of fatty acid and is involved in membrane synthesis whereas the I-FABP binds a single molecule of fatty acid and is involved in esterification of fatty acids into triacylglycerols (432). The Ileal Binding Protein is also present in the cells of the ileum and is involved mainly in bile salt interactions (370). Whether the L cell expresses either the L-FABP or the Ileal Binding Protein is undetermined at the present time.
6.4 Indirect Regulation of GLP-1 Secretion

Studies in the rat have previously shown the involvement of the upper gastrointestinal hormone, GIP, in the indirect stimulation of GLP-1 secretion. The rapid time course of GLP-1 secretion after the ingestion of a meal in humans is similar to the observed time to peak secretion in the rat, suggesting that a proximal mediator exists that indirectly stimulates the secretion of GLP-1 in the human. However, human studies have failed to demonstrate a stimulatory link between GIP and GLP-1 secretion (180). It is also possible that the infusions of GIP in the human studies were insufficient to mimic concentrations of GIP at sites adjacent to the vagal afferent nerves, even though the plasma levels reached were significantly elevated. Thus, the possibility still exists that GIP infusions, if given at the appropriate dose to mimic concentrations achieved at nerve endings, are capable of indirectly stimulating the secretion of GLP-1 in humans. Alternatively, this may be mediated by GRP in humans as unpublished studies have been cited to indicate that infusion of a GRP antagonist prevents nutrient-induced GLP-1 secretion (404). This is consistent with similar experiments conducted in the rat, which demonstrated that the GRP antagonist also prevented fat-induced GLP-1 secretion (326). The proximal limb may also involve no peptide intermediate at all, but rather, may be organized in such a way as vagal afferents sense luminal contents directly and deliver the stimulatory information to the distal L cells. Vagal afferents are capable of sensing different fat types in the cat (412). Interestingly, infusions of atropine in the human may (297) or may not (404) decrease the secretion of GLP-1, indicating that the elements of the indirect mechanism of nutrient-induced GLP-1 secretion in the human are not clearly delineated. This finding may represent a consequence of the evolution of control over nutrient homeostasis that has occurred in higher primates and
humans. In addition to GIP, humans likely possess other signals of nutrient ingestion that are conveyed to the L cell resulting in rapid secretion of GLP-1. The balance of regulation over L cell secretion may be shifted away from endocrine factors as in the rat, toward neural factors in the human, consistent with the development of higher brain functions and enhanced coordination of the nervous system characteristic of higher life forms.

6.5 The Proximal-Distal Loop in Disease

GLP-1 is an important factor in the control of nutrient intake and metabolism. It will therefore be interesting to investigate the control of GLP-1 secretion in pathological models of abnormal glucose homeostasis. One such model is the Zucker (fa/fa) rat, which is characterized by an elevation in the activity of the parasympathetic nervous system (433,434). In the young Zucker (fa/fa) rat, hyperinsulinemia ultimately leads to insulin resistance and obesity (435-437). The principal defect in the Zucker fatty rat is the mutation of the leptin (ob) receptor (438,439); in this way, Zucker fatty rats are analogous to the db/db mouse (440,441). Indeed, the lack of a functional leptin receptor causes the upregulation of a critical hypothalamic neuropeptide, NPY (442,443). NPY potently stimulates food intake and therefore contributes to hyperphagia and resultant obesity (444). This also results in the upregulation of the parasympathetic nervous system for unknown reasons and leads to the hypersecretion of insulin (433,435,442) and glucagon (442,445). Interestingly, the effect of NPY on insulin secretion is also dependent on the vagus nerve (433,434,446,447). As a role for the parasympathetic nervous system and more specifically the vagus nerve, has been demonstrated in the proximal-distal loop model of GLP-1 secretion (refer to Section 5.4), it can be hypothesized that the Zucker (fa/fa) rat, due to its overactive parasympathetic tone, may also present with elevated GLP-1 responses. GLP-1 measurements in Zucker rats have
been performed in the past (448). However, the study in question failed to demonstrate a glucose-stimulated GLP-1 response in lean Zucker rats but reported that glucose-induced GLP-1 responses were elevated in obese littermates (448).

Furthermore, as insulin secretion patterns from patients with type 2 diabetes may be either elevated, normal or decreased depending on the duration of the disorder (259,260), fat-induced GLP-1 responses from Zucker rats of young and old age may also exhibit a similar pattern over the duration of this disorder. Since the L cell is thought to be innervated directly or indirectly by the vagus nerve (refer to Section 5.4), and acetylcholine has been shown to stimulate GLP-1 secretion (311,333,334,343,404), it is possible that the vagal hyperactivity observed in Zucker (fa/fa) rats may also cause increased release of GLP-1 at early stages in the lifespan of the Zucker rat. The possibility also exists that the L cell becomes downregulated over an extended period by the hyperinsulinemic conditions prevalent in the Zucker fatty rat. An effect of insulin on the L cell is suggested by observations of elevated tissue levels of GLP-1 in conditions of insulin-deficiency (322,449). Furthermore, a regulatory effect of insulin on the L cell has been described as insulin treatment of streptozotocin diabetic rats normalized the levels of the PGDPs that were elevated in this diabetic setting (322). Alternatively, acquired L cell resistance may also result in decreased GLP-1 secretion. Indeed, the elevated parasympathetic tone observed in the Zucker rat may result in chronic stimulation of the L cell, which may lead to L cell resistance. Neurotransmitters such as acetylcholine (311,333,334,343,404) and GRP (311,326,333,334) have been shown to stimulate GLP-1 secretion and thus are potential candidates involved in L cell downregulation. In type 2 diabetes there is a reduction of the incretin effect and GIP
receptors on the β cell are known to undergo desensitization; perhaps the same occurs on the L cell.

6.6 GiP and GLP-1 as Incretins

GiP and GLP-1 share similar functions particularly with respect to their roles as incretin molecules; once they are released from the gastrointestinal tract upon nutrient ingestion, they potently stimulate the glucose-dependent secretion of insulin. These gastrointestinal hormones are both released within minutes of nutrient ingestion and act in similar ways. Specifically, they share the ability to inhibit gastric emptying and increase the secretion of insulin. The presence of two molecules that possess similar biological functions may seem to indicate a degree of redundancy within this system. However, further examination of the secretion patterns and functions of these molecules indicates that there are subtle differences between the two molecules that may point to a higher degree of complexity in the entero-insular axis. For example, secretion of both GiP and GLP-1 is regulated by nutrients such as carbohydrate and fat, however, GiP secretion is not regulated by neural factors to a large extent (450). Conversely, GLP-1 secretion is controlled to a great degree by complex neural patterns (refer to Section 5.4). GiP secretion is predominately stimulated by intraluminal nutrients and levels remain elevated in correlation with the presence of nutrients in the lumen. GiP levels are stimulated to the same extent regardless of the load of glucose administered to the lumen (230). In this way, GiP levels act as an on-signal stimulated by the presence of luminal nutrients. On the other hand, GLP-1 secretion is controlled by a complex system, of which GiP secretion is of primary importance in the rat. Furthermore, the set point for glucose-induced secretion differs between the two incretin hormones (230). GiP secretion is stimulated at low levels of luminal glucose whereas GLP-1 secretion requires
glucose concentrations to exceed a threshold amount. Furthermore, different glucose loads result in different amounts of GLP-1 secretion in contrast to the relatively stable pattern of GIP secretion induced by glucose stimulation (230). Therefore, GIP secretion is, in effect, a pattern generator for GLP-1 secretion from the ileum. Subtle differences also exist in the functions of these two peptides. GIP and GLP-1 exert similar effects on the β cell but have opposing functions at the level of the glucagon-secreting α cell; GIP is stimulatory to glucagon secretion whereas GLP-1, indirectly, exerts an inhibitory influence on the α cell. Therefore, although the two incretins share some similar characteristics in specific functions of the gastroenteropancreatic system, when the overall processes of digestion, absorption and nutrient metabolism are taken into account, GIP and GLP-1 can be seen as separate but complementary regulatory elements in the coordination of the complex biological systems governing nutrient homeostasis.

GIP, by being stimulated predominately by the presence of nutrients within the lumen of the duodenum, can be considered to be a signal of nutrient ingestion to the L cell. In contrast, GLP-1 can be viewed as being a signal of nutrient absorption in the light of its functions as an enterogastrone. Thus, in the rat, nutrient ingestion is tied to the signal of nutrient absorption in an intricate balance operating between GIP and GLP-1. These two gastrointestinal peptides deliver this information to the islets of Langerhans, which operate as the integration center governing nutrient metabolism through the modulation of insulin and glucagon secretion. Therefore, GLP-1 can be considered to be a peptide enrolled in a fine-tuning function capable of modulating the secretions of the endocrine pancreas.
6.7 Direct and Indirect Stimulation of GLP-1 Secretion by Fatty Acids

Fatty acids, as has been previously discussed, can regulate the secretion of GLP-1 both directly, at the level of the L cell, and indirectly, through the proximal-distal loop involving the intermediate peptide, GIP and the vagus nerve. Therefore, fatty acids, unlike carbohydrates, are poised at a critical position governing L cell secretion. This phenomenon gives GLP-1 the potential to operate as an index of nutrient absorption. When the concentration of fatty acids rises in the ileum, as in certain disease conditions affecting the absorption of fatty acids [e.g. the short bowel syndrome (451) and the dumping syndrome (170)], the delivery of fatty acid to the GLP-1-secreting cells is increased resulting in increased release of GLP-1. This triggers an inhibitory signal on gastric motility, resulting in a negative feedback loop, which compensates for the inadequate absorption of ingested nutrients. Thus, the direct stimulation of GLP-1 secretion by fatty acids is capable of signaling the rate of absorption to the system, using the L cell as a sensor and the ability of GLP-1 to inhibit gastric motility as an effector in this feedback loop. The distal location of the L cell, when viewed in this manner, can be seen as a sensor placed at the end of the absorptive surface. In this manner, the distribution of the L cell in an increasing gradient from the ileum to rectum would operate in the way of increasing the intensity of the signal, in conditions in which the absorption of fat is vastly compromised.

6.8 Reasons to Increase GLP-1 Secretion in Diabetes

GLP-1 secretion has been reported to be increased, decreased or normal in some patients with type 2 diabetes. It would therefore seem to be counterproductive to follow efforts designed to further increase the secretion of GLP-1 in some of these scenarios.
Furthermore, the demonstration that the GLP-1 receptor undergoes homologous desensitization would seemingly argue against the need to increase the secretion of GLP-1. However, it has been demonstrated that supraphysiological doses of GLP-1 are still capable of enhancing insulin secretion and benefiting glycemic control in patients with type 2 diabetes, despite the fact that endogenous GLP-1 levels are elevated (162). Therefore, there is clear evidence to suggest that there is benefit to increasing the secretion of GLP-1 in type 2 diabetes. Furthermore, the peripheral effects of GLP-1 as well as the inhibitory effects on glucagon secretion make GLP-1 an attractive adjuvant therapy in patients with type 1 diabetes. Increasing the endogenous secretion of GLP-1 would circumvent the compliance issues and bioavailability concerns common with exogenous administration of peptide hormones. Furthermore, the other effects of GLP-1 such as the gastric emptying and peripheral insulin sensitivity and satiating effects of this hormone have not been well studied with regard to intensive administration of supraphysiological levels of GLP-1. Therefore, it is unclear whether these processes are affected by receptor desensitization.

6.9 Future Studies

The studies described in this body of work lay the foundations for future strategies aimed at increasing the endogenous levels of GLP-1. However, much more investigation is required in order to fully understand the complex physiology of GLP-1 secretion. The intracellular mechanisms that control secretion from the L cell need to be precisely understood and the manner in which diseases such as type 2 diabetes affect GLP-1 secretion need to be critically reviewed as well. Furthermore, the mechanisms that govern the indirect secretion of GLP-1 in humans should be carefully investigated, as there appear to be specific differences between the rodent and human.
Specifically, the precise role of both I-FABP and the atypical PKC-ζ in fat-induced GLP-1 secretion should be determined through in vitro analyses. Overexpression and antisense deletion studies in the GLUTag cell line may be beneficial in determining the role of I-FABP in this process. Furthermore, experiments that examine the translocation of PKC-ζ will aid in confirming the role of this enzyme in fat-induced GLP-1 secretion. In addition, as fatty acids are known stimulators of the PPAR family of transcriptional factors, and as this family is an important regulator of cellular levels of FABP, experiments that are designed to specifically investigate the role of the PPARs in the secretion of GLP-1 will be potentially beneficial to the understanding of the mechanisms governing L cell stimulation.

Knowledge of how the L cell responds to disease processes is also relatively scarce. Initiatives should be taken in the future to further understand the interaction between the disease mechanisms manifested in conditions such as type 2 diabetes and obesity and the mechanisms governing secretion from the L cell. The varied reports of elevated or decreased GLP-1 secretion in type 2 diabetes need to be confirmed with experiments examining the GLP-1 secretion profile over the course of the disease. In addition, the role of the reported over-activity of the parasympathetic nervous system in the Zucker model of insulin resistance should be examined in relation to GLP-1 secretion, given the finding that the vagus nerve is a key mediator in fat-induced L cell stimulation. In addition, the role of the polymorphic form of I-FABP should be further analyzed through in vitro and in vivo experiments, given the finding that the L cell expresses this molecule and the recent association of the fabp2 gene with insulin resistance by linkage analysis in Pima Indians. The GLP-1 secretory patterns should be examined in cell lines and humans that express the polymorphic version of this molecule. Furthermore, the dietary studies with MUFAAs should be extended to diabetic
animals and humans in order to establish whether the benefits to glycemic tolerance mediated by increases in the secretion of GLP-1 are reproduced in type 2 diabetes.

Finally, investigations into the indirect mechanisms governing the secretion of GLP-1 from humans should be performed since previous experiments have demonstrated that GIP, the indirect mediator of GLP-1 secretion in rodents, does not mediate this function in humans. Efforts should be placed on examination of the neural mechanisms involved in sensing fat ingestion from the proximal regions of the gastrointestinal tract. For example, experiments examining GLP-1 secretion in patients who have undergone selective vagotomies will aid in the mapping of the complex regulation of GLP-1 secretion in humans. Furthermore, administration of molecules specifically designed to increase the neural stimulation to the L cell may also be implemented to benefit the secretion patterns of GLP-1 in patients with diabetes.

In conclusion, GLP-1 is a multifaceted regulatory peptide with the potential to impact several processes involved in nutrient homeostasis. By enhancing the endogenous secretion of this peptide, therapeutic advantages to the treatment of diabetes may be realized.
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