THE EFFECT OF INSULIN AND INSULIN RESISTANCE ON GLUCAGON-LIKE PEPTIDE-1 SECRETION FROM THE INTESTINAL L CELL

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

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The effect of insulin and insulin resistance on glucagon-like peptide-1 secretion from the intestinal L cell.

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Doctorate of Philosophy

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Abstract

Glucagon-like peptide-1 (GLP-1) is secreted from the enteroendocrine L cell following nutrient ingestion. Although GLP-1 regulates several aspects of nutrient homeostasis, one important function is to enhance glucose-dependent insulin secretion. In type 2 diabetes, post-prandial GLP-1 secretion is impaired. Insulin resistance, which is required for the pathogenesis of type 2 diabetes, is also associated with impaired GLP-1 secretion. I, therefore, hypothesized that insulin modulates GLP-1 secretion from the intestinal L cell and, furthermore, insulin resistance directly impairs the function of the endocrine L cell. In well-characterized L cell models, I established that insulin stimulates GLP-1 secretion through the MEK1/2-ERK1/2 pathway, and induction of insulin resistance in vitro attenuated insulin- and heterologous secretagogue-induced GLP-1 release. Furthermore, glucose-stimulated GLP-1 secretion was decreased in hyperinsulinemic-insulin resistant MKR mice, demonstrating that insulin resistance is associated with impaired L cell function.

I next examined the role of the actin cytoskeleton in insulin-stimulated GLP-1 secretion. Insulin treatment transiently induced actin depolymerization, and depolymerization of the actin cytoskeleton potentiated insulin-stimulated GLP-1 release from the L cell, demonstrating that the cytoskeleton functions as a permissive barrier. Central to
insulin’s effects on actin dynamics is the Rho GTPase, Cdc42, as siRNA-mediated knockdown and over-expression of a dominant-negative mutant, prevented insulin-stimulated actin remodeling and GLP-1 release. Insulin also promoted activation of PAK1, the downstream kinase of Cdc42, and over-expression of a kinase-dead PAK1 mutant attenuated insulin-stimulated GLP-1 release. In cells that expressed dominant-negative Cdc42 or kinase-dead PAK1, activation of ERK1/2 following insulin treatment was attenuated, demonstrating that the Cdc42-PAK1 axis regulates the activity of the canonical ERK1/2 pathway.

In summary, this thesis demonstrates, for the first time, that insulin is a GLP-1 secretagogue, and this effect of insulin is mediated through the canonical ERK1/2 pathway and the Cdc42-PAK1 axis. Insulin resistance in the L cell impairs the responsiveness of the L cell to heterologous secretagogues. Collectively, these findings suggest that an alternative approach to treat type 2 diabetes and/or insulin resistance may be to directly improve the function of the L cell, thereby enhancing endogenous GLP-1 release.
**Acknowledgements**

It has been quite an adventure over the years, ultimately culminating in the completion of my PhD thesis. This adventure in life and learning would not have been possible without the help and support from numerous people, who have affected me in so many ways. First and foremost, I would like to thank my parents, William and Helen, and my brother, Darren, for encouraging and supporting my decisions in life, and without their sacrifices, it is obvious that I would not be where I am today. I am eternally indebted to my wife, Melissa, who has stood by my side since our undergraduate years at McMaster University. She has always given me unconditional love and support to deal with the struggles of life, to keep me grounded, and to help me balance life as a student and as a father to our daughter, Clara, and our furry daughter, Chloe Sausage. Although Clara was born on Boxing Day, 2008, she has taught me so much about myself and enabled me to realize that there is more to life than completing this degree. I never understood how life would change when she was born, but she has made me grow so much as a person, and more importantly, as a father. Furthermore, I cannot forget to express my gratitude to my in-laws, Joe and Cathie, as well as my siblings-in law, Adam, Charisse, and David.

Academically, I owe my chosen career path to Drs. Warren Foster and Alison Holloway at McMaster University for giving me the opportunity to work with them for my undergraduate thesis. Without Alison’s suggestions for potential graduate supervisors, who knows where I would have ended up? Ultimately, none of this would be possible without the guidance of my supervisor, Dr Patricia Brubaker, who has helped to foster my growth through out graduate school. To this day, I am still surprised that she took in a Biology/Pharmacology major from McMaster University and trusted that I would not run for the hills when confronted with the possibility of actually having to learn some basic
physiology! All joking aside, she has always encouraged me to pursue my own scientific interests and made me realize my own personal potential.

Life in the lab has always been fun and enjoyable due to interesting lab members, past (Lixin Li, Phil Dubé, Sean McDonagh, and Tanvi Talsania) and present (Angelo Izzo, Jason Leen, Katie Rowland, Lina Lauffer, Roman Iakoubov, and Victor Wong), and without their helpful suggestions or acting as a sounding board, the past five years would have been torturous and to put it bluntly, boring. I also can not forget my project students, Nina Flora, Guan Jane Huang, and Molie Xu, for helping me with experiments, allowing me to practice teaching/mentoring, and putting up with my often erratic behavior and antics on those frustrating days that are common during graduate school. There are so many other people whom I have met over the years and at the University of Toronto that I would like to thank, but I think all of these acknowledgements could take up as much space as this thesis. I am sure that these people know who they are and will forgive me for not putting their names in writing. In fact, I bet that some of them are glad that I have spared them from immortal embarrassment by not listing their names in this thesis.

The past 10 years of school, spanning from McMaster University to the University of Toronto, have been a blast, and none of would be possible without people who have helped me along the way. I cannot imagine what the future holds for me, but I know that with the friends that I have made over the years, things will always work out. There are mornings and nights where I wake up or go to bed in shock, as it is sometimes hard to fathom that I have completed a PhD, but none of this could have been possible without the help and support of friends.
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<td>Acetylcholine</td>
</tr>
<tr>
<td>Adv:</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>Analysis of variance</td>
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<td>GDI:</td>
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<td>Glucose-dependent insulinotropic peptide</td>
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<td>Glucose-dependent insulinotropic peptide receptor</td>
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<td>Hank’s balanced salt solution</td>
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<td>Inositol-1,4,5-phosphate</td>
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<td>Messenger RNA</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>OEA</td>
<td>Oleoylethanolamide</td>
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<td>PAK1</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PCR</td>
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<td>Protein kinase C</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<td>PYY</td>
<td>Peptide YY (tyrosine/tyrosine)</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>Son of sevenless</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCF4</td>
<td>T-cell factor-4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
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</tbody>
</table>
WASP: Wiskott-Aldrich syndrome protein
WAVE: WASP family Verpoline-homologous

**Units:**

- g/kg: Gram/kilogram
- hr: Hour
- M: Molar
- in: Minute
- μM: Micromolar
- PFU/ml: Plaque forming units/milliliter
- pg/ml: Picogram/milliliter
- pM: Picomolar
- U/kg: Units/kilogram

**Amino Acids**

- Gly: Glycine
- His: Histidine
- Ile: Isoleucine
- Phe: Phenylalanine
- Ser: Serine
- Thr: Threonine
- Tyr: Tyrosine
CHAPTER 1: INTRODUCTION

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Author contribution: G. Lim produced all text and figures present in this chapter
1. Introduction

Glucagon-like peptide-1 (GLP-1) is secreted from the intestinal endocrine L cell following nutrient ingestion. GLP-1, along with glucose-dependent insulinotropic peptide (GIP), is an incretin hormone, which regulates nutrient handing primarily through enhancement of glucose-stimulated insulin secretion from the pancreatic beta cell. Aside from its insulinoactive actions, GLP-1 also decreases gastric emptying and intestinal motility and inhibits both glucagon secretion and food intake. Collectively, these biological actions highlight the importance of GLP-1 as an endogenous anti-diabetic hormone, and enhancing GLP-1 action through pharmacological means is now being used as a treatment for type 2 diabetes.

The incretin effect is diminished in type 2 diabetes, partially due to decreased biological activity of GIP on the beta cell and impaired GLP-1 secretion (1-3). Currently, it is not clear as to why GLP-1 release from the L cell is affected. Though not a pre-requisite condition to type 2 diabetes, obesity is characterized by increased adipose mass, elevating circulating fatty acids, a proinflammatory state, and insulin resistance, and in rodents and humans, obesity is also associated with decreased nutrient-induced GLP-1 secretion (4, 5). Furthermore, insulin resistance that is not dependent on the obese state correlates highly with impaired GLP-1 secretion from the L cell (6). These findings suggest that insulin has a direct effect on L cell function. While a recent study has demonstrated that insulin promotes GLP-1 synthesis, it is not known if insulin has a direct effect on GLP-1 secretion (7). The aim of this thesis was to elucidate the effects of insulin on GLP-1 secretion from the L cell and to ascertain if insulin resistance is a direct cause of impaired GLP-1 release. A better understanding of the defects associated with insulin resistance-associated GLP-1 release may lead to novel approaches to treat type 2 diabetes.
1.1 Glucagon-like peptide-1

1.1.1. Proglucagon and proglucagon-derived peptides

Proglucagon is a 160 amino acid prohormone that is expressed primarily in the pancreatic alpha cells and intestinal L cells, in addition to select neurons in the brain (8, 9). L cells are open-type endocrine cells that sense nutrients in the intestinal lumen and secrete peptide hormones accordingly. Like other endocrine cells, they contain dense-core secretory granules beneath the plasma membrane, although these are localized to the basolateral membrane and close to blood vessels (10, 11). L cells increase in density along the intestinal axis from the duodenum to the colon, but are predominantly localized to the ileum and colon (9, 11).

Proglucagon expression is dependent on the paired homeobox transcription factor, Pax6, as Pax6-deficient transgenic mice fail to develop proglucagon-expressing cells in the intestine and pancreatic islets (12, 13). Furthermore, mice that express dominant-negative Pax6 (SeyNeu) have decreased intestinal proglucagon mRNA levels (13). Several studies have further determined that, unlike the pancreatic alpha cell, Pax6, but not the transcription factor Cdx2/3, is solely required for proglucagon expression in the intestine (14, 15). Nutrient ingestion is also a potent regulator of proglucagon expression (16), and in rodent L cells, expression is enhanced by activation of the protein kinase A (PKA) pathway (17) and via β-catenin-mediated activation of the bipartite transcription factor, TCF4 (18), components of the canonical Wnt signaling pathway.

Tissue-specific expression of prohormone convertase isoforms directs the synthesis of specific proglucagon-derived peptides (PGDPs) in the L cell and alpha cell (Figure 1-1). Hence, cleavage of proglucagon by prohormone convertase 1/3 (PC1/3), which is expressed in the L cell, liberates the incretin
Figure 1- 1: Tissue-specific posttranslational processing of proglucagon liberates different proglucagon-derived peptides (PGDPs).

Prohormone convertase 2 in the alpha cell (solid arrows) releases glicentin-related pancreatic peptide (GRPP), glucagon, intervening peptide-1 (IP-1), and the major proglucagon fragment from proglucagon. In the L cell, cleavage of proglucagon by prohormone convertase 1/3 (dashed arrows) yields glicentin, oxyntomodulin, GLP-1, IP-2, and GLP-2. Early studies that examined PGDP secretion utilized antibodies that measured mid-sequence glucagon-like immunoreactivity (black circle), as opposed to C-terminal immunoreactive glucagon (black diamond). Since the gut-derived PGDPs are co-secreted in a stoichiometric ratio from the L cell, these studies that measured mid-sequence glucagon-like immunoreactivity also indirectly examined GLP-1 secretion.
hormone glucagon-like peptide-1 (GLP-1), in addition to GLP-2, glicentin and oxyntomodulin (19). In contrast, activation of prohormone convertase 2 (PC2) in the alpha cell generates glucagon, glicentin-related pancreatic peptide, and the major proglucagon fragment, which contains within its sequence both GLP-1 and GLP-2 (20). Among vertebrate species, including humans, rats, and mice, the peptide sequence of GLP-1 is completely conserved (21), and structure activity studies have identified His1, Gly4, Phe6, Phe22, and Ile23 as critical amino acids that confer specificity of the receptor for GLP-1 (22). Furthermore, GLP-1 circulates as two bioactive forms, GLP-1\(^{7-36NH_2}\) and GLP-1\(^{7-37}\), of which the former is predominant, although both forms have equal biological activity (23).

**1.1.2. Metabolism and clearance of GLP-1**

GLP-1 has short half-life of approximately 2 minutes due to its rapid degradation by the ubiquitous enzyme, dipeptidyl peptidase-IV (DPP-IV)/ CD26, which generates the metabolites, GLP-1\(^{9-36NH_2}\) or GLP-1\(^{9-37}\) (21). DPP-IV is a serine protease that cleaves peptides with alanine or proline at position 2 from the N-terminus, including GLP-1 and the related hormone GIP. It is widely expressed in multiple tissues and circulates systemically as a soluble protein (24, 25), although DPP-IV activity is highest in the kidney (25). Moreover, DPP-IV is also expressed on capillaries that line the gastrointestinal mucosa, where it may directly metabolize GLP-1 upon secretion from the L cell (26). The physiological role of this enzyme in metabolizing GLP-1 has been demonstrated in DPP-IV deficient Fisher-344 rats, as plasma from these rats display markedly elevated levels of intact, bioactive GLP-1 and GIP (27). Moreover, mice that lack DPP-IV demonstrate marked improvements in glucose and insulin tolerance when compared to wild-type mice due to significantly higher levels of the incretin hormones (28). *In vitro* studies have also demonstrated that GLP-1 is a substrate for neutral endopeptidase 24.11, which is a membrane-bound zinc metalloproteinase (29).
the pig, it appears that this enzyme may account for up to 50% of GLP-1 metabolism (30), but the role of this enzyme in human GLP-1 physiology remains undefined. Clearance of GLP-1 and its metabolites occurs at the kidneys, and the half-life of GLP-1\textsuperscript{9-36NH\textsubscript{2}} and GLP-1\textsuperscript{9-37} is approximately 5 minutes (31). The role of the kidney in clearance of GLP-1 metabolites has also been demonstrated in patients with renal insufficiency, in whom plasma levels of GLP-1\textsuperscript{9-36NH\textsubscript{2}} are higher than in control subjects (32).

1.1.3. GLP-1 receptor: localization, structure, signaling

The GLP-1 receptor (GLP-1R) was cloned from a rat pancreatic islet cDNA library and belongs to the class B family of G-protein-coupled receptors (GPCRs), which includes receptors for glucagon, GLP-2, GIP, and secretin (33). Expression of the GLP-1R has been detected in pancreatic islets, lung, heart, intestine, and distinct neurons in the hypothalamus (33, 34). GLP-1R mRNA expression in the mouse hypothalamus is similar to that of proglucagon expression (34), which suggests the existence of a local neuronal GLP-1 circuit. Furthermore, the GLP-1R is expressed on sensory afferent nerves that innervate the portal vein, and rodent studies utilizing vagotomies or capsaicin treatment, which destroys unmyelinated sensory afferent fibers, have demonstrated that the pancreatic and extra-pancreatic effects of GLP-1 are mediated in part by vagal-dependent pathways (35-38).

The rodent GLP-1R is composed of 463 amino acids (33), and although the receptor displays homology to other members of the glucagon-secretin superfamily, only GLP-1 binds with high affinity to the GLP-R (39). The GLP-1R was initially shown to be coupled to the production of cAMP (40), suggesting activation of adenylyl cyclase. Numerous studies have now demonstrated the promiscuity of the GLP-1R, as functional coupling to \(G\alpha_s, G\alpha_q11,\) \(G\alpha_{i1,2,}\) and \(G\alpha_c\) has been detected (41-43). A critical component of the GLP-1R is the third intracellular loop, as this region contains a domain that is required for coupling to \(G\alpha_s\) and
subsequent downstream activation of adenylyl cyclase and PKA (43, 44). Similar to other GPCRs, GLP-1R activity is regulated by receptor internalization through interactions with caveolin-1 or via receptor phosphorylation by G-protein receptor kinase (GRK)-5 (45-48).

**1.1.4. Biological actions of GLP-1**

GLP-1 and GIP are both classified as incretins, which are defined as gut-derived factors that augment insulin release from the pancreatic beta cell in a glucose-dependent manner (2, 49-52). Following a meal, GLP-1 levels increase from 5-10 pM to 10-40 pM (11, 53), and both GLP-1 and GIP account for up to 60% of glucose-induced insulin secretion (54).

GLP-1 has numerous biological actions, which are not solely limited to insulin secretion (Table 1-1). The classical mechanism of GLP-1-induced insulin secretion is that of GLP-1R activation leading to an increase in cAMP and PKA activity. Stimulation of PKA leads to inhibition of the $K_{ATP}$ channel, increases in intracellular calcium, and subsequent insulin granule exocytosis (55, 56). However, recent studies have now demonstrated that GLP-1-stimulated insulin secretion may also function through cAMP-regulated guanine nucleotide exchange factors (cAMP GEFs/Epac) (57), $\beta$-arrestin-1 (58), and protein kinase C (PKC) isoforms (59). Furthermore, GLP-1R activation also increases insulin gene expression and insulin biosynthesis in primary cells and immortalized rodent cell lines through a PKA-dependent mechanism (40). Aside from its effects on insulin secretion, GLP-1 has also been demonstrated to tonically inhibit glucagon secretion from the alpha cell (60), although this effect is most likely mediated by an indirect mechanism through secreted beta cell
Table 1-1: Pleiotropic actions of GLP-1

<table>
<thead>
<tr>
<th>Target tissue/cells</th>
<th>Biological Effect(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic beta cells</td>
<td>-stimulates insulin secretion</td>
<td>49-53</td>
</tr>
<tr>
<td></td>
<td>-induces insulin gene expression and biosynthesis</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>-promotes proliferation and neogenesis</td>
<td>58-62</td>
</tr>
<tr>
<td></td>
<td>-prevents apoptosis</td>
<td>60, 63-68</td>
</tr>
<tr>
<td>Pancreatic alpha cell</td>
<td>-inhibits glucagon secretion</td>
<td>54, 55</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>-decreases gastric emptying and intestinal motility</td>
<td>69-72</td>
</tr>
<tr>
<td>Brain</td>
<td>-inhibition of food intake</td>
<td>73, 74</td>
</tr>
<tr>
<td></td>
<td>-stimulates hepatic glycogen synthesis and inhibits hepatic gluconeogenesis and muscle glycogenolysis</td>
<td>75-78</td>
</tr>
<tr>
<td></td>
<td>-increases heart rate and blood pressure</td>
<td>81, 82</td>
</tr>
<tr>
<td>Heart</td>
<td>-prevents apoptosis in cardiomyocytes</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>-promotes myocardial glucose uptake</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>-increases blood flow</td>
<td>86, 87</td>
</tr>
</tbody>
</table>
factors, such as insulin or zinc, since the GLP-1R is not detected in isolated primary alpha cells (61, 62). Alternatively, GLP-1 may inhibit glucagon release by stimulating somatostatin release from pancreatic delta cells (63, 64).

Numerous studies have now demonstrated that GLP-1 is able to promote beta cell proliferation, neogenesis, and survival. Following partial pancreatectomy in rats, treatment with the degradation resistant GLP-1R agonist, exendin-4 (Ex-4), for 10 days stimulated beta cell regeneration and prevented the onset of diabetes (65). Furthermore, pancreatectomy in GLP-1R knockout (KO) mice is not associated with adaptive re-growth of beta cell mass, which highlights the importance of endogenous GLP-1 in this process (66). In a separate study, chronic GLP-1R activation delayed the onset of diabetes in leptin receptor-deficient db/db mice through increases in beta cell mass (67). The effects of GLP-1 on beta cell proliferation may be mediated via increased activation of Akt/PKB, by transactivation of the EGF receptor through the tyrosine kinase c-Src or by activation of the canonical Wnt signaling pathway (67-69). Treatment of beta cells with GLP-1 prevents apoptosis, and studies have now determined that the anti-apoptotic effects of GLP-1 are mediated by Akt, Jun NH(2)-terminal kinase (JNK), and FOXO1 (67, 70-75).

Aside from its predominant effects on the beta cell, GLP-1 also exhibits numerous extra-pancreatic effects to regulate nutrient homeostasis. Several studies on GLP-1 action revealed that injection or infusion of GLP-1 significantly inhibited gastric emptying and motility (76-78), which slows down the rate of post-prandial nutrient absorption. Interestingly, the effect of GLP-1 on gastric emptying occurs at a lower concentration than that needed for its insulinotropic activity (76, 79), which has led to speculation that GLP-1’s primary physiological role is to act as an “ileal brake” to slow down nutrient absorption rather than as an incretin. This concept is further supported by the distal localization of L cells (9, 10), which are activated by the presence of luminal nutrients in the ileum or colon.
Nevertheless, post-prandial release of GLP-1 stimulates glucose-dependent insulin secretion, which partially fulfills its definition as an incretin. GLP-1 also potently inhibits food intake through activation of peripheral and central GLP-1Rs (80, 81). Moreover, central GLP-1R activation also stimulates hepatic glycogen synthesis and inhibits both hepatic gluconeogenesis and muscle glucose utilization (82-85). While there are reports of GLP-1 directly stimulating glucose uptake in myocytes and adipocytes, these findings are controversial as the receptor has not been identified in these cells (86, 87).

Finally, GLP-1 increases heart rate and blood pressure in rodents, and it is hypothesized that these effects are mediated though a central pathway (88, 89). Moreover, recent studies have also determined that central or peripheral administration of GLP-1 increases blood flow, myocardial glucose uptake, and protects cardiomyocytes from apoptosis (90-93). Interestingly, following the use of GLP-1R KO mice to study the cardiovascular effects of GLP-1, it appears that some of these cardiovascular actions are mediated through a second unidentified GLP-1R and possibly via GLP-19-36NH2 (94).

### 1.1.5. Biological effects of other proglucagon-derived peptides

Interestingly other PGDPs from the alpha and L cells have overlapping or opposing actions to GLP-1, depending on the site of action (Table 1-2). Glucagon is a well-established counter-regulatory hormone that is secreted under during hypoglycemic conditions. The primary site of action of glucagon is the liver where receptor activation promotes hepatic gluconeogenesis and glycogenolysis through PKA-dependent pathways (95, 96). Recent studies have demonstrated that glucagon also regulates hepatocyte survival, lipid metabolism, and protein catabolism (97-100). Surprisingly, while glucagon generally antagonizes the anabolic effects of insulin, activation of the glucagon receptor promotes
Table 1-2: Distinct and overlapping biological effects of proglucagon-derived peptides

<table>
<thead>
<tr>
<th>Proglucagon-derived Peptide</th>
<th>Target tissue/cells</th>
<th>Biological Effect(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td>Liver/ hepatocytes</td>
<td>-promotes gluconeogenesis. glycogenolysis, protein catabolism, lipid metabolism</td>
<td>88, 89, 91-93</td>
</tr>
<tr>
<td></td>
<td>Beta cell</td>
<td>-regulates hepatocyte survival</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal tract</td>
<td>-stimulates insulin secretion</td>
<td>94, 95</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>-decreases motility</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-inhibits/promotes food intake</td>
<td>97, 98</td>
</tr>
<tr>
<td>Glicentin</td>
<td>Gastrointestinal tract</td>
<td>-increases intestinal growth and inhibits gastric acid secretion and intestinal motility</td>
<td>99-101</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>Beta cell</td>
<td>-stimulates insulin secretion</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>-inhibits food intake</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal tract</td>
<td>-inhibits gastric acid secretion and intestinal motility</td>
<td>101, 104</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Gastrointestinal tract</td>
<td>-promotes growth</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-stimulates intestinal adaptation, glucose absorption, proliferation, barrier function, and blood flow</td>
<td>9, 107-111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-decreases apoptosis in epithelial cells</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-inhibits food intake</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-stimulates glucagon release</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pancreatic alpha cell</td>
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</tr>
</tbody>
</table>
insulin secretion from the beta cell (101, 102), which suggests a regulatory role for this hormone in the islet. Similar to GLP-1, glucagon slows down gastrointestinal motility, although pharmacological concentrations are required to induce this effect, which questions its physiological importance as an ileal brake (103). In contrast, while central and peripheral administration of GLP-1 inhibits food intake, only intracerebroventricular administration of glucagon is able to inhibit food intake (104). Instead, peripheral glucagon administration actually promotes food intake in rodents (105), which suggests that peripheral sensory afferent nerves that express the glucagon receptor activate orexigenic centers in the brain.

Glicentin and oxyntomodulin are co-secreted with GLP-1 from the L cell. No functional receptor of glicentin has been identified, but tropic effects of glicentin on the small intestine have been demonstrated, in addition to inhibition of gastric acid secretion and gastrointestinal motility (106-108). While oxyntomodulin has insulintotropic actions, it is also able to inhibit gastric acid secretion, gastrointestinal motility, and food intake (108-111). Furthermore, the use of GLP-1R KO mice has demonstrated that oxyntomodulin increases heart rate via a GLP-1R-independent mechanism (112). Similar to glicentin, oxyntomodulin has no identified receptor, although recent findings suggest that some of its actions are mediated through the GLP-1R (109). Cleavage of proglucagon in the L cell also liberates the 33 amino acid peptide, GLP-2, which was initially identified as a potent intestinotropic factor (113). Several studies have also demonstrated that GLP-2 stimulates intestinal adaptation following fasting, proliferation, glucose absorption, barrier function, and intestinal blood flow and decreases apoptosis in villus and crypt cells (16, 114-118). Although GLP-1 and GLP-2 have different sites of action, GLP-2 also has inhibitory effects on food intake (119). In rodent pancreatic islets, GLP-2 has no effect to enhance glucose-stimulated insulin release, but surprisingly, expression of the GLP-2R is localized to alpha cells where it stimulates
glucagon release (120). When taken together, all proglucagon-derived peptides, including GLP-1, have both distinct and overlapping roles in the regulation of nutrient homeostasis.

### 1.1.6. Systemic regulation of GLP-1 secretion

Nutrient ingestion is the primary physiological stimulus of GLP-1 secretion, resulting in a biphasic pattern of GLP-1 release. In humans, the first phase of GLP-1 secretion occurs 15-30 min after a meal, while a second, sustained phase occurs at 90-120 min (6). Based on published reports, there are differences in the kinetics of GLP-1 secretion among species, such that in mice, the first phase of GLP-1 secretion occurs as early as 2 min after nutrient ingestion (121), while the early phase of GLP-1 release in rats follows a pattern similar to that of humans (122, 123).

L cell-derived PGDPs are co-secreted in an equal stoichiometric 1:1 ratio (124, 125). Early studies on PGDP secretion utilized antibodies that targeted a mid-sequence epitope of glucagon as opposed to the C-terminus of pancreatic glucagon, and this resulted in detection of glicentin/oxynotomodulin (*aka* enteroglucagon or gut glucagon-like immunoreactivity), as well as of glucagon (Figure 1-1). However, since GLP-1 is co-secreted in equal amounts, such studies therefore indirectly also examined GLP-1 release. Several recent studies have also begun to use total GLP-1 (C-terminus-specific antisera) assays as a surrogate marker of bioactive GLP-1 secretion, because over a short time period, the plasma profile of either form of GLP-1 is identical (126).

Glucose and fat have been found to be potent stimulators of early GLP-1 secretion, as demonstrated when either nutrient is ingested or is placed directly into the duodenal lumen (125, 127-129). In contrast, proteins do not consistently stimulate GLP-1 secretion (128-131), and to date, only peptones, or meat hydrolysates have been found to enhance GLP-1 release (129, 132). Essential amino acids are also able to stimulate GLP-1 secretion *in vitro*
but the relative contribution of either peptones or essential amino acids to meal-induced GLP-1 secretion is relatively small, as luminal levels of either stimuli would be expected to be low in ileum. In the same study, Reimer also determined that non-essential amino acids do not stimulate GLP-1 secretion from human L cells in vitro (133), although Reimann et al. demonstrated that murine L cells secrete GLP-1 in response to glutamine, a highly abundant non-essential amino acid (134). A recent study has also demonstrated that ingestion of large amounts of glutamine stimulates GLP-1 secretion in humans (135), although it is unlikely that dietary concentrations of glutamine are able to stimulate the L cell.

Consistent with the close proximity of L cells to neurons and the microvasculature of the intestine (26, 136), the L cell is affected by both neural and hormonal signals. Several studies have demonstrated that, in rodents, the early phase of nutrient-induced GLP-1 secretion is mediated by a neuro-endocrine loop (Figure 1-2). Following ligation of the distal duodenum, placement of glucose or fat directly into the proximal duodenum induced an immediate and prolonged stimulation of PGDP release from the L cell that was comparable in magnitude to that induced by placement of nutrients directly into the ileum (127). This increase in PGDPs is associated with a concomitant increase in circulating levels of GIP. As infusion of GIP or treatment of primary rat L cells in culture with GIP stimulates PGDP secretion, it was suggested that GIP was involved in the regulation of GLP-1 secretion by proximal nutrients (125, 127, 137). Coordination of this “proximal-distal loop” was demonstrated to be mediated by the vagus nerve, as stimulation of the L cell by placement of fat into the duodenum or by infusion of physiological concentrations of GIP was completely abrogated by subdiaphragmatic vagotomy. Furthermore, activation of the efferent celiac branch was demonstrated to increase GLP-1 secretion (125).
Figure 1-2: Regulation of GLP-1 secretion by ingested nutrients.

Following a meal, nutrients in the duodenum activate a proximal-distal neuroendocrine loop, which stimulates GLP-1 secretion from L cells in the ileum and colon. In rodents, glucose-dependent insulinotropic peptide (GIP), which is released from K cells, activates vagal afferents, which subsequently causes GLP-1 secretion through vagal efferents and enteric neurons that release acetylcholine (Ach) and gastrin releasing peptide (GRP). Movement of nutrients towards more distal sections of the intestine also stimulates GLP-1 secretion through direct interaction of nutrients with L cells. Direct effect of GIP on the L cell, as indicated by the dashed line, are only seen at supraphysiological concentrations (119).
Acetylcholine (Ach) and gastrin-releasing peptide (GRP) have now been identified as the neurotransmitter and neuropeptide, respectively, which mediate the “proximal-distal loop.” *In vitro* studies have determined that human and rodent L cells are sensitive to the stimulatory effects of Ach (136, 138). Furthermore, when rodents were infused with the cholinergic antagonist, atropine, or pirenzipine, an M1 muscarinic receptor antagonist, duodenal nutrients were unable to induce GLP-1 release (136). Finally, GRP is locally released from GRPergic neurons in the enteric nervous system, and *in vitro* and *in vivo* studies have demonstrated GRP to be a potent GLP-1 secretagogue (137, 139). Furthermore, the importance of GRP in the “proximal-distal loop” has been demonstrated using a GRP receptor antagonist, as well as GRP null mice (139, 140). Taken together, these studies demonstrate that in rodents, the regulation of GLP-1 release by proximal nutrients is mediated via GIP actions on cholinergic fibers of the vagus nerve, and by local secretion of GRP from the enteric nervous system.

Although the early rise of GLP-1 does occur in humans (6, 128), this event is likely not to be mediated by GIP, as GIP infusions do not stimulate GLP-1 release (141). While some GLP-1-expressing cells have been identified in the duodenum, their exact role in nutrient-induced GLP-1 release is unknown and still controversial (10, 142). Nonetheless, the human L cell is responsive to Ach *in vitro* (138), and administration of atropine prevents GLP-1 secretion following nutrient ingestion (143). Moreover, the human L cell is also responsive to GRP *in vitro* (132). While components of the rodent “proximal-distal loop” are present in humans, further work is clearly required to identify the early signal that promotes the rapid release of GLP-1 secretion following nutrient ingestion.

The second, later peak of GLP-1 secretion is believed to occur when nutrients transit into distal regions of the intestine and directly interact with L cells. Placement of nutrients, specifically long chain fatty acids and glucose, directly into the ileal lumen stimulates GLP-1
release (127, 144), while treatment of rodent and human L cells in culture with either glucose or fatty acids induces dose-dependent increases in GLP-1 secretion (132, 145-148). As glucose does not reach the distal gut in high concentrations (149), it has been proposed that fat, which does transit to the ileum (150), is the more physiological direct regulator of GLP-1 release (151).

Recently, GLP-1-expressing cells in the human and rodent intestinal epithelium have been demonstrated to express the TIR and T2R families of taste receptors, which recognize sweet and bitter substances, respectively (152-154). The ability of artificial sweeteners to elicit a GLP-1 response was originally detected in an immortalized human L cell model, but recent studies have demonstrated that primary L cells are not responsive to these substances (9, 153, 155, 156), which raises questions as to the role of the T1R receptors on the L cell. Harmful (bitter) tasting substances are detected by T2Rs (157), and activation of T2Rs \textit{in vitro} stimulates GLP-1 secretion (152). GLP-1 is co-secreted from the L cell with peptide YY (PYY), and as both peptide hormones have been demonstrated to induce conditioned taste aversion in mice (158, 159), it has been suggested that this mechanism is an evolved mechanism to avoid harmful, noxious substances (152, 160). Further work is required to clarify the role of the bitter taste receptors in GLP-1 secretion \textit{in vivo}.

1.1.7. Intracellular signaling pathways regulating GLP-1 secretion

The development of in vitro models of the murine (GLUTag, STC-1), rat (FRIC), and human (NCI-H716) L cell has now permitted more detailed examination of signaling pathways expressed in the L cell. Some signaling pathways of secretagogues were also defined through the use of isolated canine L cells and perfused porcine ileum models. GLUTag cells were originally derived as a single cell clone from a colonic tumor in mice that expressed the oncogene Simian virus 40 (SV40) large T antigen under the control of the
proglucagon promoter (161). STC-1 cells were derived from duodenal secretin-secreting tumor cells in mice expressing the SV40 and polyoma small T antigen oncogenes under the rat insulin promoter (162). NCI-H716 cells are a human L cell line that was derived from a cecal adenocarcinoma (163). Lastly primary FRIC cultures are generated from fetal rat intestinal cells collected from term pregnant Wistar rats (164). The recent development of transgenic mice that express a fluorophore in proglucagon-expressing cells now permits the isolation of primary L cells, which will be an invaluable tool to study gene and protein expression (9). All current L cell models to study GLP-1 secretion are a heterogeneous mixture of cells, including, now, the GLUTag cells (121). Nonetheless, they are excellent models to study GLP-1 secretion, as they respond to known physiological secretagogues. However, in some instances, mechanisms of action of various secretagogues have been inferred from other endocrine cell models.

1.1.7.1. Glucose

In both immortalized and primary murine L cells, a dose-dependent effect of glucose on GLP-1 secretion has been observed (9, 147, 148). It appears that the classic stimulus-secretion coupling pathway of glucose-induced insulin secretion in the beta cell is also present in the L cell (Figure 1-3A). Electrophysiological studies have shown that glucose alters the membrane potential of the murine L cell through closure of $K_{ATP}$ channels (147), as well as via depolarization due to co-transport of sodium and glucose through the sodium-glucose co-transporter (SGLT) (148). Alternatively, glucose may also enter the L cell via the facilitative glucose transporters, GLUT1 and GLUT5, of which mRNA transcripts have been detected (147). Recent reports have indicated that the $K_{ATP}$ channel cannot be detected in the mouse L cell in vivo (165), and $K_{ATP}$ null mice do not show alterations in circulating GLP-1 levels (166). In contrast, closure of the $K_{ATP}$ channel in immortalized and
Figure 1-3: Signaling components involved in direct nutrient-induced GLP-1 secretion.

(A) Glucose entry into L cells via sodium glucose transporters (SGLT) and facilitative glucose transporters 1 and 5 (GLUT1/5) causes an increase in ATP, resulting in $K_{\text{ATP}}$ channel closure. Together with $Na^+$ entry via SGLT, this results in a decrease in membrane potential ($\psi$), which opens L-type voltage-gated calcium channels and leads to GLP-1 release. (B) Long chain fatty-acid (LCFA)-induced GLP-1 secretion is mediated by GPR40 and GPR120 and is associated with increases in intracellular calcium and phosphorylation of Akt and ERK1/2 via $G_{\alpha_q}$. Alternatively, LCFAs may be transported via fatty acid transport proteins (FATP), leading to activation of $PKC_{\zeta}$ through an unknown mechanism to stimulate GLP-1 secretion. Fatty-acid derivates, such as oleoylethanolamide (OEA), signal through GPR119 to stimulate GLP-1 secretion. Activation of GPR119 induces the production of cAMP, which increases the activity of PKA and subsequent GLP-1 release. (Solid arrows = known pathways; dashed arrows = unknown pathways)
isolated primary mouse L cells results in a potentiation glucose-induced GLP-1 release (9, 147). The differences in these findings results can be reconciled by two explanations: L cells from different regions (i.e. small intestine vs colon) may differentially express the KATP channel, or the relative contribution of KATP channel closure in vivo is too small to mediate to glucose-induced GLP-1 release. Furthermore, glucose also elicits transient increases in intracellular calcium in both primary and immortalized L cells, which are also required for glucose-induced GLP-1 release (9, 147, 167). Expression of the glucose sensor, glucokinase, has also been detected in the mouse intestinal L cell in vivo (168), but does not appear to be required for GLP-1 secretion from the human L cell (169). Taken together, the electrogenic response of the murine L cell to glucose is similar to the stimulus-secretion coupling events that occur during glucose-stimulated insulin secretion (170). However, unlike the beta cell, the absence of GLUT2 mRNA expression in the primary and immortalized murine L cells suggests that glucose transport is primarily facilitated by SGLTs, which leads to cell depolarization via co-transport of Na+ with glucose (9).

1.1.7.2. Fatty acids

Long-chain fatty acids (LCFAs) directly stimulate GLP-1 secretion from murine, rat and human L cells through distinct mechanisms (Figure 1-3B). Recently, LCFAs have been found to interact with two, recently de-orphanized GPCRs, GPR40 and GPR120. As was first described in the beta cell, GPR40 is coupled to both Goq and Got, as demonstrated by increases in cytosolic calcium and inhibition of forskolin-induced cAMP production, respectively, in response to LCFAs. Furthermore, siRNA-mediated knock-down of GPR40 prevents FA-induced insulin secretion (171). Generation of GPR40 KO mice permitted the evaluation of GPR40’s role in FFA-induced GLP-1 secretion, such that GLP-1 secretion in response to high-fat feeding was impaired. However, the lack of detailed diet composition in
the study prevents one from identifying which FAs are responsible for the GPR40-dependent effects on GLP-1 release (172). GPR120 is also expressed in most GLP-1-positive cells in the mouse colon (173). In STC-1 cells, activation of GPR120 by unsaturated long-chain FAs stimulates GLP-1 release and is associated with increases in cytosolic calcium as well as ERK1/2 phosphorylation. However, it appears that in these cells, neither of these pathways are required for FA-induced GLP-1 release (174). Finally, GPR119 was recently de-orphanized and identified as a receptor for fatty acid derivatives such as oleoylethanolamide (OEA) and lysophosphatidylcholine (175). GPR119 was discovered to be required for secretion of FA derivate-dependent secretion of GLP-1 and GIP (121). In GLUTag cells, OEA directly activates GPR119, which stimulates GLP-1 release through a Gα₅ pathway involving PKA (175-177).

The murine L cell expresses all three classes of PKCs (conventional, novel, and atypical) (178). While activation of conventional and novel PKCs stimulates GLP-1 secretion, oleic acid-induced GLP-1 release is mediated via activation of the atypical isoform, PKC zeta (178). The mechanism by which oleic acid activates PKC zeta has yet to be determined, nor is it clear how oleic acid enters the cell. RT-PCR has confirmed the expression of fatty acid transport proteins (FATPs), but their functional significance in oleic acid-induced PKC zeta activation and GLP-1 secretion is unclear (R. Iakoubov and P.L. Brubaker, personal communication). Further studies are clearly required to elucidate the exact mechanism of action of this important physiological regulator of GLP-1 release.

**1.1.7.3. Sweet and bitter substances**

*In vitro* studies have demonstrated that sweet and bitter substances induce GLP-1 secretion through T1Rs and T2Rs, respectively. Both receptors are coupled to Gα_{gust} or Gαₐ
(Figure 1-4) (153, 179), which decreases intracellular cAMP levels through activation of phosphodiesterases (PDEs) and inhibition of adenylyl cyclase (179, 180). While increasing cAMP with forskolin and IBMX augments GLP-1 secretion, it is not clear how decreasing cAMP induces GLP-1 release. However, activation of these receptors also increases cytosolic calcium and phosphorylation of ERK1/2 (153, 180, 181), although the role of the latter pathway in GLP-1 release is not fully known (182). Further work is required to determine which pathways are required for taste receptor-induced GLP-1 release and for the exact role of these receptors in nutrient homeostasis.

1.1.7.4. Acetylcholine

As discussed above, Ach stimulates GLP-1 secretion in vitro and in vivo. M1 muscarinic receptor agonists increase GLP-1 secretion from rat L cells, and inhibition of M1 muscarinic receptors prevents release of GLP-1 induced by bethanechol, a non-selective muscarinic agonist (136). In human L cells, inhibition of M1 and M2 muscarinic receptors also prevents bethanechol-induced GLP-1 release (138). M1 muscarinic receptors are GPCRs that are linked to Gαq/11, and ligand binding results in the activation of phospholipase C which cleaves phosphatidylinositol(4,5)biphosphate into IP₃ and DAG, leading to increases in intracellular calcium and activation of both conventional and novel PKC isoforms (Figure 1-5) (183). M2 receptors are thought to be coupled to Gαi, which inhibits adenylyl cyclase (183); hence, the enhanced GLP-1 secretory response to M2 receptor activation in human L cells suggests the existence of an alternative intracellular pathway.
Figure 1-4: Downstream pathways mediating sweet and bitter taste receptors.
Sweet and bitter substances signal through the T1Rs and T2Rs, respectively, to induce GLP-1 secretion from the L cell. Both type of receptors utilize the G\textsubscript{\gamma13} subunit to activate PLC, which results in IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release. Alternatively, G\alpha\textsubscript{gust} mediates the actions of T1Rs, and sweet substances have been found to decrease intracellular levels of cAMP, which suggests that G\alpha\textsubscript{gust} couples to a phosphodiesterase (PDE). (Solid arrows = known pathways; dashed arrows = unknown pathways)
Figure 1-5: Intracellular pathways of GLP-1 secretagogues activated by proximal nutrients.

Binding of GRP or Ach to Gαq-linked GRP- or M1- receptors (R), respectively, is associated with phospholipase C (PLC) activation and subsequent activation of conventional and novel isoforms of protein kinase C (c/nPKC). Activation of these receptors is also associated with increases in intracellular calcium and the phosphorylation of ERK1/2. Glucose-dependent insulinotropic peptide (GIP) receptor activation causes the activation of adenylyl cyclase (AC) via Gαs. This leads to an increase in cAMP and activation of protein kinase A (PKA). The somatostatin receptor (ssTR5) is coupled to Gαi, which inhibits AC, therefore inhibiting GLP-1 secretion. Binding of GABA to GABA_A receptors depolarizes the membrane potential (ψ) by channel opening leading to chloride efflux. (Solid arrows = known pathways; dashed arrows = unknown pathways)
1.1.7.5. Gastrin-releasing peptide (GRP)

GRP is a potent stimulator of the intestinal L cell in vivo and in vitro (137, 139), but the signal transduction cascade that occurs in response to GRP treatment in the L cell has yet to be defined. Based on studies utilizing other endocrine cell models, GRP binds to a GPCR that is coupled to $\text{G}_q$ (Figure 1-5) (184). In STC-1 cells, treatment with GRP stimulates the activation of ERK1/2 and PKC, which subsequently leads to hormone secretion (185), while in the beta cell, GRP stimulates insulin secretion via increasing intracellular calcium (186). Since increases in intracellular calcium levels promote GLP-1 release in the rodent L cell (167, 187), it is possible that GRP-mediated calcium influxes stimulate GLP-1 secretion. Further work is required to examine if these events facilitate GRP-mediated GLP-1 release.

1.1.7.6. Gamma-amino butyric acid (GABA)

GABAergic neurons are components of the enteric nervous system located primarily in the myenteric plexus of the colon. Three isoforms of the GABA receptor exist (GABA$_A$R, GABA$_B$R, and GABA$_C$R), and their expression and distribution is tissue-specific. Of the three isoforms, GABA$_A$R and GABA$_C$R are ion-channel linked receptors, while the GABA$_B$R is a metabotropic GPCR (188). Gameiro et al. confirmed the expression of GABA$_A$R in the murine L cell, and GABA treatment of these cells caused an efflux of chloride ions from the cell, leading to depolarization, opening of voltage-gated calcium channels, and GLP-1 secretion (Figure 1-5) (189). These in vitro findings suggest that GABA from GABAergic neurons may act in a paracrine manner to modulate hormone secretion. Nonetheless, the physiological role of GABA modulation of GLP-1 secretion in vivo still remains to be demonstrated.
1.1.7.7. Glucose-dependent insulinitropic peptide (GIP)

GIP mediates its biologic effects through a GPCR belonging to the secretin-glucagon receptor super-family, which includes receptors for other structurally related, gut-derived peptides, including GLP-1, GLP-2, glucagon, and secretin (33). GIP receptor activation leads to the activation of adenylyl cyclase through $G_s$, resulting in increases in cAMP and PKA-dependent GLP-1 secretion from the L cell (Figure 1-5) (137, 146, 176, 190). In the beta cell, GIP has also been reported to stimulate hormone secretion through cAMP-dependent, PKA-independent activation of the cAMP-GEFII/Epac pathway (57). A recent study by Islam et al demonstrated that direct activation of Epac has no effect on GLP-1 secretion (191); therefore, it is unlikely that GIP stimulates GLP-1 secretion from the rodent L cell via an Epac-dependent mechanism.

1.1.7.8. Somatostatin

Two distinct forms of somatostatin are produced in the intestine, SS14 by enteric neurons, and SS28 by enteroendocrine D cells. However, in both rats and pigs, SS-28 is a more potent inhibitor of GLP-1 secretion (137, 192, 193). Somatostatin receptors exist as five isoforms (sstR1-sstR5), of which sstR5 is expressed by the rat L cell (192). These receptors are coupled to a pertussis toxin-sensitive $G_i$ protein, and activation both inhibits adenylyl cyclase and decreases intracellular calcium levels (Figure 1-5) (194). Since GLP-1 stimulates the secretion of both forms of somatostatin from the intestine, these findings suggest the existence of a feedback loop through which locally-produced intestinal somatostatin can modulate GLP-1 release following the ingestion of nutrients (137, 192, 193, 195).
1.1.7.9. Leptin

Leptin is a cytokine derived from adipocytes with potent effects on food intake (196). When administered to rats and mice *in vivo*, leptin demonstrates stimulatory effects on GLP-1 secretion, and these effects have also been observed in rodent and human L cells in vitro (197). The leptin receptor is a product of the *Ob-R* gene, which is related to other class I cytokine receptors. *Ob-R* is spliced into five isoforms, but only the Ob-Rb isoform, which is the long form of the receptor, has the necessary intracellular motifs for leptin signaling (196). Following leptin binding to Ob-Rb, Janus kinases (JAK) phosphorylate residues on Ob-Rb, which then serve as docking sites for signal transducers and activators of transcription (STAT) molecules (Figure 1-6). Once phosphorylated, STAT molecules dimerize and enter the nucleus to mediate effects on gene transcription (196). Consistent with these findings, human and rodent L cells express Ob-Rb, and leptin treatment increases STAT-3 phosphorylation (197); however, it remains unknown as to how this results in enhanced GLP-1 release. Nonetheless, stimulatory effects of leptin have also been reported in heterogeneous STC-1 cells, such that ERK1/2 phosphorylation leads to CCK release (198). Whether the effect of leptin on GLP-1 secretion from the L cell is mediated by ERK1/2 remains to be determined.

1.1.8. Clinical relevance of GLP-1

Type 2 diabetes is classically defined as the combination of insulin resistance and failure of beta cells to adequately secrete insulin. It is also associated with a reduction in the incretin effect, hyperglucagonemia, decreased beta cell mass, and obesity (2, 199-202). Numerous factors increase the risk of developing type 2 diabetes, including family history, pre- and post-natal environmental factors, low birth weight, inactivity, obesity, gestational diabetes, and increasing age (203). It is predicted that from 2000 to 2030, the prevalence
Figure 1-6: Potential mechanism of leptin-induced GLP-1 secretion.

Binding of leptin to its receptor (Ob-Rb) in the L cell results in signal transducer and activator of transcription (STAT)-3 phosphorylation, likely through janus kinase (JAK). Leptin receptor activation may also result in ERK1/2 phosphorylation. (Solid arrows = known pathways)
of type 2 diabetes will rise from 2.8% to 4.0% worldwide (204). Early studies using GLP-1R antagonists and neutralizing antibodies demonstrated the importance of endogenous GLP-1 in maintaining glucose homeostasis (51, 52, 60). In mice, genetic ablation of the GLP-1R is associated with glucose intolerance and impaired insulin secretion in response to oral and intraperitoneal glucose challenges (205). Furthermore, chronic GLP-1/Ex-4 treatment delays the onset of hyperglycemia in rodent models of both type 1 and type 2 diabetes (67, 206, 207). Taken together, the pleiotropic actions of GLP-1 on nutrient homeostasis have led to the use of GLP-1R agonists or DPP-IV inhibitors as novel approaches to treat type 2 diabetes.

In early studies, acute infusions of GLP-1 improved fasting and post-prandial blood glucose levels and reduced appetite in subjects with type 2 diabetes (208, 209). The ground-breaking study by Zander and colleagues determined that over a 6-week period, continuous infusion of native GLP-1 decreased fasting blood glucose, HbA1c levels (by 1.3%), and body weight, and improved insulin sensitivity and beta cell function (210). Similar effects were also observed with chronic Exenatide (synthetic Ex-4) treatment on HbA1c levels and blood glucose (211-213), although nausea appears to be a common side effect, consistent with the GLP-1-induced conditioned taste aversion in rodents (158). DPP-IV inhibition is also being used as an alternative approach to enhance endogenous GLP-1 and GIP action, and long-term studies in humans have demonstrated that this class of drugs has similar lowering effects on HbA1c levels and blood glucose (214-216). While both GLP-1R agonists and DPP-IV inhibitors have profound effects on glucose homeostasis, they have contrasting effects on weight loss, such that administration of native GLP-1 or GLP-1R agonists has been shown to induce weight loss (210, 211, 217). In contrast, treatment with DPP-IV inhibitors appears to prevent weight gain in subjects with type 2 diabetes without lowering body weight (216, 218). The differing effects on weight could be attributed to pharmacological activation of
GLP-1R by the exogenous agonist, whereas DPP-IV inhibition merely prolongs the biological activity of endogenous GLP-1. Nonetheless, due to the glucose-dependence of GLP-1’s actions in the beta cell, the risk of treatment-related hypoglycemia is mild using either class of drug, unless used in combination with sulfonylureas (219).

DPP-IV is closely related to other serine proteases, namely DPP-VII, DPP-VIII, and DPP-IX (25). Early studies that investigated the role of DPP-IV utilized non-selective inhibitors that also had effects on DPP-VIII/ IX. Since DPP-IV is also a marker for activated T-cells (220), it was postulated that DPP-IV inhibition could compromise immune function; however, inhibition of DPP-VIII or –IX, but not DPP-IV, is associated with increased mortality in dogs (221). Nonetheless, long-term safety reports from prolonged usage of either degradation-resistant GLP-1R agonists or DPP-IV inhibitors are required.

Unexpectedly, GLP-1R activation has beneficial cardiovascular effects in subjects with chronic heart failure or coronary artery disease, such that improvements in left ventricular ejection fraction and endothelial function were observed, respectively (222-224). The mechanism by which GLP-1 exerts its beneficial effects on cardiovascular function are not fully understood, but these findings suggest a potentially novel use for GLP-1 as a treatment for heart disease or as a combination therapy for cardiovascular disease in subjects with type 2 diabetes.
1.2 Insulin resistance, obesity, type 2 diabetes and the incretin effect

Reduced biological activity of GIP and impaired GLP-1 secretion contribute to the impaired incretin effect seen in type 2 diabetes (1-3, 225-230). While it is unclear if DPP-IV activity is altered in type 2 diabetes (231, 232), there do not appear to be any differences in the levels of GLP-1 and GIP metabolites between healthy subjects and those with type 2 diabetes (233, 234). Furthermore, the observed reduction in GLP-1 levels is not due to increased clearance of GLP-1(233).

In humans, GIPR mutations have been identified, and while expression of these mutants in cell lines is associated with impaired GIPR function, genome-wide analysis from two different cohorts of diabetic subjects revealed no association between these variants and type 2 diabetes (235, 236). Furthermore, based on rodent studies, hyperglycemia or obesity are postulated to be responsible for decreasing GIPR expression and, therefore, the biological activity of GIP (237-240). While the majority of published reports describe impaired post-prandial GLP-1 secretion in type 2 diabetes (3, 226-230), two recent studies have found no association between GLP-1 release and type 2 diabetes (241, 242). In studies where defects in secretion were observed, all subjects were matched for body-mass index (BMI), which removes the separate impact of obesity on L cell function (3, 226-230). It has been suggested that the effects of type 2 diabetes on GLP-1 release could be attributed to poor glycemic control (i.e, HbA1c >8.0%) or due to the duration of T2DM (3, 226, 227). However, a recent study by Musceli and colleagues demonstrated subjects with HbA1C of <6.5% also had decreased GLP-1 release after an oral glucose challenge (229), which demonstrates that glycemic control is not a predictor of decreased L cell function. Currently, there is no explanation for why there are differing observations on the effects of type 2 diabetes on GLP-1 release, but the general trend is that post-prandial GLP-1 levels are reduced.
Risk factors for type 2 diabetes include gestational diabetes, low birth weight, and familial association (243, 244), but reductions in nutrient-induced GLP-1 secretion are not associated with these factors (245-248). In 2006, it was discovered that a polymorphism in intron 3 on the TCF7L2 gene conferred increased susceptibility to type 2 diabetes (249). As TCF7L2 is a transcription factor that is involved in intestinal proglucagon expression (7, 18), Grant et al. speculated that the increased susceptibility was due to altered proglucagon expression or GLP-1 secretion (249). However, it was recently determined that this polymorphism was associated with impaired GLP-1-induced insulin release from the pancreatic beta cell and not with decreased circulating levels of GLP-1 (250, 251).

Obesity is a multi-faceted syndrome associated with leptin resistance, elevated circulating fatty acids, increased adiposity, and a proinflammatory state (4). While obesity does not automatically predispose an individual to type 2 diabetes, studies from separate groups have determined that BMI is negatively associated with nutrient-induced GLP-1 secretion (5, 197, 229, 252-254). Additionally, two previous studies have shown that elevated circulating non-esterified fatty acids, as a consequence of obesity, impair GLP-1 release in humans (252, 255). In mice, diet-induced obesity in mice decreases glucose-stimulated GLP-1 release (197), and it was suggested that leptin resistance could impair the function of the L cell. Once obesity has been established, conventional methods of weight loss (i.e. diet and exercise) do not appear to restore GLP-1 secretion to levels seen in lean individuals (253, 256). In contrast, Roux-en-Y gastric bypass surgery, which is currently being used to induce weight loss and treat type 2 diabetes in obese individuals (257, 258), increases GLP-1 secretion from the L cell due to expedited delivery of nutrients to the ileum and colon. This effect is not observed following gastric banding surgery, which is another surgical option to promote weight loss (257-259), likely due to the absence of enhanced nutrient transit to the L cells.
Unlike obesity, insulin resistance is a prerequisite condition for the pathogenesis of type 2 diabetes, and a hallmark characteristic of this condition is chronically elevated insulin levels, or hyperinsulinemia. Rask et al. recently determined that insulin resistance is associated with impaired GLP-1 release in humans subjects (6, 260), which suggested that hyperinsulinemia has deleterious effects on L cell function. Recently, Yi et al demonstrated that the intestinal L cell is a target of insulin due to its stimulatory effects on proglucagon expression and GLP-1 synthesis (7). However, to further understand the possible effects of insulin on L cell function and GLP-1 secretion, a deeper understanding of insulin’s effects on the intestine and downstream signaling pathways of the receptor under normal and pathophysiological conditions is required.

**1.2.1. Non-intestinal actions of insulin**

Similar to GLP-1, insulin is derived from posttranslational processing of its prohormone, proinsulin, by PC-1/3 and PC-2. This liberates the mature, 51 amino acid insulin molecule that is co-secreted with C-peptide upon stimulation of the beta cell (261). The actions of insulin on glucose homeostasis and metabolism have been well documented, such that insulin promotes protein synthesis and glucose uptake in muscle and fat, while inhibiting gluconeogenesis and glycogenolysis in the liver. Moreover, insulin promotes fatty acid synthesis and storage, and inhibits lipolysis and beta oxidation of fatty acids.

Molecular techniques have permitted a detailed investigation into the systemic actions of insulin (Table 1-3). In insulin receptor (IR) knockout mice, a slight retardation of growth was observed at birth, which highlights the importance of insulin during fetal growth.
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<th>Phenotype</th>
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<td>- impaired insulin-stimulated glucose uptake and glycogen synthesis</td>
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<td>Fat (FIRKO)</td>
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and development. As expected, these mice die postnatally from severe diabetic ketoacidosis (262). The use of the Cre-Lox system to generate tissue-specific IR knockout mice, and numerous studies have now elucidated the specific role of insulin in target tissues (263). Deletion of the IR in beta cells (βIRKO) causes impaired glucose tolerance due to a loss of first-phase insulin secretion. Moreover, it appears that endogenous insulin regulates beta cell mass, as these mice have smaller islets when compared to wild type controls (264). These findings were further corroborated in recent studies in which treatment of mouse and human islets with physiological concentrations of insulin induced beta cell proliferation and survival (265-267). Several lines of evidence from primary and immortalized rodent beta cells suggest that insulin can also stimulate insulin secretion from the beta cell through increases in intracellular calcium. However, treatment of human beta cells with physiological concentrations of insulin failed to stimulate insulin release (268-271), which either suggests species-dependent effects or a non-physiological effect in rodent beta cells.

In liver-specific IR knockout (LIRKO) mice, fasting and fed hyperglycemia is observed due to inability of insulin to suppress hepatic glucose production. Moreover, LIRKO mice have decreased hepatic triglyceride secretion due to a loss of insulin function to promote triglyceride synthesis (272). A recent study that utilized an inducible IR knockout in the liver demonstrated that insulin-like growth factor-1 (IGF-1) levels are elevated; furthermore, increased circulating IGF-1 is associated with an expansion of beta cell mass (273). In mice that lack the IR in the vascular endothelium and endocardial cushion, no defects in glucose and lipid homeostasis were observed; however, blood vessels in these mice have increased protection from hypoxia (274, 275).

Skeletal muscle is the main site of glucose disposal in vivo. Hence, it was unexpected that no effects on fasting or post-prandial glucose levels were detected following deletion of muscle-specific IRs (MIKRO) in mice, despite impaired insulin-stimulated glucose uptake,
glycolysis and glycogen synthesis in muscle. These findings suggested that other target-tissues were compensating for the inability of muscle to facilitate glucose uptake. Furthermore, these mice had decreased muscle mass as a result of the inability of insulin to promote protein synthesis (276). In fat-specific IR knockout (FIRKO) mice, loss of the insulin receptor led to improvements in glucose and lipid metabolism and insulin sensitivity. Interestingly, these mice have an increased life span, due to unknown mechanisms, and are leaner than wild type litter mates (277, 278). In the brain, deletion of the IR was performed by targeting neuroepithelial stem cells or by injecting anti-sense oligonucleotides into the third ventricle (279, 280). Through either method, loss of the IR led to hyperinsulinemic-insulin resistance, hyperphagia, hypertriglyceridemia, and dysfunction in endogenous glucose production. Moreover, in separate studies, injection of insulin directly into the third ventricle directly inhibited hepatic glucose production, independent of circulating levels of insulin, and reduced food intake (281, 282). In summary, numerous tissues and organs, as demonstrated by transgenic mouse models, facilitate the metabolic actions of insulin, but in the literature, there is little emphasis given to the role of insulin in the intestine.

1.2.2. Insulin actions on the intestine

The insulin receptor is expressed throughout the gastrointestinal tract from the stomach to the colon (283). Expression of the receptor is localized to the brush border and the basolateral membranes of all cells along the crypt-villus axis, although expression is primarily in the crypts where it promotes cell proliferation (284, 285). The intestinal epithelium is composed of four distinct cell types: absorptive enterocytes, mucin-producing goblet cells, lysozyme-containing paneth cells, and endocrine cells. In the epithelial absorptive cells, insulin regulates the expression and activity of brush border enzymes (286). Glucose transport from the intestinal lumen into the circulation is dependent on SGLT1
expression on the brush border membrane and the facilitative glucose transporter, GLUT2, on the basolateral membrane, and under conditions of high luminal glucose, GLUT2 is actively recruited to the basolateral membrane (287, 288). Following an increase in plasma insulin levels, glucose absorption is reduced through sequestration of GLUT2 below the plasma membrane (287, 288). Additionally, glycolysis in the intestine is also transiently regulated by insulin-induced activation of pyruvate dehydrogenase and phosphofructokinase (289). Lipid and cholesterol absorption in the intestine is facilitated by enterocytes, which are responsible for packaging lipids and cholesterol into ApoB48-containing chylomicrons prior to secretion into the lymph system (290). Studies in animal models have demonstrated that insulin regulates intestinal-derived lipoprotein synthesis and secretion from enterocytes, and this is impaired under conditions of insulin resistance (291). Intestinal endocrine cells, such as L, K, D, I, and X/?, in the intestinal epithelium secrete hormones in response to enteral stimuli. Under pathophysiological conditions such as insulin resistance, hormone release can be augmented or inhibited (292-298). Taken together, these findings demonstrate that in the intestine, insulin predominantly has a metabolic function, which is similar to other insulin-sensitive tissues.

1.2.3. The insulin receptor

The IR is a widely-expressed heterotetrameric receptor that belongs to the subclass II family of receptor tyrosine kinases (RTKs), which also includes the IGF-1 receptor (IGF-1R), and the insulin receptor-related receptor (299). The IR is composed of two identical monomers held together by disulfide bonds, with each monomer consisting of a largely extracellular alpha-subunit and a beta-subunit with intrinsic kinase activity (300). The alpha subunit is made up of 723 amino acids and has a molecular weight of approximately 130 kDa, while the beta subunit contains 620 amino acids and has a molecular weight of 95 kDa.
Alternative splicing of the IR mRNA at exon 11, which encodes 12 amino acids, generates isoforms A (Ex11+) and B (Ex11-). The absence of exon 11 (IR-B) increases the binding affinity for insulin by two-fold (301-304).

In mammals, insulin signaling is mediated by insulin and the structurally-related peptides, IGF-1, and IGF-2 (299). IR and the IGF-1R share approximately 50% overall amino acid homology and 85% homology in the tyrosine kinase domain. Due to these structural similarities, all three members of the insulin family are able to bind to homologous IRs and IGF-1Rs, as well to hybrid insulin/IGF-1 receptors, with varying degrees of affinity (301). Biochemical studies have demonstrated insulin and IGF-1 bind with the highest affinities to their respective receptors. However, all three ligands bind IR-A/IGF-1R hybrids, with increasing affinity from insulin to IGF-2 to IGF-1. IGF-1, but not insulin, binds to hybrid IR-B/IGF-1 receptors with the highest affinity (301), and IR-B has a ten-fold higher binding affinity than IR-A for IGF-1 (305). The endogenous receptor for IGF-2 has been identified as the mannose-6-phosphate receptor, which is assumed to be involved in IGF-2 degradation (306), but IGF-2 binds with equal affinity to hybrid IR/IGF-1Rs, IGF-1R, and IR-A (301). Finally, at supra-physiological concentrations, insulin and IGF-1 are able to bind and activate both the IR and IGF-1R. While insulin and IGF-1 generally regulate metabolism and growth, respectively, recent studies have suggested overlapping roles of these hormones in cancer progression and growth (307).

Following binding of insulin to its receptor, the intrinsic kinase activity of the receptor is activated, stimulating cis- and trans-phosphorylation of tyrosine residues in the beta subunits in the juxtamembrane region (Tyr$^{953}$, Tyr$^{960}$, and Tyr$^{972}$), regulatory region (Tyr$^{1146}$, Tyr$^{1150}$, and Tyr$^{1151}$), and the C-terminus (Tyr$^{1316}$ and Tyr$^{1322}$) (299, 308). Unlike other RTKs, like the platelet-derived growth-factor and epidermal growth-factor receptors (PGDFR and EGFR, respectively), the IR does not directly bind to effector molecules.
through src-homology-2 (SH2) domains (299), but rather through insulin receptor substrate (IRS) molecules, which interact with phosphorylated Tyr$^{960}$ and Tyr$^{972}$ residues in the juxtamembrane region of the IR (299, 309). Phosphorylation of all three Tyr residues in the regulatory region increases the kinase activity of the IR by 10-20-fold, resulting in the phosphorylation of IRS molecules and activation of downstream signal transduction pathways (310).

1.2.4. Canonical insulin signaling pathways: PI3K-Akt and MEK1/2-ERK1/2

IRS molecules facilitate most of the actions of the insulin receptor, and although six IRS isoforms have been identified, it appears that IRS1 primarily mediates the metabolic actions of the IR, while IRS2 is involved in the mitogenic effects of insulin and IGF-1 (311-313). Tyrosine phosphorylation of IRS molecules by the IR promotes the association of IRS with other proteins that contain SH2 domains, which include the classical downstream adaptor molecules of the insulin signaling cascade, phosphoinositide 3-kinase (PI3K) and Grb2 (Figure 1-7) (313). Whole-body knockouts of IRS1 or IRS2 have revealed different roles of each molecule in insulin signaling and whole body homeostasis. IRS1 knockout mice display a phenotype of insulin resistance, impaired glucose tolerance, and hypertriglyceridemia; however, these mice do not manifest diabetes, potentially due to compensatory signaling through IRS2 (314). Furthermore, IRS1 knockout mice display severe growth retardation, most likely consequent to the inability of IGF-1 to mediate its mitogenic effects. In contrast, IRS2 mice exhibit a progressive deterioration of glucose homeostasis due to skeletal muscle and hepatic insulin resistance and a lack of compensatory growth by the beta cell (315). As yet, the expression of IRS1 or IRS2 in the intestine has not been determined, nor is it known which isoforms are required for intestine-specific insulin effects.
Figure 1- 7: Canonical insulin signaling pathways and their roles in insulin action. Binding of insulin to its receptor (IR) induces receptor autophosphorylation, which serve as docking sites for insulin receptor substrate (IRS) molecules and subsequently stimulate phosphorylation of IRS on tyrosine residues. Binding of IRS to the regulatory subunit of PI3K via SH2 domains promotes the synthesis of PIP3, which recruits Akt to the plasma membrane and induces PDK1-dependent activation of Akt. The PI3K-Akt pathway facilitates the metabolic actions of insulin, such that Akt activation is associated with glucose uptake, glycogen synthesis, inhibition of gluconeogenesis, and protein synthesis. Alternatively, IRS molecules or the adaptor protein Shc interact with Grb2, which is complexed with the guanine nucleotide exchange factor (GEF) SOS. Once SOS is activated, it leads to the downstream activation of Ras, followed by Raf-1, MEK1/2, and ERK1/2 cascade. This pathway predominantly mediates the mitogenic effects of insulin through regulation of protein synthesis and proliferative gene expression.
1.2.4.1. The PI3K-Akt pathway

Phosphoinositide-3 kinases (PI3Ks) are a diverse family of lipid kinases. The class IA PI3Ks, consisting of a p85α regulatory subunit and a p110α catalytic subunit, mediate the biological actions of insulin (316). Activation of PI3K catalyzes the formation of phosphatidylinositol-3,4,5-phosphates (PIP₃), which is required for recruiting Akt to the plasma membrane and activating 3-phosphoinositide-dependent protein kinase 1 (PDK1), a kinase responsible for Akt activation (313). Regulation of PI3K activity is facilitated by PTEN (phosphatase and tensin homology) and SHP2 (SH2-containing inositol 5’-phosphatase-2) phosphatases, which dephosphorylate PIP₃ into PIP₂ (313). The regulatory subunit P85α and its closely related isoforms p55α and p50α are all encoded by the same gene, Pik3r1, and disruption of this gene results in early postnatal lethality (317). In contrast, heterozygous Pi3kr1 mice display improved insulin sensitivity in mice, through an undetermined mechanism (318). Finally, homozygous mice that are deficient in the catalytic subunit, p110α, die embryonically, but heterozygous mice are glucose tolerant, despite fasting hyperinsulinemia and decreased insulin action in target tissues (319, 320).

Akt consists of three isoforms that are expressed in variety of tissues. Of the three isoforms, Akt2 appears to be required for glucose homeostasis, as Akt2 knock out mice have impairments in insulin action in skeletal muscle and the liver (321). In addition to phosphorylation by PDK1 on Thr308 in the catalytic domain, phosphorylation at Ser473 in the regulatory domain by PDK2/mTOR-Rictor is required for full activation (322, 323). Akt activity is essential for insulin-associated metabolic effects: inhibition of hepatic gluconeogenesis through inhibition of FOXO1 activity in the liver (324, 325), promoting glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3) (326), increasing protein synthesis through the actions of the mammalian target of rapamycin (mTOR) on p70 ribosomal S6 kinase and the translation initiation factor, 4E-BP1 (327), and
GLUT4 translocation and insertion into the plasma membrane for glucose transport (313). Moreover, Akt activity is also required to phosphorylate and inhibit the Rab GAP AS160 to allow for GLUT4 vesicle translocation to the plasma membrane (328).

1.2.4.2. The mitogen-activated protein kinase pathway

Extracellular-regulated kinase-1/2 (ERK1/2) belongs to the family of mitogen-activated protein kinases (MAPKs), which also includes p38 MAPK and c-jun N-terminal kinase /stress-activated protein kinase (JNK/SAPK). All MAPKs are activated through a protein phosphorylation cascade such that MAPK kinase kinases (MAP3Ks) phosphorylate MAPK kinases on serine or threonine residues, followed by tyrosine and threonine phosphorylation of MAPKs by MAPK kinases (329). Of the three MAPK pathways, activation of the MEK1/2-ERK1/2 pathway by insulin is best characterized.

In the MEK1/2-ERK1/2 pathway, Raf-1 (c-Raf) has been identified as the MAP3K responsible for ERK1/2 activation. Following IR phosphorylation, IRS molecules or the adaptor protein, Shc, recruit Grb2/SOS to the plasma membrane. SOS is a guanine nucleotide exchange factor (GEF) that exchanges GTP for GDP on Ras, which leads to the downstream activation of Raf-1 (330-333). Raf-1, in turn, phosphorylates and activates MAPK/ERK1/2 kinase (MEK1/2) on Ser^{217} and Ser^{221} (334). In vitro studies have demonstrated that MEK1/2 is the dedicated kinase for ERK1/2, as kinases structurally-related to ERK1/2 are poor substrates of MEK1 (335). ERK1/2 activation is dependent on the sequential phosphorylation of Tyr^{185} followed by Thr^{183}, in the activation loop, which opens the active site of the kinase (336). Ultimately, insulin-induced activation of ERK1/2 regulates gene expression via the transcription factor Elk-1, as well as protein synthesis through RS6K1 (337-339), and its effects on growth have recently been suggested to be due to enhancement of growth hormone-induced effects (340).
Deletion of Raf-1, MEK1, or ERK2 results in embryonic lethality (341-343), but the viability of ERK1 knockout mice has allowed some insight into its metabolic function in vivo. In ERK1 knockout mice, ERK2 expression is upregulated as a compensatory mechanism for ERK1 deficiency (344), although these mice demonstrate fewer adipocytes than wild type counterparts, in addition to resistance to diet-induced obesity and improved insulin sensitivity (345).

1.2.5. Insulin signaling and actin remodeling

One of the best-established actions of insulin is the stimulation of GLUT4 translocation and insertion into the plasma membrane in skeletal muscle and adipocytes to promote glucose uptake. While numerous studies have demonstrated that this process is dependent on the activation of the PI3K-Akt pathway, it has become evident that insulin-stimulated actin remodeling is also a pre-requisite event (346-348). In skeletal muscle and adipocytes, actin remodeling is dependent on the activation of the Rho GTPases, which include Cdc42, Rac1, and TC10 (349, 350). Studies on these tissues and cells have demonstrated that PI3K promotes Cdc42 and Rac1 activation, which suggests a bifurcation point at the level of PI3K: actin remodeling via Rho GTPases and GLUT4 translocation by Akt activation (Figure 1-8) (351, 352).

Following insulin treatment of myotubes or adipocytes, Rho GTPases are activated by GEF proteins. In contrast, GAPs or guanine-nucleotide dissociation inhibitors (GDIs) catalyze GTP to GDP or prevent GDP to GTP exchange, respectively, to inhibit Rho GTPase activity (353). With respect to PI3K-induced activation of Cdc42 and Rac1, the interaction of the p85 regulatory subunit promotes Cdc42 activation while binding of PIP3 to GEF protein complexes promotes Rac1 activity (354-356). As there are numerous GEF proteins, it is possible that insulin may also activate Cdc42 and/or Rac1 through a PI3K-independent
Figure 1-8: Pathways mediating insulin-stimulated actin remodeling.

Insulin induces the activation of the phosphatase, slingshot 1 (SSH1). SSH1 then de-phosphorylates and activates the actin-severing enzyme, cofilin. Insulin also promotes actin polymerization through the Rho GTPases, Cdc42, Rac1, and/or TC10. Activation of Cdc42 or Rac1 is mediated by an unidentified GEF. Conversely, CAP-dependent activation of Cbl promotes its translocation to lipid rafts where it interacts with the CrkII-C3G complex to activate TC10. Downstream of the Rho GTPases is the serine/threonine kinase PAK1, which activates LIM kinase, an inhibitor of cofilin. Insulin-regulated actin polymerization is also facilitated by Rho GTPase-dependent activation of the Arp2/3 complex via WASP/WAVE proteins.
mechanism, although such data has yet to be reported. Finally, in adipocytes, TC10 is activated following the recruitment and phosphorylation of Cbl to the plasma membrane by the adaptor protein CAP. Activated Cbl then translocates to lipid rafts where it interacts with the adaptor protein CrKII and the GEF, C3G, to activate TC10, thereby promoting actin remodeling and GLUT4 insertion to the plasma membrane (349, 352, 357). Although TC10 is also expressed in skeletal muscle, its role in GLUT4 translocation and glucose uptake is currently unclear, as conflicting results have been reported (352, 358).

Actin remodeling is a balance of actin depolymerization and polymerization. Downstream of the Rho GTPases are Wiskott-Aldrich Syndrome Protein (WASP) and WASP family Verpoline-homologous (WAVE) proteins, which facilitate Rho GTPase-induced actin polymerization through interaction with Actin-related proteins 2/3 (Arp2/3) that are responsible for actin nucleation (359). Overexpression of a dominant-negative WASP mutant prevents insulin-stimulated actin polymerization and GLUT4 translocation (349, 358, 360). However, a recent study has also demonstrated that insulin activates the phosphatase Slingshot (SSH1), which promotes the activity of cofilin by dephosphorylating Ser³ (361). Insulin has been further demonstrated to activate PAK1, a serine/threonine kinase downstream of Cdc42, Rac1, and TC10, that has recently been identified as a mediator of insulin-induced changes in actin morphology (362-364). Through Rho GTPase-mediated activation of PAK1, activation of LIM kinase leads to the phosphorylation of the actin-severing protein cofilin on Ser3, which inhibits its activity (362, 365). Collectively, these findings demonstrate that insulin may promote actin reorganization through multiple effects, including both depolymerization and polymerization.
1.2.6. Insulin signaling during pathophysiological conditions

Insulin resistance can be defined as decreased responsiveness to insulin in a target tissue. Insulin resistance in vivo is associated with hyper-insulinemia, -glycemia, and -lipidemia, in addition to elevations in circulating adipokines such tumor necrosis-factor alpha (TNF-α) (366-368). These diverse factors are all able to induce insulin resistance via different mechanisms, but it appears that they induce defects primarily at the level of the IR or IRS molecules (Figure 1-9) (366-368).

The IR is the most proximal component in the signaling pathway, and regulation of its function and expression are altered through diverse mechanisms. Hyperinsulinemia directly reduces biosynthesis of IR mRNA and protein (369-371), and recently, it has been determined that IR expression is regulated through a FOXO1-dependent mechanism (371). Furthermore, insulin receptor expression is also regulated by increased degradation through the ubiquitin-proteasome pathway (372). Autophosphorylation of the insulin receptor is a key event that coordinates activation of downstream signaling pathways, and numerous proteins that inhibit this process have been identified. Plasma cell membrane glycoprotein-1 (PC-1/ENPP-1) is upregulated in insulin resistance and type 2 diabetes (373), and following binding to the connecting domain of the insulin receptor, PC-1 induces a conformational change that prevents autophosphorylation of tyrosine residues in the beta subunit (373). Tyrosine phosphatases such as PTP-1B and SHP-1 have also been implicated in insulin resistance, and their expression is increased by the adipokine, TNF-α (374). The regulatory role of these enzymes in insulin signaling has been recently elucidated, such that knockout of either protein enhances insulin sensitivity in rodents (375, 376),

Phosphorylation of different serine residues on the IR is associated with decreased insulin-induced autophosphorylation or downstream phosphorylation of IRS molecules
Multiple defects in insulin signaling have been demonstrated in type 2 diabetes and insulin resistance. At the level of the insulin receptor, binding of plasma cell membrane glycoprotein-1 (PC-1/ENPP-1) to the insulin receptor alpha subunit prevents autophosphorylation of the receptor. Furthermore, phosphorylation of serine residues (black-filled circles) on the beta subunit prevents docking of IRS molecules with the insulin receptor. The activity of phosphatases such as PTP1B and SHP1 and expression of suppressor of cytokine signaling-3 (SOCS-3) molecules impair insulin signaling by dephosphorylating or binding to tyrosine residues, respectively, preventing IRS molecules from interacting with the insulin receptor. As with the insulin receptor, the activity of IRS molecules is decreased via serine phosphorylation (filled circles) by numerous intracellular kinases. However, in contrast, SOCS-3 binding to IRS promotes its ubiquitination, leading to degradation via the 26S proteasome.
In obesity-related insulin resistance, the activity of PKCs is increased as a result of elevated intracellular lipid content or hyperglycemia (374, 378), which leads to phosphorylation of serine residues in the IR beta subunit (377, 379). Additionally, serine phosphorylation of the IR is mediated by circulating adipokines such as TNF-α via a p38 MAPK-dependent manner (380). Lastly, suppressors of cytokine signaling (SOCS)-1 and -3 are two related proteins that are up-regulated in the presence of adipokines, such as TNF-α and interleukin-6 (IL-6), or chronic exposure to insulin. Binding to the IR at Tyr\textsuperscript{960} via their SH2 domains prevents IRS molecules from interacting with the insulin receptor and becoming phosphorylated due to steric hindrance (381, 382).

As previously mentioned, the IR exists as two isoforms with different affinities for insulin. Early studies that utilized semi-quantitative RT-PCR to measure mRNA transcripts for IR-A and IR-B found no differences in expression in skeletal muscle mRNA from control subjects and those with type 2 diabetes (383). However, later studies using \textsuperscript{125}I-insulin binding assays and Western blotting determined that muscle and fat from subjects with type 2 diabetes expressed higher levels of the lower affinity isoform, IR-A (384, 385). Furthermore, increases in insulin-insensitive IR/IGF-1R hybrids have been detected in skeletal muscle and adipose tissue from subjects with type 2 diabetes (386, 387).

IRS1 is a key mediator of insulin action, and reductions in IRS1 protein levels are associated with insulin resistance and type 2 diabetes (388). Reduced IRS1 activity is attributed to phosphorylation of serine residues, namely Ser\textsuperscript{307} in rodents and Ser\textsuperscript{312} in humans (389). Numerous causes of IRS-1 serine phosphorylation have been identified, including hyperinsulinemia, over-nutrition, stress, hyperlipidemia, hyperinsulinemia, increased TNF-α, and obesity (390, 391). In rodents, phosphorylation of Ser\textsuperscript{307} prevents IRS1 from interacting with the IR and, as a result, uncouples IRS1 from PI3 kinase (392, 393). Insulin itself promotes Ser\textsuperscript{307} phosphorylation as a negative feedback mechanism
involving PI3 kinase activation of other downstream kinases including PKC\textsubscript{c}, IKK\textbeta{}, JNK, mTOR, and S6K1 (394), and IRS1 and IRS2 expression is regulated by a PI3K-dependent mechanism, which promotes degradation through the ubiquitin-proteasome pathway (395, 396). 14-3-3 proteins are a ubiquitous group of proteins that bind to phosphorylated serine or threonine residues and act as molecular scaffolds that regulate protein-protein interactions (397). While there are no reports of increased 14-3-3 protein expression in insulin resistance or obesity, the increase in serine phosphorylation of IRS1 molecules could promote 14-3-3 binding, thereby sequestering IRS1 in the cytosol and uncoupling the insulin signaling pathway (398-400). Finally, as with the IR, SOCS-3 binds to IRS molecules, which prevents tyrosine phosphorylation, and a recent study has also demonstrated that SOCS-3 can reduce IRS1 and IRS2 expression by promoting their ubiquitination and subsequent degradation (401).

Defects in insulin signaling are primarily attributed to the PI3K-Akt pathway and not the MEK1/2-ERK1/2 pathway (366, 402), and modulation of PI3K activity occurs during insulin resistance through distinct mechanisms. Under normal physiological conditions, there exists a ratio between free p85\textalpha{} and the p85\textalpha{}-p110 heterodimer, but increased expression of free p85\textalpha{} and its related isoforms has been reported in insulin resistance and type 2 diabetes (402). Since free p85\textalpha{} and the heterodimer compete for the same phosphorylated tyrosine residues on IRS molecules, increasing or decreasing p85\textalpha{} would, therefore, impair or enhance insulin-stimulated activation of PI3K and downstream actions of insulin, respectively (403, 404). Furthermore, PTEN expression is increased in insulin resistance (405), and since PTEN inactivates PIP\textsubscript{3} by dephosphorylating the phospholipid, alterations in the activity of the phosphatase could contribute to insulin resistance. Consistent with this notion, mice with a genetic deletion of PTEN in skeletal muscle exhibit increased insulin sensitivity and resistance to diet-induced obesity and insulin resistance.
When taken together, numerous mechanisms contribute to cellular insulin resistance, which decreases insulin action. As there is a paucity of literature as to whether any of these mechanisms contribute to impaired post-prandial GLP-1 secretion in insulin resistance and type 2 diabetes, further work is required to examine L cell-specific insulin signaling under pathophysioloical conditions.

1.3. Hypothesis

Impaired meal-induced GLP-1 secretion in humans occurs in type 2 diabetes and insulin resistance. The mechanism behind this defect in GLP-1 secretion is not known; however, diet-induced leptin resistance is associated with attenuated oral nutrient-induced GLP-1 secretion, which suggests that circulating hormonal factors are able to alter the responsiveness of the L cell to secretagogues. I, therefore, hypothesize that insulin modulates GLP-1 secretion from the intestinal L cell and, furthermore, that insulin resistance directly impairs the function of the endocrine L cell. This hypothesis will be examined in this thesis with the use of well-characterized L cell models and rodent models of insulin resistance to determine whether insulin and insulin resistance modulate GLP-1 release from the L cell and if so, to establish the intracellular signaling pathways by which insulin acts in the L cell.
CHAPTER 2: INSULIN REGULATES GLUCAGON-LIKE PEPTIDE-1 SECRETION FROM THE ENTEROENDOCRINE L CELL

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Author contributions:
Studies involving the role of insulin in fetal rat intestinal cell cultures and human NCI-H716 cells were conducted by the undergraduate project students G.J. Huang and N. Flora, respectively, while under the direct supervision of G. Lim. G.J. Huang generated data for figure 2-9, while N.Flora generated data for figures 2-5 and 2-6. Transgenic mice and adenoviruses were provided by D. LeRoith and C.J. Rhodes, respectively. All data analysis and writing were completed by G. Lim.
2.1. Abstract

Insulin resistance and type 2 diabetes mellitus are associated with impaired post-prandial secretion of glucagon-like peptide-1 (GLP-1), a potent insulinotropic hormone. The direct effects of insulin and insulin resistance on the L cell are unknown. I, therefore, hypothesized that the L cell is responsive to insulin and that insulin resistance impairs GLP-1 secretion. The effects of insulin and insulin resistance were examined in well-characterized L cell models: murine GLUTag, human NCI-H716, and fetal rat intestinal cells. MKR mice, a model of chronic hyperinsulinemia, were used to assess the function of the L cell in vivo.

In all cells, insulin activated the PI3 kinase-Akt and MEK-ERK1/2 pathways and stimulated GLP-1 secretion by up to 275 ± 58%. Insulin resistance was induced by 24 hr pretreatment with 10^{-7} M insulin, causing a marked reduction in activation of Akt and ERK1/2.

Furthermore, both insulin-induced GLP-1 release and secretion in response to glucose-dependent insulinotropic peptide and phorbol-12-myristate-13-acetate, were significantly attenuated. While inhibition of PI3 kinase with LY294002 potentiated insulin-induced GLP-1 release, secretion was abrogated by inhibiting the MEK-ERK1/2 pathway with PD98059 or by over-expression of a kinase-dead MEK1-ERK2 fusion protein. Compared to controls, MKR mice were insulin resistant and displayed significantly higher fasting plasma insulin levels. Furthermore, they had significantly higher basal GLP-1 levels, but displayed impaired GLP-1 secretion following an oral glucose challenge. These findings indicate that the intestinal L cell is responsive to insulin and that insulin resistance in vitro and in vivo is associated with impaired GLP-1 secretion.
2.2. Introduction

Several reports have indicated that post-prandial or nutrient-induced GLP-1 secretion is impaired in subjects with type 2 diabetes, independent of obesity, suggesting a role for this peptide in the etiology and/or pathophysiology of this disease (3, 135, 226, 229, 230, 233). As the rate of clearance of bioactive GLP-1 between normal subjects and those with type 2 diabetes is not different (233), these findings suggest a secretory defect in the L cell. DPP-IV is the enzyme responsible for degrading GLP-1, and to date, there is no clear evidence that indicates the activity of DPP-IV between healthy subjects and those with type 2 diabetes is altered (231, 232, 241), which further suggests a secretory defect.

Rask et al. have demonstrated that insulin resistance is negatively correlated with GLP-1 secretion in a cohort of normal male subjects with varying degrees of insulin sensitivity (6, 260). The notion that impaired insulin action reduces secretion by the endocrine intestine is not without precedence, as release of the orexigenic hormone, ghrelin, is also decreased in non-obese subjects with insulin resistance (407). Yi et al recently demonstrated that the L cell is sensitive to insulin, as demonstrated by increased proglucagon expression and GLP-1 synthesis (7), but the effect of insulin on GLP-1 release was not examined. Therefore, I hypothesized that insulin modulates GLP-1 secretion from the intestinal L cell and that insulin resistance in the L cell directly impairs the secretion of GLP-1. These hypotheses were tested with the use of several well-characterized models of GLP-1 secretion; murine GLUTag cells, human NCI-H716 cells, and fetal rat intestinal cultures (136, 138, 145, 178, 197). Additionally, non-obese, insulin resistant MKR mice were used to examine the effects of chronic hyperinsulinemia on GLP-1 secretion from the enteroendocrine L cell in vivo (408, 409).
2.3. Materials and Methods

2.3.1. In vitro models

The mouse L cell model, GLUTag, was generated from a large bowel tumor in mice carrying a proglucagon/simian virus 40 large T-antigen transgene, while human NCI-H716 L cells were derived from a poorly differentiated adenocarcinoma of the cecum (138, 178, 197). Both cell models were grown in high glucose media, unless otherwise indicated. Fetal rat intestinal cell (FRIC) cultures are a heterogeneous primary L cell model, cultured from fetal intestines collected from term pregnant Wistar rats, as previously described in detail (136, 145, 178, 197). Furthermore, GLUTag cells also release CCK, while FRIC cultures have been shown to secrete PYY and somatostatin, albeit at levels insufficient to alter L cell secretion (410-412).

For all in vitro studies, experiments were performed on different batches of cultures to ensure reproducibility of results. Insulin resistance (InsRes) was induced by a 24 hr pretreatment with media containing 10^{-7} M insulin (Eli Lilly, Toronto, ON, Canada). Following pretreatment, cells were washed for 3 x 40 min with media containing 1% bovine serum albumin before addition of test agents for 2 hr. Previous studies have demonstrated that similar conditions are sufficient to decrease insulin action in adipocytes and myotubes (347, 413-416). This protocol did not alter total GLP-1 content in any cell model (data not shown).

To study GLP-1 secretion, cells were washed with HBSS and treated with insulin, IGF-1 (Long R3 IGF-1, Novozymes GroPep, Adelaide, Australia), GIP (10^{-6} M; Bachem Inc, Torrance, CA - positive control), or phorbol 12-myristate 13-acetate (PMA, 10^{-6} M; Sigma-Aldrich - positive control), and treatments were prepared, as previously described (138, 178, 197). Some cells were also pre-treated with the pharmacological inhibitors, LY294002 (Sigma-Aldrich) or PD98059 (Calbiochem, La Jolla, CA; each at 50 μM) for 15 min. After the 2 hr treatment,
peptides in supernatants or cell extracts were collected by reversed-phase extraction, as previously described (125, 136, 138, 178, 197).

Total GLP-1 was assayed in cell and medium samples by radioimmunoassay with a GLP-1 antiserum (Affinity Research Products, Nottingham, UK) that targets the carboxy-terminus of GLP-1<sup>7-36NH<sub>2</sub></sup>, as previously described (125, 136, 138, 178, 197). Secretion was expressed as the total amount of GLP-1 in the medium, normalized to the total cell content of GLP-1 (media plus cells) and expressed as a percent of control. Basal media content and cell content in GLUTag cells were 28.3 ± 2.8 pg/ml and 273.1 ± 76.9 pg/ml (n=17), respectively. In NCI-H716 cells, basal media content and cell content were 40.5 ± 8.1 pg/ml and 5201.7 ± 1274.3 pg/ml (n=18), respectively, while in FRIC cultures, basal media content and cell content were 50.6 ± 9.8 pg/ml and 394.3 ± 56.1 pg/ml (n=8), respectively. The cause of differences in basal secretion among cell models is not known, but may be attributed to species differences and/or specific culture conditions for each model. Nonetheless, all cell models release GLP-1 appropriately in response to a variety of known secretagogues, making them ideal models to study GLP-1 secretion from the L cell (as described in chapter 1.1.8.).

2.3.2. In vivo studies

MKR mice (a kind gift from Drs. D. LeRoith, New York, NY and M.B. Wheeler, Toronto, ON) were generated by the expression of a dominant-negative IGF-1 receptor in skeletal muscle, resulting in insulin resistance not only in skeletal muscle, but also in fat and liver (408, 409). In all experiments, age- and sex-matched FVB mice (Charles River, St. Laurent, QC), were used as wild type controls. All mice were housed four per cage with ad libitum access to food and water under a 12:12 light:dark cycle. At 11 weeks of age, insulin sensitivity was assessed by intraperitoneal insulin tolerance test (1 U/kg) following a 3 hr fasting period, and blood glucose concentrations were determined with a glucometer (OneTouch Ultra,
LifeScan Canada, Burnaby, BC). After two days of recovery, mice were fasted for 24 hr and divided into two groups for the measurement of basal, fasting glucose, insulin, and GLP-1 levels at t=0 and 10 min after an oral glucose load. Pilot studies with 1.5 g/kg and 3 g/kg glucose loads did not elicit detectable responses in GLP-1 secretion; as such, 6 g/kg (10 μl/g gavage volume) was used for all experiments. Mice from each group were anesthetized with isofluorane and immediately exsanguinated by cardiac puncture. Blood was collected into Trasylol, EDTA, and Diprotin-A, and plasma was collected and stored at -80°C (197). Plasma levels of insulin were measured by ELISA (Crystal Chem, Downers Grove, IL). Bioactive GLP-1<sup>1-36NH<sub>2</sub></sup> was measured using an ELISA (Meso Scale Discovery, Gaithersburg, MD) that is specific to GLP-1<sup>1-36NH<sub>2</sub></sup>, and does not recognize the closely related GLP-1<sup>1-37</sup> or the degradation product GLP-1<sup>9-36NH<sub>2</sub></sup>. The detection limit of this assay was 2 pg/ml. As no significant differences in any of these biochemical parameters were detected between male and female MKR or FVB mice, with the exception of slightly reduced basal blood glucose levels in FVB females, all data were combined for analysis. All animal procedures were approved by the Animal Care Committee of the University of Toronto.

### 2.3.3. Immunohistochemistry

Sections of mouse ileum or jejunum were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut to 5 μm thickness. Mouse and 5 μm human jejunal sections (a kind gift from Dr. S.L. Asa, Toronto, ON) and all cell models were subjected to immunohistochemistry or –cytochemistry, as previously described (138, 178, 197, 417). In brief, tissues and cells were incubated overnight at 4 °C with primary antibodies against GLP-1 (mouse anti-human, a generous gift from Dr. D.A. D’Alessio, Cincinnati, OH), the alpha- or beta-subunits of the insulin receptor (rabbit anti-mouse, Santa Cruz Biotechnology, Santa Cruz, CA), or PIP₃ (mouse anti-PIP₃, Echelon Biosciences, Salt Lake City, UT). The absence of primary antisera
was used as a negative control. Furthermore, mouse and human intestinal sections were examined in the presence of only the secondary fluorescent-labelled secondary antibodies for the insulin receptor and GLP-1 to examine the contributions of each secondary to non-specific staining. To further test the specificity of the insulin receptor antibodies, pancreatic sections from wild-type and beta cell-specific insulin receptor knock out (βIRKO) mice (a kind gift from Dr. R.N. Kulkarni, Boston, MA) were also stained for the insulin receptor using the insulin receptor α-subunit antibody. Images were visualized with a Carl Zeiss Axioplan Deconvolution Microscope (Carl Zeiss Canada, Ltd., Don Mills, ON).

2.3.4. RNA isolation and semi-quantitative RT-PCR

Total RNA extracted from white adipose tissue of CD1 mice, GLUTag and NCI-H716 cells (RNeasy kit, Qiagen Inc., Mississauga, ON), along with human jejunal (Ambion, Austin, TX), and human placental RNA (a generous gift from Dr. J.R.G. Challis, Vancouver, BC) were reverse-transcribed and amplified with a OneStep RT-PCR kit (Qiagen Inc.). As a negative control, template RNA was replaced with RNase-free water. Identification of mouse and human insulin receptor transcripts was based on primers and conditions reported to generate PCR products of 500 bp or 600 (isoform A)/636 (isoform B) bp, respectively (301, 418). Primers used to amplify IGF-1 receptor mRNA transcripts in mouse and human RNA samples generated PCR products of 71 bp and 300 bp, respectively (419, 420).

2.3.5. Construction of adenoviruses and adenoviral infection

Recombinant adenoviruses expressing green fluorescent protein (GFP) or a dominant-negative form of ERK-2 (Ad-MEK1-ERK2 KR) (a kind gift from Dr. C.J. Rhodes, Chicago, IL) (421), were generated and purified as previously described (422, 423). Adenoviral titers were determined by infecting GLUTag cells (~70% confluency in 6-well plates) with Adv-GFP or Adv-MEK1-ERK2 KR (0.5 - 5.0 x 10⁹ PFU/ml) for 2 hr. After viral infection, media was
replaced with complete growth medium, and cells were incubated for a further 48 hr prior to extraction for western blot of phospho- and total ERK1/2. For secretion experiments, cells were infected, as above, with 5.0 x 10^9 PFU/ml of the constructs, followed by treatment with GLP-1 secretagogues, as above.

2.3.6. **Cell lysis, immunoprecipitation, and immunoblotting**

Following treatment with insulin, IGF-1, pharmacological inhibitors, and/or adenovirus constructs, cell lysates were subjected to insulin receptor immunoprecipitation and/or Western blot analysis, as previously described (138, 178, 197, 418). Proteins of interest were detected with antibodies targeted against phospho-tyrosine (clone 4G10, 1:1000; Upstate, Lake Placid, NY); insulin receptor (β-subunit, 1:400; Calbiochem); phospho-Akt (Ser^473^), Akt, phospho-ERK1/2 (Thr^{202} / Tyr^{204} ), ERK1/2 (1:1000; Cell Signaling, Danvers, MA); and actin (1:5000; Sigma-Aldrich).

2.3.7. **Statistical analysis**

All data are expressed as mean ± SEM. In some experiments, data were log10 transformed to normalize variance for statistical analysis. Data were analyzed by Student’s t-test or by one- or two-factor ANOVA, followed by appropriate post-hoc testing (SAS, Cary, NC). Factors for ANOVA analysis consisted of the effects of doses of insulin or IGF-1 for one-way analyses and the effects of glucose, insulin, 24 hr insulin pretreatment, LY294002, PD98059, adenovirus infections, or genotype for two-way analyses. Statistical significance was assumed at p<0.05.
2.4. Results

2.4.1. Expression of the insulin receptor in L cells

Both mRNA transcripts and protein for the insulin receptor were detected in GLUTag and NCI-H716 cells, as determined by semi-quantitative RT-PCR and immunocytochemistry (Figure 2-1A). To establish whether primary L cells also express the insulin receptor, double-immunofluorescence staining for the insulin receptor and GLP-1 was performed on sections of mouse ileum and human jejunum and on FRIC cultures. Positive immunoreactivity for the insulin receptor α-subunit was observed on all cells along the crypt-villus axis in the mouse and human intestinal epithelium (Figure 2-1Bd,j); moreover, positive staining for the insulin receptor was also observed along the basolateral membrane in human jejunal sections (Figure 2-1Bj and Figure 2-2A), but not in beta cells lacking the insulin receptor (βIRKO mice, negative control, Figure 2-3). Pancreatic sections from βIRKO mice were used because analysis of DNA by single cell PCR has determined that beta cells from these mice completely lack DNA for the insulin receptor (264). The presence of positive immunoreactivity within βIRKO islets may represent glucagon-containing alpha cells (424). Furthermore, L cells that stained positive for GLP-1 were also found to express insulin receptor alpha-subunit protein (Figure 2-1B). When an antiserum against the insulin receptor beta-subunit was used, a similar colocalization of the insulin receptor protein and GLP-1 was found (Figure 2-2B).

2.4.2. Insulin signaling and actions on the mouse L cell

Treatment of GLUTag cells with $10^{-7}$ M insulin for 5 min induced a 21.9-fold increase in insulin receptor phosphorylation (Figure 2-4A), in addition to a robust increase in cytosolic PIP$_3$, demonstrating PI3 kinase activation (Figure 2-1C). No change in total insulin receptor protein was observed (Figure 2-4B); however, Akt and ERK1/2 phosphorylation were increased by 3.4-
Figure 2-1: Expression of the insulin receptor and activation of PI3 kinase in L cells.

(A): Semi-quantitative RT-PCR analysis for insulin receptor mRNA transcripts in GLUTag (a: lane 2) and NCI-H716 cells (c: lane 2). Mouse fat (a: lane 3) and human placenta (c: lane 3) were used as positive controls, respectively, while the omission of template RNA was used as negative controls (a: lane 1, c: lane 1). Murine GLUTag (b) and human NCI-H716 (d) cells were subjected to immunofluorescent staining for the insulin receptor β-subunit (red); DAPI (blue) was used to visualize nuclei. Arrowheads indicate L cells, and arrows indicate basolateral staining for the insulin receptor β-subunit. Colocalization of the insulin receptor and GLP-1 is indicated by yellow in the overlay (f,l,r). (B): Mouse (a-f) and human (g-l) intestinal sections and FRIC cultures (m-r) were subjected to double-immunofluorescent staining for the insulin receptor α-subunit (green) and GLP-1 (red); DAPI (blue) was used to visualize nuclei. The absence of primary antisera was used as negative controls (mouse: a-c; human: g-i; FRIC: m-o). Arrowheads indicate L cells, and arrows indicate basolateral staining for the insulin receptor α-subunit. Colocalization of the insulin receptor and GLP-1 is indicated by yellow in the overlay (f,l,r). (C): Immunofluorescent detection of PIP_3 production (red) in response to treatment of GLUTag cells with media alone (a) or 10^{-7} M insulin (b) for 5 min. Identical camera exposure times were used. (scale bars = 10 μm)
Figure 2-2: Confirmation of insulin receptor immunoreactivity in L cells.

(A): Mouse and human intestinal sections were stained for the insulin receptor α-subunit alone, followed by secondary antibodies for insulin receptor (green) and GLP-1 (red) antisera; DAPI (blue) was used to visualize nuclei. Arrows indicate basolateral staining for the insulin receptor α-subunit. (B): Antiserum for the insulin receptor β-subunit (green) was used in addition to an antibody for GLP-1 (red) on mouse and human intestinal sections. DAPI (blue) was used to visualize nuclei. Insulin receptor α-subunit immunoreactivity was localized to the cell membrane and expressed diffusely throughout the cytoplasm. Arrowheads indicate L cells that express the insulin receptor. (scale bars= 10 μm)
Figure 2-3: Specificity of the insulin receptor antibody.

Pancreatic sections from wild-type and βIRKO mice were subjected to immunofluorescent staining for the insulin receptor α-subunit (red); DAPI (blue) was used to visualize the nuclei. In βIRKO mice, which do not express the insulin receptor in pancreatic beta cells, a decrease in positive immunoreactivity was observed when compared to wild-type mice. Identical exposure times were used for all images. (scale bars= 10 μm)
Figure 2-4: Effects of insulin and insulin resistance on murine GLUTag cells.

Cells were exposed for 24 hr to either media alone or to high insulin to induce insulin resistance and, following 3 x 40 min washes with serum-free media containing 1% BSA, were treated acutely with $10^{-7}$M insulin for 5 min for Western blot analysis or with the indicated concentrations of insulin or GIP for 2 hr to measure GLP-1 secretion by radioimmunoassay. (A-D): Crude protein lysates or immunoprecipitated protein extracts were subjected to immunoblot analysis for (A) insulin receptor phosphorylation (n=3 per group), (B) total insulin receptor expression (n=4 per group), (C) Akt phosphorylation (n=4 per group), or (D) ERK1/2 phosphorylation (n=7 per group). Representative blots are shown, and all values were expressed relative to the untreated control. (*: p<0.05, **: p<0.01, ***: p<0.001 when compared to untreated control. #: p<0.05 as indicated). (E): GLP-1 secretion in response to medium alone (control), insulin (10^{-8} M), and GIP (10^{-6} M) in the presence of 5 or 25 mM glucose. GLP-1 secretion was determined by radioimmunoassay, and all data were expressed as a percent of the untreated control (n=3-4 per group; *: p<0.05, **: p<0.01) (F): GLP-1 secretion in response to medium alone (control), insulin, or GIP from normal (open bars) or insulin resistant (closed bars) GLUTag cells. GLP-1 secretion was determined by radioimmunoassay, and all data were expressed as a percent of the untreated control (n=5-6 per group). (*: p<0.05, **: p<0.01, ***: p<0.001 when compared to untreated control. #: p<0.05, ##: p<0.01, ###: p<0.001 as indicated.)
and 2.5-fold (p<0.05), as determined by Western blot analysis (Figure 2-4C,D). In preliminary studies (Figure 2-4E), it was observed that GLUTag cells required high glucose (25 mM) conditions in order to demonstrate insulin-induced GLP-1 secretion (p<0.05), and all subsequent experiments with these cells were, therefore, performed under these conditions. In response to an acute treatment of GLUTag cells with 10^{-11}-10^{-6} M insulin, a hyperbolic pattern of GLP-1 secretion was observed, such that 10^{-9}-10^{-7} M insulin significantly stimulated GLP-1 release, to 144.5 ± 9.1% of control (p<0.05), while 10^{-6} M insulin significantly inhibited GLP-1 secretion, to 45.6 ± 3.1% of control (p<0.001) (Figure 2-4F), suggestive of IR desensitization. GIP, used as a positive control for the rodent L cell, increased GLP-1 secretion to 286.5 ± 13.8% (p<0.001). However, no additive effects of insulin and GIP on GLP-1 release were detected (data not shown).

Insulin resistance was induced in GLUTag (InsRes-GLUTag) cells by 24 hr pretreatment with media containing 10^{-7} M insulin. Insulin-induced insulin receptor phosphorylation in InsRes-GLUTag cells was decreased from 21.9-fold to 7.5-fold as a result of the pretreatment, and total insulin receptor protein levels were decreased by 64.5 ± 4.0% (p<0.001) (Figure 2-4A,B). Moreover, in InsRes-GLUTag cells, insulin-induced Akt phosphorylation was decreased to 1.6-fold (p<0.05) and insulin-induced ERK1/2 phosphorylation was completely abolished (Figure 2-4C,D). A small but significant 13.4 ± 2.6% decrease in basal GLP-1 release was observed in InsRes-GLUTag cells (p<0.05), while the stimulatory effect of insulin on GLP-1 secretion was completely abrogated. Unexpectedly, insulin resistance also caused a significant decrease in GIP-mediated GLP-1 release as compared to normal GLUTag cells (from 286.9 ± 13.8% to 202.5 ± 24.3%, p<0.001) (Figure 2-4F).
2.4.3. Insulin and the human L cell

To confirm the findings in the murine GLUTag L cell model, human NCI-H716 cells were similarly treated with $10^{-7}$ M insulin, resulting in 7.6- and 3.0-fold increases in Akt and ERK1/2 phosphorylation, respectively ($p<0.05-0.01$) (Figure 2-5A,B). Increased GLP-1 secretion was also observed following acute treatment with insulin, whereby concentrations greater than $10^{-8}$ M insulin significantly stimulated GLP-1 release by up to $275.2 \pm 57.6\%$ of control ($p<0.05$) (Figure 2-5C). As found in InsRes-GLUTag cells, InsRes-NCI-H716 cells displayed a marked impairment in activation of downstream insulin signaling pathways after 24 hr pretreatment with $10^{-7}$ M insulin, such that insulin-induced Akt and ERK1/2 phosphorylation were reduced to 3.0- and 1.7-fold ($p<0.05$), respectively. These defects were accompanied by an inability of insulin to stimulate GLP-1 secretion (Figure 2-5A-C). Although GIP does not enhance GLP-1 secretion from the human L cell, heterologous desensitization was observed in InsRes-NCI-H716 cells in response to the known secretagogue PMA, decreasing GLP-1 secretion from $409.3 \pm 68.2\%$ to $189.4 \pm 15.4\%$ of controls ($p<0.001$).

2.4.4. Activation of the insulin-like growth factor-1 receptor does not stimulate GLP-1 secretion

As it has been reported that non-physiological concentrations of insulin may cause activation of the IGF-1 receptor, and that IGF-1 can exert similar effects to insulin (425-427), the contribution of IGF-1 receptor activation was next examined. Expression of IGF-1 receptor mRNA transcripts was detected in GLUTag and NCI-H716 cells, as determined by RT-PCR (Figure 2-6A). Unexpectedly, a higher molecular weight band was also amplified from NCI-H716 mRNA, which was not seen in control human jejunal RNA. In preliminary studies treatment of both L cell models with IGF-1 increased Akt and ERK1/2 phosphorylation (data not shown). However, IGF-1 had no effect on GLP-1 release from the GLUTag cells and exhibited
Figure 2-5: Effect of insulin and insulin resistance on human NCI-H716 cells.

Cells were exposed for 24 hr to either media alone or to high insulin to induce insulin resistance and, following 3 x 40 min washes with serum-free media containing 1% BSA, were treated for acutely with 10^{-7} M insulin for 5 min for Western blot analysis or with graded concentrations of insulin or PMA for 2 hr to measure GLP-1 secretion. (A-B): Crude protein lysates were subjected to immunoblot analysis for (A) Akt phosphorylation (n=4 per group), or (B) ERK1/2 phosphorylation (n=4 per group). All values were expressed relative to the untreated control, and representative blots are shown (*: p<0.05, **: p<0.01 when compared to untreated control. #: p<0.05 as indicated). (C): GLP-1 secretion in response to medium alone (control), graded concentrations of insulin, or PMA from normal (open bars) or insulin resistant (closed bars) NCI-H716 cells. GLP-1 secretion was determined by radioimmunoassay, and all data were expressed as a percent of the untreated control (n=5-6 per group). (*: p<0.05, **: p<0.01, ***: p<0.001 when compared to untreated control. #: p<0.05, ##: p<0.01, ###: p<0.001 as indicated.)
Figure 2-6: Contribution of IGF-1 receptor activation to GLP-1 secretion.

(A): Semi-quantitative RT-PCR was performed on isolated RNA from GLUTag (lane 2) and NCI-H716 (lane 5) cells for IGF-1 receptor mRNA transcripts. Total RNA from mouse fat (lane 3) or human jejunal tissue (lane 6) was used as a positive control, and the omission of template RNA was used as a negative control (lanes 1,4). (B-C): The effect of IGF-1 receptor activation on GLP-1 release was analyzed by treating GLUTag (n=6 per group) (B) and NCI-H716 (n=4-6 per group) (C) cells with graded concentrations of human Long-Arg3-IGF-1 for 2 hr. GLP-1 secretion was determined by radioimmunoassay, and all data are expressed as a percent of the untreated control. (*: p<0.05)
variable but inhibitory effects on GLP-1 release at $10^{-10}$-$10^{-8}$ M (p<0.05) in NCI-H716 cells (Figure 2-6B,C). These findings suggest that the stimulatory effects of insulin on the L cell are not mediated via the IGF-1 receptor.

### 2.4.5. Mechanism of insulin-induced GLP-1 secretion

To determine the mechanism of action of insulin in stimulating GLP-1 release from the L cell, PI3K-Akt and MEK1/2-ERK1/2 signaling were inhibited with LY294002 and PD98059, respectively. Inhibition of PI3K did not prevent insulin-induced GLP-1 secretion, but unexpectedly potentiated the response to insulin in both GLUTag and NCI-H716 cells (p<0.05) (Figure 2-7A,B). The PI3K-inhibitory activity of the LY294002 was confirmed in both cell lines by significant inhibition of insulin-stimulated Akt phosphorylation (p<0.05). In contrast, basal GLP-1 secretion from GLUTag cells was attenuated by 59.9 ± 1.2% in the presence of PD98059 (p<0.001), while insulin-induced GLP-1 release was decreased slightly in the GLUTag cells (p<0.001). Furthermore, insulin-stimulated GLP-1 release was completely abrogated by PD98059 pretreatment of NCI-H716 cells (p<0.01) (Figure 2-7C,D). Together, these findings suggest a role for the MEK1/2-ERK1/2 pathway in insulin-induced GLP-1 release from the L cell.

To confirm the results of the PD98059 studies, the effect of over-expressing a kinase-dead MEK1-ERK2 fusion protein on GLP-1 release was examined (421). GLUTag cells were infected with AdvMEK1-ERK2 KR or with the control adenovirus, Adv-GFP (0.5-5.0x10^9 PFU/ml) to determine optimal viral titers, and cell lysates were immunoblotted for ERK1/2 to detect expression of the fusion protein (~80kDa) (Figure 2-8A). As previously reported (421), expression of the MEK1-ERK2 KR fusion protein did not alter endogenous ERK1/2 phosphorylation or total ERK1/2 expression, nor did over-expression of GFP (Figure 2-8A). GLUTag cells infected with Adv-GFP displayed a normal, 1.6-fold (p<0.05) increase in insulin-
Figure 2-7: Effect of PI3 kinase-Akt and MEK-ERK1/2 inhibition on insulin-stimulated GLP-1 secretion.

GLUTag (A, C) and NCI-H716 (B, D) cells were pretreated with 50 μM LY294002 (A, n=4-5 per group; B, n=6 per group) or 50 μM PD98059 (C, n=6-9 per group; D, n= 5-6 per group) for 15 min and subsequently treated with 10^{-8} or 10^{-7} M insulin, respectively, for 2 hr. GLP-1 secretion was determined by radioimmunoassay, and all data were expressed as a percent of the untreated control. Insets: PI3K inhibitory activity of the LY294002 was confirmed by immunoblot analysis of Akt phosphorylation in response to insulin in both cell models (n=4 per group). (*: p<0.05, ***: p<0.001 when compared to untreated control. #: p<0.05, ##: p<0.01, ###: p<0.001 as indicated)
Figure 2-8: Effect of over-expression of kinase-dead MEK1-ERK2 on insulin-induced GLP-1 secretion.

(A): GLUTag cells were infected with different titers of AdvGFP or AdvMEK1-ERK2 KR, and expression of the fusion protein (~80kDa) was detected by immunoblotting for phospho- and total ERK1/2 (representative of n=4 per group). (B) GLUTag cells infected with 5 x 10^9 PFU/ml AdvGFP or AdvMEK1-ERK2 KR were treated with media alone (control), insulin (10^-8 M), and PMA (10^-6 M) for 2hr, and GLP-1 secretion was measured by radioimmunoassay. All data were expressed as a percent of the untreated Adv-GFP control (n=5-6 per group). (*p<0.05 when compared to the untreated control).
stimulated GLP-1 secretion. Over-expression of MEK1-ERK2 KR resulted in a slight, but non-significant increase in basal GLP-1 secretion (p=0.19); however, insulin-induced GLP-1 release was completely abrogated (Figure 2-8B). Cells that over-expressed GFP or MEK1-ERK2 KR were also treated with 10^-6 M PMA, as a positive control (178), and demonstrated no differences in their GLP-1 secretory responses (up to 1.6-fold, p<0.05). Taken together these findings highlight the importance of the MEK1/2-ERK1/2 pathway in insulin-stimulated GLP-1 secretion.

2.4.6. Effect of insulin on the primary rodent L cell

To further confirm the effects of insulin and insulin resistance on the L cell, the role of insulin in GLP-1 secretion was determined in primary FRIC cultures and in MKR mice. Similar to the findings with both GLUTag and NCI-H716 cells, insulin treatment increased Akt phosphorylation by 2.8-fold and ERK1/2 phosphorylation by 3.3-fold (p<0.05) in FRIC cultures (Figure 2-9A,B). However, insulin significantly increased GLP-1 secretion only at 10^-6M, by 50.6 ± 18.6% over control (p<0.05) (Figure 2-9C). Furthermore, although insulin receptors were detected on the fetal rat L cells (Figure 2-1Bm-r), no staining of the insulin receptor could be detected on the adult rat L cell (data not shown), suggesting that the rat may not be a good model to study insulin-induced GLP-1 secretion.

Therefore, to directly assess the effects of insulin resistance on GLP-1 secretion in vivo, MKR mice were used as a model. These mice are insulin resistant due to over-expression of a dominant negative human IGF-1 receptor in skeletal muscle and, as a consequence, develop chronic hyperinsulinemia, in addition to insulin resistance in liver and fat (408, 409). MKR mice were slightly lighter than their wild type counterparts (20.9 g vs 22.7 g, p<0.01) (Figure 2-10A). However, an insulin tolerance test revealed a profound metabolic difference between the two strains, such that following a 3 hr fast, basal blood glucose levels were 1.5-fold higher than in
Figure 2-9: Effects of insulin on FRIC cultures.

(A-B): FRIC cultures were treated with 10^{-7} M insulin for 5 min, and cell lysates were collected for analysis of Akt (n=4 per group) (A) and ERK1/2 (n=4 per group) (B) phosphorylation by immunoblot (*p<0.05). All data were expressed as a fold over control, and representative blots are shown. Separate FRIC cultures were treated for 2 hr with different concentrations of insulin, and GLP-1 secretion was determined by radioimmunoassay. (n=8 per group, *: p<0.05, when compared to the untreated control).
Figure 2-10: GLP-1 secretion in MKR mice, a non-obese model of chronic hyperinsulinemia.

(A) Age- and sex-matched MKR mice and wild type FVB mice were analyzed for body weights at 11 weeks of age (n=31-36 per group, **: p<0.01). (B) Following a 3 hr fast, insulin sensitivity of MKR (black squares, n=9) and FVB (white circles, n=14) mice were assessed by intraperitoneal insulin tolerance test (n=9-14, *: p<0.05, ***: p<0.001). (C-E): Mice were fasted overnight and administered an oral glucose tolerance test (6 g/kg). Blood glucose (C), plasma insulin (D), and plasma bioactive GLP-1 (E) levels were determined at t=0 min and 10 min after oral glucose administration (n= 14-17 per group, *: p<0.05, **: p<0.01, ***: p<0.001).
WT controls (p<0.05) and were not suppressed over the 60 min period following i.p.
administration of insulin (p<0.001) (Figure 2-10B).

To measure GLP-1 secretion in vivo, wild type and MKR mice were fasted overnight and
administered an oral glucose challenge (6 g/kg). Fasting blood glucose levels were similar
between the two groups, and no difference in the glucose excursion was observed 10 min
following the glucose load (Figure 2-10C). Basal insulin levels were elevated by 1.6-fold
(p<0.05) in the MKR mice and increased normally in response to oral glucose (Figure 2-10D).
In contrast, basal GLP-1 levels were increased 1.7-fold (p<0.05), but the L cell response to the
glucose load was significantly impaired in MKR mice when compared to wild type controls,
with GLP-1 release reduced from 2.6-fold to 1.6-fold (p<0.05) (Figure 2-10E). These findings
indicate that chronic hyperinsulinemia is associated with impaired GLP-1 secretion from the
intestinal L cell in response to an oral nutrient load.

2.5 Discussion

Given the demonstrated importance of endogenous GLP-1 release to the maintenance of
normal insulin secretion and glucose homeostasis (52, 205), it is, therefore, somewhat
paradoxical that postprandial GLP-1 release is decreased in subjects with type 2 diabetes and
insulin resistance, independent of obesity (3, 6, 226, 229, 230, 233, 260). It has been proposed
that the release of GLP-1 from the intestinal L cell is impaired in such individuals, but direct
evidence demonstrating a modulatory effect of insulin on the L cell has yet to been described. In
this chapter, I demonstrated, for the first time, that insulin exerts a direct stimulatory effect on
GLP-1 secretion from the intestinal L cell, and that insulin resistance in the L cell impairs both
homologous and heterologous secretagogue-induced GLP-1 secretion in vitro and in vivo.

The pancreatic beta cell is a known target of GLP-1 action; however, there is no evidence
that demonstrates a reciprocal effect of insulin on the L cell. Nonetheless, normal murine,
human, and fetal rodent intestinal L cells were found to express the insulin receptor, as were both the GLUTag and NCI-H716 L cell lines. Furthermore, treatment of the L cells in vitro with insulin resulted in the phosphorylation of both Akt and ERK1/2, two classical effectors of insulin action. Insulin has also been reported to stimulate proglucagon gene expression, as well as GLP-1 synthesis, in GLUTag cells through an Akt-glycogen synthase kinase-3 pathway that involves the bipartite transcription factor, TCF-4 (7). Collectively, these findings indicate that the intestinal L cell should be included amongst the known targets of insulin action.

Slightly different patterns of insulin-induced GLP-1 release were observed for the GLUTag and NCI-H716 cells and FRIC cultures; however, similar differences have been reported for the dose-dependent responses of these in vitro L cell models to leptin (197). These findings, nevertheless, indicate that the L cell secretes GLP-1 in response to insulin, thereby suggesting the existence of a positive feedback loop in vivo. Such interactions are not common in physiology, but are well established to occur during the potentiation of luteinizing hormone release during the menstrual cycle. Moreover, a positive feedback loop between the duodenal hormone, cholecystokinin (CCK), and leptin secreted from the gastric mucosa, has been reported (198). Although previous studies have demonstrated that insulin does not stimulate GLP-1 secretion in fasting human subjects undergoing hyperinsulinemic clamps or from the isolated, perfused porcine ileum (428-431), I determined that primary murine and human L cells express the insulin receptor and that insulin stimulates GLP-1 release from immortalized mouse and human L cell lines. I speculate that the absence of an insulin effect in the aforementioned studies may be due to different experimental paradigms wherein low levels of nutrients in the lumen due to fasting or experimental conditions do not permit the effect of insulin on GLP-1 secretion. Therefore, just as GLP-1 requires the presence of elevated glucose levels to stimulate insulin secretion by the beta cell (49), insulin may not stimulate GLP-1 release from the enteroendocrine L cell unless high glucose levels are present in the intestinal lumen. Alternatively, the lack of
effect of insulin in the L cell in vivo may also be due to hormonal control by somatostatin. GLP-1 stimulates somatostatin secretion from intestinal cultures and perfused ileum, while somatostatin inhibits GLP-1 secretion, thereby suggesting a feedback loop between GLP-1 and local somatostatin secretion (137, 195, 431). The apparent lack of effect of insulin in the previously mentioned studies could, therefore, have been consequent to intestinal-derived somatostatin production in these experiments, which may then be alleviated under post-prandial conditions. Finally, while other studies have demonstrated that insulin inhibits GIP secretion during conditions of euglycemia, this inhibitory effect is reversed when blood glucose levels are elevated, suggesting either a loss of the inhibitory effect of insulin on GIP release or a possible stimulatory effect of insulin on the K cells during hyperglycemic conditions (432, 433).

Immunohistochemistry determined that the adult rat L cell does not express the insulin receptor, which precludes the use of this animal as a model to study insulin-induced GLP-1 secretion, but this hypothesis should be directly testable in future human or mouse studies using carefully controlled fasting vs. fed hyperinsulinemic plus high luminal glucose conditions.

In the present study, the importance of the MEK1/2-ERK1/2 pathway in mediating insulin-induced GLP-1 secretion from the L cell was demonstrated through pharmacological and adenoviral over-expression studies. MEK1/2-ERK1/2 signaling has also been found to play a role in the release of other hormones, including insulin (434, 435) and the catecholamines (436, 437); moreover, an essential role for this pathway in the human L cell has been demonstrated for meat hydrolysate- and amino acid-induced GLP-1 secretion (133). In contrast to these findings, inhibition of PI3K did not prevent insulin-induced GLP-1 release in either of the L cell lines but, instead, potentiated both basal and insulin-induced GLP-1 secretion, possibly due to non-specific inhibition of inward rectifying Kv channels (438). When taken together, therefore, the present findings indicate a role for the MEK1/2-ERK1/2 pathway in mediating insulin-induced GLP-1 release from the intestinal L cell.
Surprisingly, IGF-1 was found to have no effect on the murine L cell and a slight inhibitory effect on GLP-1 secretion from the L human cell, even though both insulin and IGF-1 receptors share high sequence homology and activate similar downstream components. Studies performed with over-expression models of either receptor or chimeric receptors have revealed differential effects of these hormones on other cell types (439-441), which may account for the contrasting effects of IGF-1 and insulin on the L cell. Nonetheless, further studies are required to examine the role of IGF-1 in the L cell.

The finding that induced-cellular insulin resistance impaired the GLP-1 secretory response to insulin is consistent with the concomitant down-regulation that was observed in both insulin receptor protein expression and insulin receptor phosphorylation. Moreover, desensitization to heterologous secretagogues, most notably GIP and PMA, was also observed in the insulin resistant L cells in vitro, suggesting that insulin resistance affected the actions of these GLP-1 secretagogues downstream of the changes in insulin receptor activation and expression. Decreased insulin-mediated GLP-1 secretion in insulin resistance may be partly explained by the down-regulation observed in MEK1/2-ERK1/2 activation, as seen with decreased ERK1/2 phosphorylation, possibly in association with known effects of insulin to upregulate the expression and activity of dual-specificity MAPK phosphatases (442, 443). Interestingly, although GIP and PMA stimulate GLP-1 secretion through the activation of PKA and PKC isoenzymes, respectively (178, 444), recent studies have demonstrated that these secretagogues can also activate the MEK1/2-ERK1/2 pathway in other cell models (445, 446). Therefore, as insulin resistance decreased the response to GIP and PMA, as well as to insulin, a decrease in the activity of the MEK1/2-ERK1/2 pathway in the L cell may account for the diminished responses to all of these secretagogues.

High fat feeding-induced obesity results in altered basal GLP-1 levels in mice, as well as impairments in oral glucose-induced GLP-1 release (197). As obesity is associated with a
multitude of metabolic impairments, the exact cause of the impaired GLP-1 release in that study was not known. In this study, I utilized MKR mice as a non-obese model of hyperinsulinemic-insulin resistance and demonstrated that chronic hyperinsulinemia is associated with increased basal levels of circulating GLP-1 in these mice, but decreased GLP-1 secretion in response to an oral glucose load. These findings are in accordance with reports that decreased insulin sensitivity is associated with impaired GLP-1 release in response to a mixed-meal (6, 260). Moreover, based on the \textit{in vitro} findings of the present study whereby insulin resistance impaired GLP-1 secretion in response to heterologous secretagogues, these data suggest that chronic hyperinsulinemia may decrease the responsiveness of the L cell to nutrient and hormonal secretagogues.

In summary, enteroendocrine L cells secrete GLP-1 in response to insulin in a glucose-dependent manner and, in the mouse and human L cell, this effect is mediated through a MEK1/2-ERK1/2-dependent pathway. Insulin resistance \textit{in vitro} is associated with loss of both of these actions of insulin, as well as with an impaired response to heterologous secretagogues, suggesting that insulin resistance induces a defect in a distal exocytotic process that governs GLP-1 release. Consistent with these findings, GLP-1 secretion is also attenuated in response to an oral glucose load in MKR mice, further demonstrating the deleterious effect of chronic hyperinsulinemia on the function of the L cell. Knowing that insulin stimulates GLP-1 release, I, therefore, decided to further examine the distal secretory mechanisms that govern insulin-stimulated GLP-1 secretion, as described in chapter 3.

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CHAPTER 3: THE RHO GTPASE, CDC42, IS REQUIRED FOR
INSULIN-INDUCED GLP-1 SECRETION AND ACTIN
REMODELING IN THE INTESTINAL ENDOCRINE L CELL

Some of the text in this chapter has been submitted for publication:

insulin-induced GLP-1 secretion and actin remodeling in the intestinal endocrine L cell.


Author contributions:

M. Xu was an undergraduate project student under the direct supervision of G. Lim and
generated data for figures 3-1 and -2. Lim. J. Sun and T. Jin provided reagents, and J. Sun
performed Western blots with samples provided by G. Lim for figure 3-8. G. Lim completed all
data analysis and writing.
3.1. Abstract

Rho GTPases, such as Cdc42 and Rac1, have recently been identified as critical regulators of actin dynamics and hormone secretion from several endocrine cell types; however, the function of these proteins in secretion of the incretin hormone, GLP-1, by the intestinal L cell is unknown. Treatment of the murine GLUTag and human NCI-H716 L cell lines with insulin stimulated transient depolymerizations of cortical F-actin as demonstrated by up to 1.6-fold increases in the ratio of monomeric G-actin to polymerized F-actin (p<0.05). Under normal conditions, insulin induced a 1.4-fold increase in GLP-1 secretion (p<0.05); however, F-actin depolymerizing and stabilizing agents augmented insulin-induced GLP-1 release up to 2.1-fold (p<0.05 vs the absence of either agent), suggesting that F-actin functions as a permissive barrier in the L cell. In GLUTag cells, insulin-induced F-actin depolymerization occurred simultaneously with activation of Cdc42 (p<0.05), but not of Rac1, while transfection of siRNA against Cdc42 and of dominant-negative Cdc42(T17N) completely abrogated insulin-induced GLP-1 release and impaired actin remodeling. Insulin also promoted the association and activation of PAK1 via Cdc42, which was also prevented by Cdc42(T17N), and over-expression of kinase-dead PAK1(K299R) similarly attenuated insulin-induced GLP-1 secretion. Cdc42(T17N) and PAK1(K299R) also prevented insulin-induced activation of the MEK1/2-ERK1/2 pathway, which was previously identified to mediate insulin-stimulated GLP-1 release. Taken together, these data identify a novel signaling pathway in the endocrine L cell, whereby Cdc42 regulates actin remodeling, activation of the canonical ERK1/2 pathway, and GLP-1 secretion induced by insulin.
3.2. Introduction

The L cell is an open-type epithelial cell that is able to sense nutrients in the intestinal lumen through microvilli on the apical membrane. The presence of luminal nutrients stimulates release of GLP-1 from numerous secretory granules that are situated beneath the basolateral plasma membrane. The membrane localization of such granules is considered a hallmark of endocrine secretory cells, including the pancreatic beta cell (10, 447). Recently, it has become apparent that a number of the stimulus-secretion coupling pathways that exist in the pancreatic beta cell are also present in the intestinal L cell (138, 146-148). It is, therefore, plausible that other proximal or distal events regulating insulin secretion from the endocrine beta cell may also function in the intestinal endocrine L cell (448-450). Presently, numerous secretagogues and their cognate receptors have been identified in the L cell [as described in chapter 1.1.8. and (138, 197)]. To date, only a single study to date has determined the expression of SNARE proteins in the L cell (451), and no studies have elucidated the distal events leading to GLP-1 granule exocytosis.

In non-endocrine and endocrine cells, the actin cytoskeleton regulates various cellular functions, including cell migration, granule/vesicle transport, and exocytosis. Moreover, polymerized (F-) actin, which forms the cytoskeleton, is known to undergo reorganization in response to various stimuli (452-455). Studies from endocrine cells, such as pancreatic beta and adrenal chromaffin cells, have demonstrated that cortical F-actin functions as a barrier to granule exocytosis. Following stimulus-induced remodeling of the actin cytoskeleton, it is believed that granules translocate from a sub-plasma membrane pool to the plasma membrane in order to undergo exocytosis (435, 456-459). Actin remodeling in the brush border of intestinal absorptive cells is also required for nutrient absorption (460, 461); however, the role and/or
requirement for actin remodeling in hormone secretion from endocrine cells of the intestine has yet to be examined.

The actin cytoskeleton is regulated by numerous actin-binding proteins, and it has recently been established that Rho GTPases, such Cdc42 and Rac1, facilitate actin remodeling in endocrine cells. Moreover, numerous studies have also demonstrated that Rho GTPases also regulate hormone secretion from these cells (456, 458, 462-466). In the intestinal epithelium, Cdc42 has been demonstrated to regulate gene expression and cell survival, as well as cytokine secretion (467-470); however, its function in the L cell is unknown. In other cell types, insulin activates Rho GTPases and their downstream kinases (350, 351, 363). **I, therefore, hypothesized that insulin requires Cdc42 and/or Rac1 to regulate GLP-1 secretion; moreover, remodeling of the actin cytoskeleton is also required for insulin-induced GLP-1 release from the L cell.**

### 3.3. Materials and Methods

#### 3.3.1. Cell Culture and transfection

The murine GLUTag and human NCI-H716 L cell lines were grown and maintained under high glucose conditions (25 mM) with 10% FBS, as previously described (138, 197). MIN6 beta cells were maintained as described previously (471). Two days prior to experimentation, L cell lines were seeded on 24-well plates that were coated with poly-D-lysine (Sigma-Aldrich, Oakville, ON) in order to promote cell adherence. Independent batches of cells were used in all experiments to ensure reproducibility. For transient transfection studies, GLUTag cells were washed once with HBSS, incubated with Opti-Mem media (Invitrogen, Carlsbad, CA), and subsequently treated with either 1 μg of plasmid cDNA or 100 nmol of
Cdc42-specific siRNA or scrambled siRNA (control) oligonucleotides in Lipofectamine 2000 (Invitrogen) for 5 hr. All transfected cells were allowed to recover for 48 hr prior to treatment.

3.3.2. Plasmids and siRNA oligonucleotides

A plasmid encoding myc-tagged, dominant-negative Cdc42(T17N) and the corresponding pcDNA3 empty vector control were kindly provided by Drs. Gregory Downey (National Jewish Medical and Research Center, Denver, CO) and Andras Kapus (St. Michael’s Hospital, Toronto, ON) (472). pCMV6 (control) and myc-tagged PAK1(K299R) plasmids were provided by Drs. Jeffrey Field (Penn Medicine, Philadelphia, PA) and Tianru Jin (University Health Network, Toronto, ON), and were prepared as previously described (473, 474). siRNA oligonucleotides against Cdc42 and scrambled control siRNA were purchased from Ambion (Austin, TX) and have previously been found to significantly reduce Cdc42 expression in MIN6 beta cells (465). Immunoblots were performed on lysates from siRNA- or plasmid-transfected cells to ensure efficient protein knockdown or over-expression.

3.3.3. RNA isolation and RT-PCR

Total RNA was isolated and purified from GLUTag and MIN6 cells (RNeasy kit, Qiagen Inc., Mississauga, ON) for RT-PCR analysis. Briefly, 1 μg of RNA was reverse-transcribed and amplified with a One-step RT-PCR kit (Qiagen, Inc.) with primers targeting murine Cdc42 and Rac1. As a negative control, template RNA was omitted and replaced with RNase-free water. Primers for Cdc42 (Forward: 5’-CGACCGCTAAGTTATCCACAG, Reverse: 5’GCAGCTAGGATAGCCTCATCA) and Rac1 (Forward: 5’-GGACACAGCTGGACAAGAAGA-3’, Reverse: 5’-GGACAGAGAAACCGCTCGGATA) were expected to yield PCR products of 325 bp and 485 bp, respectively (475).
3.3.4. GLP-1 secretion studies

GLUTag and NCI-H716 cells were washed with HBSS and treated with media alone (control), insulin (10⁻⁸ M; Eli Lilly) or phorbol 12-myristate 13-acetate (PMA, 10⁻⁶ M; Sigma-Aldrich) for up to 2 hr. Some cells were pretreated with 10 μM latrunculin B or 1 μM jasplakinolide (Calbiochem, La Jolla, CA) for 30 min prior to the addition of insulin or PMA. Following treatment, peptides in the supernatant and cells were removed and collected by reversed-phase extraction, and total GLP-1 secretion was measured by radioimmunoassay, as described previously (125, 136, 138, 178, 197). Secretion was expressed as the amount GLP-1 in the supernatant relative to the total amount of GLP-1 (media plus cells). Basal media content and cell content were 28.0 ± 3.8 and 344.1 ± 20.7 pg/ml, respectively, in GLUTag cells (n=8), and 206.8 ± 20.1 and 11,958.1 ± 2110.1 pg/ml, respectively, in NCI-H716 cells (n=16). The cause of differences in basal secretion between cell models is unknown, but may be due to species differences and/or basal media formulations. Alternatively, when cell protein was collected for immunoblot, cells were lysed with RIPA buffer, and GLP-1 secretion was expressed as a ratio of the GLP-1 in the media to the total cell protein per well, as determined by Bradford assay.

3.3.5. Analysis of F-actin

To visualize stimulus-induced changes in F-actin, cells were treated with media alone (control), 10⁻⁸ M insulin, or 10⁻⁶ M PMA and subsequently fixed with 4% paraformaldehyde for 10 min at 4 °C. Following permeabilization with TBS/triton X-100 (0.1% v/v), cells were incubated with TRITC-conjugated phalloidin (Sigma-Aldrich) for 1 hr at room temperature. After successive washes with TBS/triton X-100, DAPI (Vector labs, Burlingame, CA) was added to visualize nuclei, and slides were immediately cover-slipped. Images were visualized with a Carl Zeiss Axioplan Deconvolution Microscope (Carl Zeiss Canada, Ltd., Don Mills, ON).
Equal exposure times were used to normalize the intensity of TRITC-phalloidin and DAPI staining, and all images were captured at the equatorial plane of focus, which was the mid-point of the cell. Fluorescence intensity was measured with ImageJ software (NIH, Bethesda, MD), as previously described (476). Briefly, a line was drawn across the center of the cell along which the fluorescence intensity of TRITC-phalloidin was measured. The edge of the cell was defined as the first detectable increase in fluorescence intensity. All values were normalized to a percentage of overall cell size, in order to account for differences in cell diameters. On average, GLUTag and NCI-H716 cells were 16.8 ± 0.9 μm and 13.4 ± 0.4 μm, respectively. With all images, one pixel represented 0.078 μm.

In separate experiments, cells were treated with media alone (control), 10^{-8} M insulin, or 10^{-6} M PMA for various incubation times, followed by lysis with a Cell Lysis and F-actin Stabilization Buffer that was provided with a G-actin/ F-actin In vivo assay kit (Cytoskeleton, Denver, CO). This kit specifically measures the relative amount of monomeric globular (G-) actin to polymerized filamentous (F-) actin (350). Briefly, actin in cell lysates was stabilized and centrifuged at 100,000xg for 1 hr at 37 °C to separate soluble G-actin from the F-actin-containing pellet. F-actin was then depolymerized in the presence of 2 μM cytochalasin D, and the relative ratio of G- to F- (G:F) actin was quantified by immunoblot analysis using a pan-actin antibody.

### 3.3.6. Rho GTPase activation assays

To detect activation of Cdc42 or Rac1, GLUTag cell lysates from media alone (control)-, 10^{-8} M insulin-, or 10^{-6} M PMA-treated cells were analyzed using Cdc42 or Rac1 Activation Assay Biochem kits (Cytoskeleton), respectively, as per the manufacturer’s protocol. These kits specifically detect the active, GTP-loaded forms of Cdc42 or Rac1 through a pull-down assay using GST-tagged PAK1-PBD fusion proteins linked to agarose beads. Briefly, 2 mg of whole
cell lysate was incubated with PAK1-PBD beads overnight at 4 °C and, following consecutive washes with buffer, bound proteins were eluted from the beads and subjected to immunoblot analysis to determine the relative amounts of Cdc42 or Rac1 with antibodies against Cdc42 or Rac1, as appropriate. All densitometric data are expressed relative to Ponceau S staining; however, similar results were obtained when expressed relative to Cdc42 in total cell lysates.

### 3.3.7. Co-immunoprecipitation and immunoblotting

Following treatment with media alone (control), 10⁻⁸ M insulin, or 10⁻⁶ M PMA, cell lysates were collected in RIPA buffer, resolved by 12% SDS-PAGE, and transferred to PVDF membranes, as previously described (138, 178, 197). In separate experiments, the association between Cdc42 and PAK1 was detected by co-immunoprecipitation experiments. Briefly, 2 mg of cleared cell lysates was incubated with PAK1 or Cdc42 antibodies (Cell Signaling Technologies or Santa Cruz Biotechnologies, respectively) overnight at 4 °C, followed by a 4 hr incubation with protein A Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 4 °C. After 5 successive washes with lysis buffer, immunoprecipitated complexes were subjected to 12% SDS-PAGE. Proteins of interest were detected with antibodies targeted against phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), total ERK1/2, phospho-MEK1/2 (Ser²¹⁷/²²¹), phospho-MEK1 (Ser²⁹⁸), total MEK1/2, phospho-PAK1 (Thr⁴²³)/PAK2 (Thr⁴⁰²), PAK1 (Cell Signaling Technologies), and Cdc42, Rac1, and Actin (Cytoskeleton). Proteins of interest were visualized with Amersham Enhanced Chemiluminescence (ECL) Western blotting reagent (GE Healthcare UK Ltd, Buckinghamshire, England) and quantification was performed with Kodak Molecular Imaging software (Carestream Molecular Imaging, New Haven, CT).

### 3.3.8. Statistical analysis

All data are expressed as mean ± SEM. In some experiments, data were log10 transformed to normalize variance for statistical analysis. Data were analyzed when appropriate
by Student’s t-test or by one- or two-factor ANOVA, followed by appropriate post-hoc tests (SAS, Cary, NC). Statistical significance was assumed at p<0.05.

3.4 Results

3.4.1. Regulation of GLP-1 secretion by the actin cytoskeleton

Previous studies have demonstrated that the actin cytoskeleton acts as a permissive barrier to regulate hormone secretion from several endocrine cell types (435, 456-459); however, this function has yet to be described in intestinal endocrine cells. Murine GLUTag and human NCI-H716 intestinal L cells were thus pretreated with the actin depolymerizing and stabilizing agents, latrunculin B (LB, 10 μM) or jasplakinolide (JP, 1 μM), respectively. In the absence of either LB or JP, insulin stimulated 1.4-fold increase (p<0.05) in GLP-1 release from both GLUTag and NCI-H716 cells. Following pretreatment with LB, insulin-induced GLP-1 secretion was potentiated at t=2 hr from 1.4-fold to 1.7-fold (p<0.05 vs the absence of LB in GLUTag cells (Figure 3-1A) and in NCI-H716 cells (Figure 3-1B). Although both cell lines were also treated with 10^{-6} M PMA as a positive control, a potentiated response was only observed in the NCI-H716 cells following LB pretreatment (3.6 vs 5.0-fold, p<0.05). When both cell models were pretreated with jasplakinolide, insulin-induced GLP-1 secretion was potentiated in GLUTag cells from 1.4-fold in control, insulin-treated cells to 2.1-fold following JP pretreatment (p<0.05, Figure 3-1A); conversely, PMA-, but not insulin-induced GLP-1 release was potentiated in NCI-H716 cells (p<0.05, Figure 3-1B).

As it has been demonstrated that remodeling or depolymerization of F-actin is a prerequisite event for granule-vesicle interaction with the plasma membrane (347, 458, 459), the effects of insulin (10^{-8} M) and PMA (10^{-6} M) on F-actin dynamics was next explored. Treatment of GLUTag and NCI-H716 cells with insulin (Figure 3-2A, B, respectively) induced transient F-
Figure 3-1: Depolymerization or stabilization of the actin cytoskeleton modulates insulin-induced GLP-1 secretion.

Murine GLUTag (A, n=4-8 per group) or human NCI-H716 (B, n=6-16 per group) cells were pretreated with 10 μM latrunculin B or 1 μM jasplakinolide for 30 min and subsequently treated with media alone (control), 10^{-8} M insulin, or 10^{-6} M PMA for 2 hr. GLP-1 secretion was determined by radioimmunoassay, and all data were expressed as a percent of the untreated control. (*: p<0.05, ***: p<0.001 when compared to respective control; #: p<0.05 when compared to the delta increase for insulin treatment of control cells)
Figure 3-2: Insulin and PMA induce actin depolymerization in the L cell.
Murine GLUTag (A, C, E, G) and human NCI-H716 (B, D, F, H) cells were treated with 10^{-8} M insulin (A,B, n=3-6 per time point) or 10^{-6} M PMA (C,D, n=3-6 per time point) for t= 0, 5, 10, 15, 30, or 60 min, and cell lysates were collected and subjected to ultra-centrifugation to separate
monomeric G-actin and polymerized F-actin. Fractions were subjected to Western blot analysis to determine the G:F actin ratio as a measure of actin depolymerization, and representative blots are shown (*: p<0.05 when compared to t=0). GLUTag (E) and NCI-H716 (F) cells were stained with TRITC-phalloidin to visualize F-actin under basal conditions (t=0) and at the time corresponding to the peak of actin depolymerization after insulin or PMA treatment (Scale bars= 10 μm). Changes in TRITC-phalloidin-associated fluorescence intensity were measured following insulin or PMA treatment in GLUTag (G, n=6-12 per group) and NCI-H716 (H, n=6-13 per group) cells. Equal exposure times were used for all images, and all data were expressed relative to overall cell size. (Inset images: Total area under the curve (AUC) corresponding to relative fluorescence intensity normalized to cell size. (**:p<0.01)).
actin depolymerization, with 1.6- and 1.4-fold (p<0.05) increases in the G:F actin ratio detected 15 min after addition of the secretagogue. PMA induced similar changes in actin dynamics, with a 2.1-fold increase (p<0.05) in G:F actin detectable 15 min after addition to GLUTag cells (Figure 3-2C) and a 1.6-fold increase (p<0.05) after 5 min in NCI-H716 cells (Figure 3-2D). Staining with TRITC-conjugated phalloidin confirmed the changes in F-actin that were observed following insulin or PMA treatment in GLUTag (Figure 3-2E) and NCI-H716 (Figure 3-2F) cells, such that both secretagogues induced a disruption of F-actin around the perimeter of the cell. After 30 min treatment with insulin or PMA, the cortical ring was fully restored (data not shown).

Analysis of TRITC-phalloidin-associated fluorescence intensity across the diameter of the cell indicated the presence of a cortical F-actin ring with peaks at 10% and 80% of cell diameter in untreated (control) cells. Following insulin treatment, 77.3 ± 2.4 % and 57.3 ± 4.1 % decreases in overall fluorescence intensity were detected in GLUTag (p<0.01, Figure 3-2G) and NCI-H716 (p<0.01, Figure 3-2H) cells, respectively, as well as a diminution in peak height. In NCI-H716 cells, PMA also decreased overall TRITC-phalloidin-associated fluorescence intensity by 48.9 ± 5.8% (p<0.01), including the peripheral ring. In contrast, while an overall 38.9 ± 3.5% (p<0.01) decrease was observed in PMA-treated GLUTag cells, a redistribution of F-actin was detected into the cytoplasm (Figure 3-2E,G).

3.4.2. Insulin and PMA display different kinetics of GLP-1 release in murine GLUTag cells

The initial GLP-1 secretion experiment was conducted over a 2 hr time period (Figure 3-1), but the early effects of insulin and PMA on actin dynamics suggested a faster time course of release (Figure 3-2). Therefore, I sought to determine the kinetics of GLP-1 secretion, relative to actin remodeling. GLUTag, rather than NCI-H716 cells, were chosen for this and further analyses in this study due to similar kinetics in actin remodeling in response to insulin and PMA.
Murine GLUTag cells were, therefore, treated with $10^{-8}$ M insulin and $10^{-6}$ M PMA for 15, 30, 60, and 120 min. By $t=30$ min, PMA-induced a 3.6-fold increase ($p<0.01$) in GLP-1 secretion (Figure 3-3). Conversely, insulin-stimulated GLP-1 secretion was only observed 60 min after treatment, with a 1.5-fold ($p<0.05$) in GLP-1 release. Taken together, these results demonstrate that insulin and PMA induce GLP-1 secretion after their depolymerizing effects on the actin cytoskeleton, suggesting that activation of intracellular kinases or other signaling events are required prior to GLP-1 release.

### 3.4.3. Cdc42 regulates GLP-1 secretion and actin polymerization

Several studies have demonstrated that insulin receptor activation stimulates Cdc42 and Rac1 signaling (350, 351). mRNA transcripts for Cdc42 and Rac1 were detected by RT-PCR in mRNA isolated from GLUTag cells (Figure 3-4A). Treatment of the cells with $10^{-8}$ M insulin caused a 1.8-fold increase ($p<0.05$) in the levels of activated, Cdc42-GTP at $t=10$ min (Figure 3-4B); conversely, preliminary analyses indicated that PMA did not induce Cdc42 activation (data not shown). Furthermore, no changes in the amount of Rac1-GTP were detected in response to insulin treatment of the GLUTag cells (Figure 3-4C).

To examine the role of Cdc42 in insulin-stimulated GLP-1 secretion, siRNA oligonucleotides against Cdc42 were used to reduce Cdc42 expression in GLUTag cells, by up to 44% ($p<0.05$; Figure 3-5A). Basal GLP-1 release was not affected by reduced expression of Cdc42; however, insulin-induced GLP-1 release was completely abrogated (Figure 3-5B). Furthermore, consistent with the lack of effect of PMA on Cdc42 activation in these cells, PMA-stimulated GLP-1 release was not affect by Cdc42 knockdown (Figure 3-5B).

To corroborate the finding that reduced Cdc42 expression decreased insulin-stimulated GLP-1 secretion, GLUTag cells were transfected with a plasmid encoding a dominant-negative,
Figure 3- 3: GLP-1 secretion in response to insulin is detectable 60 min after treatment. Murine GLUTag cells were treated for 15, 30, 60, and 120 min with media alone (control), $10^{-8}$ M insulin, or $10^{-6}$ M PMA, and GLP-1 secretion was measured by radioimmunoassay. All data are expressed as the delta from the 15 min time point. (n=6-8 per time point per group, *: p<0.05, **: p<0.01, when compared to untreated control at each time point; ##: p<0.01 when compared to insulin treatment).
Figure 3-4: Insulin activates the Rho GTPase Cdc42 in murine GLUTag cells.

(A): RT-PCR for Cdc42 and Rac1 was performed on isolated RNA from GLUTag cells. The absence of template RNA was used as a negative control, while MIN6 beta cell RNA was used as a positive control. (B,C): Cdc42 (B, n=4-6 per time point) and Rac1 (C, n=4-6 per time point) activation assays were performed on insulin (10^{-8} M)-treated cell lysates. Cell lysates were incubated with GST-PAK PBD beads, which specifically bind to activated (GTP-loaded)-Cdc42 or -Rac1. Samples were subsequently analyzed by Western blot analysis with Ponceau S staining as a loading control. Representative blots are shown, and all data were expressed as a percentage of the untreated control (*: p<0.05 when compared to untreated control).
Figure 3-5: siRNA-mediated knock-down of Cdc42 expression abrogates insulin-induced GLP-1 release.

(A): Murine GLUTag cells were transfected with scrambled (Scr) or siRNAs against Cdc42 (100 nM final concentration). Immunoblot analysis was performed on cell lysates for Cdc42 and actin to determine the efficiency of knock-down, and representative blots are shown (n=3-4 per group, *: p<0.05). (B): Following transfection with siScr or siCdc42#1, GLUTag cells were treated with media alone (control), insulin (10^{-8} M) or PMA (10^{-6} M) for 2 hrs. GLP-1 that was secreted into the media was measured by radioimmunoassay, and GLP-1 secretion was normalized to total protein per well. All data were expressed as a percent of the untreated control. (n=4-6 per group, *: p<0.05, ***: p<0.001).
GDP-bound Cdc42 (T17N) protein (472). Pilot studies to determine transfection efficiency demonstrated that transfection with 1.0 μg of plasmid DNA was associated with highest expression of the myc-tagged protein, without any effects on actin expression (Figure 3-6A) or, visually, on cell viability. Immunocytochemistry for c-myc showed that approximately 70% of cells expressed the protein (data not shown). Similar to findings made with the Cdc42 siRNA, over-expression of Cdc42 (T17N) attenuated insulin-induced GLP-1 release, with no effects on PMA-stimulated secretion (Figure 3-6B). As Cdc42 has been demonstrated to regulate actin remodeling, the effect of over-expression of Cdc42 (T17N) on the G:F actin ratio was also examined. No differences were detected in the ability of insulin to induce actin depolymerization in cells transfected with the Cdc42 (T17N) plasmid as compared to the control pcDNA3 vector at t=15 min; however, an impairment in actin polymerization was detected in GLUTag cells over-expressing the mutant Cdc42 protein, such that the G:F actin ratio was 1.6-fold higher (p<0.05) than in cells transfected with the empty, control vector at t=30 min (Figure 3-6C).

3.4.4. Insulin promotes the activation of PAK1, which is required for GLP-1 release

PAK1 is an established effector kinase of Cdc42 and, in the beta cell, is required for glucose-stimulated insulin secretion (465). Under basal conditions, PAK1 is auto-inhibited due to its native conformation; however, following binding of Cdc42 to the p21-binding domain, PAK1 is released from auto-inhibition and phosphorylated at Thr423 in the kinase domain (477). An increase in PAK1-Thr423 phosphorylation was observed when GLUTag cells were treated with 10⁻⁸ M insulin, with maximal changes seen at t=20 min after treatment (Figure 3-7A). Insulin-induced PAK1 phosphorylation also coincided with a detectable increase in the association of Cdc42 with PAK1, as determined by co-immunoprecipitation analysis (Figure 3-7B). To further investigate the interaction of Cdc42 and PAK1, GLUTag cells were transfected
Figure 3-6: Over-expression of dominant-negative Cdc42 (T17N) abrogates insulin-stimulated GLP-1 release.

(A): Murine GLUTag cells were transfected with different amounts of cDNA encoding a myc-tagged dominant-negative Cdc42 (T17N), and cell lysates were subjected to immunoblot analysis to determine the expression of c-myc, Cdc42, and actin. Representative blots are shown from three independent experiments. (B): Following transfection with the empty pcDNA3 vector or plasmid encoding a myc-tagged Cdc42 (T17N), GLUTag cells were treated with media alone (control), insulin (10^-8 M), or PMA (10^-6 M) for 2 hrs. Secreted GLP-1 was measured by radioimmunoassay, and GLP-1 secretion was normalized to total protein per well. All data were expressed as a percent of the untreated control. (n=4-6 per group *: p<0.05, ***: p<0.001; ###: p<0.0001 when compared to insulin-treated empty vector cells). (C): Cell lysates from control- or insulin (10^-8 M)-treated cells that were transfected with either the pcDNA3 vector or Cdc42 (T17N) plasmid were subjected to ultra-centrifugation in order to determine the relative amounts of G- and F-actin at each time point by Western blot. A representative blot is shown (n=5-8 per time point for each treatment. *: p<0.05 when compared to respective t=0, #: p<0.05 when compared to respective treatment).
Figure 3-7: Insulin promotes the association and activation of PAK1 through Cdc42.

(A): Murine GLUTag cell lysates from $10^{-8}$ M insulin treated cells were subjected to immunoblot analysis with phospho-specific antibodies for PAK1/PAK2 (pThr423-PAK1/ pThr402-PAK2) and total PAK1. Representative blots from three independent experiments are shown. (B): Cells were treated with media alone (control) or $10^{-8}$ M insulin, and cell lysates were used for immunoprecipitation with an anti-PAK1 antibody. Immune complexes were subsequently resolved by SDS-PAGE, and membranes were probed with anti-Cdc42 and –PAK1 antibodies. Representative blots from three independent experiments are shown. (C): Cell lysates from insulin treated GLUTag cells that were transfected with either a pcDNA3 vector or Cdc42 (T17N) plasmid were subjected to immunoprecipitation using an anti-Cdc42 antibody. Immune complexes were subjected to SDS-PAGE, and membranes were probed with antibodies against PAK1, Cdc42, and c-myc. Representative blots from three independent experiments are shown.
with a plasmid encoding the dominant-negative GDP-bound Cdc42 (T17N), which should not interact with the p21-binding domain of PAK1. Following insulin treatment, GLUTag cells transfected with the control pcDNA3 vector displayed no impairment in the association of PAK1 with endogenous Cdc42; however, in cells that over-expressed Cdc42 (T17N), this interaction was not detectable (Figure 3-7C).

Finally, having established that PAK1 is activated and associates with Cdc42 in response to insulin treatment of GLUTag cells, the role of PAK1 in insulin-stimulated GLP-1 release was examined. GLUTag cells were transfected with a plasmid encoding kinase-dead PAK1 (K299R) (473, 474) or a pCMV6 empty vector control. Pilot studies demonstrated that 1 µg of plasmid DNA was required for optimal expression of PAK1 (K299R) (Figure 3-8A). Over-expression of PAK1 (K299R) abrogated insulin-induced GLP-1 secretion from the L cell (Figure 3-8B), demonstrating that PAK1, following activation by Cdc42, is required for insulin-stimulated GLP-1 release from the intestinal L cell.

### 3.4.5. Cdc42 regulates insulin-induced activation of the MEK-ERK1/2 pathway in the L cell

Activation of ERK1/2 is dependent on the activity of the upstream kinases, MEK1/2. In non-endocrine cells, the Cdc42-PAK1 axis has been demonstrated to modulate the activity of the canonical ERK1/2 pathway (478, 479); however, there is no evidence for such an interaction in the intestinal L cell. When GLUTag cells were transfected with the control vectors, pcDNA3 or pCMV6, insulin stimulated up to a 1.5-fold (p<0.05) increase in phosphorylation of MEK1/2 on Ser\(^{217/221}\) (Figure 3-9 A,B) and up to a 1.7-fold (p<0.05) increase in ERK1/2 phosphorylation (Figure 3-9 C,D). In contrast, over-expression of dominant-negative Cdc42 (T17N) or kinase-dead PAK1 (K299R) completely abrogated activation of both MEK1/2 and ERK1/2 in response to insulin (Figure 3-9).
Figure 3-8: PAK1 is required for insulin-induced GLP-1 secretion.

(A): Murine GLUTag cells were transfected with different amounts of cDNA encoding a kinase-dead, myc-tagged PAK1 (K299R). Cell lysates were subjected to Western blot to determine the expression of c-myc, PAK1, and actin. Representative blots are shown from three independent experiments. (B): Following transfection with either an empty pCMV6 vector or a plasmid encoding PAK1 (K299R), GLUTag cells were treated with media alone (control), insulin (10^{-8} M), or PMA (10^{-6} M) for 2 hrs. Secreted GLP-1 was measured by radioimmunoassay, and GLP-1 secretion was normalized to total protein per well. All data were expressed as a percent of the untreated control (n=4-6 per group, *: p<0.05, ***: p<0.001).
Figure 3-9: Cdc42 and PAK1 regulate insulin-stimulated activation of the MEK1/2-ERK1/2 pathway.

Following transfection with control vectors (pcDNA3 or pCMV6) or plasmids encoding Cdc42 (T17N) or PAK1 (K299R), murine GLUTag cells were treated with 10^{-8} M insulin for 5, 10, 15, 30, or 60 min and immediately lysed. (A,B): Cell lysates were subjected to immunoblot analysis with antibodies against pSer^{217/221}-MEK1/2, pSer^{298}-MEK1, total MEK1/2, and actin. Representative blots are shown (n=3-4 per time point, *: p < 0.05 when compared to respective t=0). (C, D): Cell lysates were subjected to immunoblot analysis with antibodies against pThr^{202}/Tyr^{204}-ERK1/2, total ERK1/2, and actin. Representative blots are shown (n=3-5 per time point, *p < 0.05 when compared to respective t=0).
In some, but not all, cell types, phosphorylation of MEK1 at Ser\textsuperscript{298} by PAK1 is postulated to prime MEK1 for activation (478-481). In GLUTag cells transfected with the pcDNA3 or pCMV6 vectors, insulin induced up to a 1.7-fold increase in Ser\textsuperscript{298} phosphorylation on MEK1 (p<0.05, Figure 3-9A,B). Surprisingly, over-expression of Cdc42 (T17N), but not PAK1 (K299R), attenuated insulin-mediated phosphorylation of MEK1 on Ser\textsuperscript{298} (Figure 3-A,B), which suggests that Pak1 is not responsible for Ser\textsuperscript{298} phosphorylation on MEK1. Furthermore, as either expression of either mutant attenuated insulin-induced activation of the MEK1/2-ERK1/2 pathway, these findings also suggest that phosphorylation of Ser\textsuperscript{298} does not influence the activation of either MEK1 or ERK1/2.

3.5. Discussion

Numerous studies have identified novel GLP-1 secretagogues and their associated signaling pathways in the intestinal L cell. In the current study, I determined, for the first time, an important role for the Rho GTPase, Cdc42 in the L cell. Through the use of siRNA oligonucleotides and dominant-negative constructs, my findings demonstrate a requirement for Cdc42 in insulin-induced actin remodeling, activation of the canonical ERK1/2 pathway, and GLP-1 secretion in the L cell. Moreover, I also determined the ability of the actin cytoskeleton to control secretagogue-induced GLP-1 secretion, through the use of the well-characterized actin depolymerizing and stabilizing agents, latrunculin B and jasplakinolide, respectively. Results from the present study also demonstrate that transient remodeling of the actin cytoskeleton is a prerequisite event prior to GLP-1 release from the L cell.

In the intestine, Cdc42 has been found to regulate expression of the brush-border enzyme, villin, in addition to controlling epithelial cell restitution following injury (467, 468). Moreover, effector proteins from \textit{Salmonella} have been shown to activate Cdc42 in intestinal epithelial cells, which promotes infection-induced actin polymerization and interleukin-8 secretion (469,
In the present study, insulin was found to activate Cdc42, but not Rac1, although both proteins were expressed in the murine L cell. To date, there are no published reports of insulin signaling through Cdc42 to mediate hormone secretion, although the importance of Rho GTPases, such as Cdc42 and Rac1, in regulated exocytosis from other endocrine cells in response to a variety of different secretagogues has been well documented (456, 458, 464-466, 482).

Over-expression of dominant-negative Cdc42 (T17N) in the murine L cell impaired actin re-polymerization following insulin treatment, such that the G:F actin ratio was significantly higher 30 min after treatment when compared to cells transfected with the empty pcDNA3 control vector. In pilot studies, treatment of murine GLUTag cells with 10⁻⁸ M insulin was found to activate the actin severing enzyme coflin (G.E. L., P.L.B., data not shown). Moreover, Cdc42/PAK1-mediated activation of LIM kinase has been demonstrated to inhibit the activity of coflin (362). Based on results from the current study, the observed impairment in actin polymerization could therefore be accounted by the failure of dominant-negative Cdc42 (T17N) to inhibit the activity of coflin via LIM kinase. Alternatively, Cdc42 is known to control actin polymerization by coupling to the WASP/neural (n-)WASP-Arp2/3 complex, which promotes actin nucleation and elongation (452, 483, 484). Hence, failure to re-polymerize following insulin treatment could also be consequent to altered association with WASP-Arp2/3. As several studies have determined that n-WASP is required for insulin-stimulated actin remodeling and exocytosis (360, 485), further work is required to examine if Cdc42-mediated actin remodeling is facilitated by either WASP or Arp2/3.

PAK1 is a serine/threonine kinase that is an established effector of Cdc42. In the current study, insulin promoted the association and activation of PAK1 through Cdc42, and over-expression of a kinase-dead PAK1 (K299R) abolished insulin-induced GLP-1 secretion from the L cell. Although over-expression of PAK1 (K299R) impaired the insulin response in GLUTag
cells, further studies should be repeated with siRNA to reduce endogenous PAK1 expression to confirm these findings. However, as PAK1 can function as a molecular scaffold (486), both over-expression of PAK1 mutants and knock down of PAK1 may disrupt protein-protein interactions within the cell. Alternatively, a recently developed PAK1 specific inhibitor could be used to inhibit endogenous PAK1 activity (487). A similar role for Cdc42/PAK1 has been described in the pancreatic beta cell, such that the activation of PAK1 via Cdc42-GTP is required for the second phase of glucose-induced insulin secretion (465). Although, PAK1 is hypothesized to prime the activation of the canonical ERK1/2 pathway by phosphorylating Raf-1 on Ser\(^{338}\) or MEK1 on Ser\(^{298}\) (478-481), it is unlikely that this occurs in the L cell, as insulin-induced phosphorylation of MEK1/2 on Ser\(^{217/221}\) or Ser\(^{298}\) and ERK1/2 occurs prior to Cdc42 and PAK1 activation. Furthermore, based on the results of the present study, Ser\(^{298}\) phosphorylation on MEK1 occurs despite PAK1 (K299R)-associated inhibition of insulin-stimulated activation of the canonical ERK1/2 pathway, suggesting that PAK1 is not responsible for Ser\(^{298}\) phosphorylation and, therefore, that another kinase downstream of Cdc42 regulates this event in the L cell. A similar uncoupling between Ser\(^{298}\) phosphorylation and PAK1 activity has been reported in fibroblasts (488), further suggesting that MEK1 activation is not dependent on Ser\(^{298}\) phosphorylation. Notwithstanding, over-expression of either Cdc42 (T17N) or PAK1 (K299R) completely abrogated insulin-induced activation of either kinase, which directly implicates Cdc42 and PAK1 as upstream regulators of the canonical ERK1/2 pathway in the L cell. Recently, modulation of Cdc42 activity by over-expression of Cdc42 (T17N) or of Rho guanine-nucleotide disassociation inhibitors (GDIs) has been found to sequester Ras, thereby preventing its interaction with Raf-1 and subsequent activation of ERK1/2 (489, 490). This may provide an additional mechanism by which Cdc42(T17N) may regulate insulin-induced activation of the canonical ERK1/2 pathway. Collectively, the results of the present study, therefore, suggest that Cdc42 and PAK1 function as mediators of insulin action in the L cell,
by regulating the activation of the MEK-ERK1/2 pathway, actin remodeling, and GLP-1 secretion (Figure 3-10).

Depolymerization of F-actin with latrunculin was found to potentiate insulin-induced GLP-1 secretion from the endocrine L cell suggesting that, similar to other endocrine cells, the actin cytoskeleton functions as a permissive barrier to regulate exocytosis (435, 457, 459). Furthermore, recent electron-micrograph data has shown that actin depolymerization increases the number of secretory granules below the plasma membrane in pancreatic beta cells (491). Interestingly, jasplakinolide pre-treatment to stabilize the actin barrier (458, 482) also potentiated secretagogue-induced GLP-1 release. However, analysis of the kinetics of jasplakinolide action has also revealed its ability to depolymerize actin and reposition granules closer to the cell membrane in the pancreatic beta cell (458, 492). Furthermore, in non-endocrine cells, F-actin may play a facilitative role in exocytosis, such that depolymerization of F-actin actually impairs insulin-stimulated GLUT4 vesicle exocytosis (346, 347, 360, 493). Thus, the findings of similar effects of actin depolymerization and stabilization on GLP-1 release are consistent with a finely-tuned role of actin to regulate exocytosis in the L cell. In summary, the results of the present study demonstrate that the Rho GTPase, Cdc42, functions as a master regulator of insulin action in the L cell to facilitate activation of the canonical ERK1/2 pathway and actin remodeling, leading to GLP-1 secretion. Furthermore, actin remodeling is a key distal process that facilitates insulin-stimulated GLP-1 secretion from the intestinal endocrine L cell. As yet, there are no indications that Cdc42 activity is altered in type 2 diabetes, and since GLP-1 secretion is impaired in this condition, it is possible that Cdc42 could be a novel target to increase endogenous GLP-1 secretion in vivo.
Figure 3-10: Schematic model of insulin-induced GLP-1 secretion from the intestinal L cell.

Insulin receptor activation in the L cell promotes the activation of two signaling pathways: canonical ERK1/2 and Cdc42. Activation of both pathways appears to be required for insulin-stimulated GLP-1 secretion. Solid arrows indicate established effects of insulin in the L cell, while dashed arrows indicate the possibility of additional downstream mediators.
3.6. Acknowledgements

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Chapter 4: Discussion and Conclusions

Author contribution:
All writing was completed by G. Lim.
4.1. Summary of results

In the L cell, insulin has been reported to stimulate proglucagon expression and synthesis (7), but it was unknown if insulin could also induce GLP-1 release from the L cell. Furthermore, given that nutrient-induced GLP-1 secretion is impaired in type 2 diabetes and insulin resistance (3, 6, 226-230, 260), it has been suggested that both conditions exert deleterious effects on L cell function. In chapter 2 of this thesis, I demonstrated for the first time that insulin stimulates GLP-1 secretion from murine and human L cells via the MEK1/2-ERK1/2 pathway. Moreover, I showed that hyperinsulinemic-insulin resistance in vitro and in vivo is associated with impaired GLP-1 release in response to insulin and heterologous GLP-1 secretagogues. In chapter 3, I examined the role of the actin cytoskeleton in GLP-1 secretion from the L cell. Remodeling of the actin cytoskeleton in other endocrine cells is a prerequisite event for granule exocytosis. Hence, just as insulin induces actin remodeling in non-endocrine cells (346-348), I determined that this process is also required for insulin-stimulated GLP-1 release. Furthermore, these events are facilitated through the actions of the Rho GTPase, Cdc42, and its downstream kinase PAK1. In the L cell, Cdc42 appears to be a critical effector of insulin action, as decreases in Cdc42 and PAK1 activity attenuate insulin-induced actin remodeling, activation of the canonical ERK1/2 pathway, and GLP-1 secretion. Despite uncovering these novel functions of insulin in the intestinal L cell, several unanswered questions still require further investigation. Of primary importance, it is necessary to establish if insulin can stimulate GLP-1 release in vivo. Furthermore, as insulin resistance and type 2 diabetes are associated with impaired nutrient-induced GLP-1 release, the contribution of impaired GLP-1 secretion to the pathogenesis of type 2 diabetes requires further assessment. Lastly, identification of the downstream effectors of insulin-stimulated GLP-1 secretion is necessary, as these proteins could serve as novel therapeutic targets to augment GLP-1 secretion as a treatment for type 2 diabetes.
4.2. Insulin: an endogenous GLP-1 secretagogue

Previously, Ryan et al. failed to demonstrate that insulin could stimulate GLP-1 release in fasted humans undergoing a hyperinsulinemic-euglycemic clamp, whereby the rate of insulin infusion was modeled to mimic a post-prandial insulin response (430). In a separate study, Hansen et al. did not detect any significant effect of insulin on GLP-1 release from the isolated, perfused porcine ileum (431). In contrast to these findings, in vitro results from chapter 2 indicate that insulin does stimulate GLP-1 secretion, but only under high glucose conditions (25 mM). The absence of an insulin effect in humans or the porcine ileum may thus be partially explained by the minimal concentrations of luminal nutrients following an overnight fast or by the low-glucose perfusate that was infused into the intestinal lumen, respectively.

While tissue-specific knock-down of the IR has enabled researchers to determine insulin’s actions in specific tissues (263), the presence of multiple insulin-sensitive cell types in the intestinal epithelium may make it difficult to interpret findings from an intestine-specific IR KO model. Furthermore, generation of L cell-specific IR deficient mice is not possible as current methodologies to target the intestinal L cell also affect the pancreatic alpha cell and hypothalamic neurons (9, 494). Nonetheless, to determine whether insulin is an endogenous GLP-1 secretagogue, modifications to the previously mentioned models could be utilized. Hence, in conjunction with the clamp settings used by Ryan et al. (430), intraluminal infusions of fat or glucose in the duodenum should be administered in order to mimic the presence of nutrients following a mixed meal. To target L cells in the distal intestine, fat should be used as the luminal nutrient due to its low insulinotropic activity [R. I. and P. L. B., unpublished observations and (176)] and because it transits to the distal ileum and colon (150). Furthermore, such placement of fat may reveal if insulin functions as a GLP-1 secretagogue during the late phase of GLP-1 release. The L cell may require the presence of glucose for the secretagogue effects of insulin (Figure 2-4), thus, infusion of glucose into the duodenal lumen should be
performed in conjunction with a hyperinsulinemic-euglycemic clamp to mimic the post-prandial state (495). This may elucidate GLP-1-containing cells in the duodenum are responsive to stimulatory effects of insulin on GLP-1 release (142).

In separate studies, high levels of glucose could be infused into the lumen of the isolated, perfused porcine ileum to mimic post-prandial conditions, since glucose will not elicit a beta cell response in this model. Moreover, this model allows for the use of a wide range of insulin concentrations, which will permit determination of whether insulin is a GLP-1 secretagogue at physiologically relevant concentrations.

4.3. Does decreased nutrient-stimulated GLP-1 release contribute to the development of type 2 diabetes?

In studies in which impaired nutrient-induced GLP-1 release was associated with insulin resistance or type 2 diabetes, up to a 30% decrease in GLP-1 secretion was observed (3, 6, 226-230, 260). It is unclear as to whether such a decrease in post-prandial GLP-1 levels directly causes or is consequent to diabetes, but a reduction in endogenous GLP-1 action by infusion of the GLP-1R antagonist, Ex-4 (9-39), or GLP-1 neutralizing antibodies promotes glucose intolerance (52, 60, 496). Furthermore, genetic deletion of the GLP-1R in mice causes elevated fasting glucose levels and impaired glucose tolerance (205). Conversely, exogenous administration of GLP-1 levels in subjects with type 2 diabetes improves glucose tolerance (210). Collectively, these findings indicate that decreased GLP-1 levels in type 2 diabetes may contribute to impaired glucose homeostasis.

It is currently not known how a 30% reduction in GLP-1 levels translates to changes in endogenous GLP-1 action or glucose homeostasis, and in normal, healthy subjects, it is difficult to reduce endogenous levels GLP-1. One potential approach would be to infuse somatostatin, which reduces GLP-1 release via the sstR5 isoform (497). However, these findings would be confounded by the ability of somatostatin to also inhibit endogenous insulin and glucagon.
secretion (498, 499). Furthermore, it is not known which somatostatin receptor isoforms are expressed in the human L cell, although the expression of sstR5 in human pancreatic alpha and beta cells (500) prevents the use of selective sstR5 agonists to inhibit endogenous GLP-1 secretion. Alternatively, Ding et al. recently demonstrated that heterozygous Pax6 deficient mice have decreased fasting GLP-1 levels and impaired glucose tolerance; however, nutrient-induced GLP-1 release was not assessed in these mice (501). Nonetheless, gain of function studies could be performed in these mice such that GLP-1 can be administered to restore levels to those seen in wild type mice, followed by metabolic studies to determine if there are any beneficial effects of GLP-1 administration on the phenotype in these mice. While humans with Pax6 mutations also display impaired glucose tolerance, it is not known if there are alterations in GLP-1 levels. Furthermore, since Pax6 is required for differentiation of the endocrine pancreas, it may be difficult to draw conclusions as to whether the impairment in glucose tolerance is due to a defect in islet function or GLP-1 secretion (13, 502).

The debate as to whether impaired GLP-1 secretion is a cause or consequence of type 2 diabetes is difficult to answer. Two studies have suggested that the impaired incretin effect is a consequence of type 2 diabetes; however, in both studies, subjects with type 2 diabetes did not present any reductions in nutrient-induced GLP-1 release, which is contradictory to other published reports (241, 242). It was suggested that any deteriorations in GLP-1 secretion are secondary to other metabolic abnormalities like hyperglucagonemia or chronically, elevated free fatty acids (126, 242). Furthermore, in normal, at-risk individuals for type 2 diabetes, post-prandial GLP-1 secretion is not altered (245-248, 251), which suggests that defective GLP-1 release is a consequence of the diabetic state. Mutations in the diabetes susceptibility gene, TCF7L2, increases the risk of type 2 diabetes (249), potentially through decreased insulinoctropic actions of GLP-1 on the beta cell (106, 251). The effect of this mutation suggests that in some
instances, a reduction in GLP-1 action, but not secretion, is involved in the pathogenesis of diabetes.

In contrast to these findings, obesity and insulin resistance, which are risk factors in the pathogenesis of type 2 diabetes, are associated with decreased nutrient-induced GLP-1 release in humans (5, 6, 229, 255, 260). However, it may prove to be difficult to distinguish which factors, GLP-1 levels, obesity, and/or insulin resistance, influence the development of type 2 diabetes. Rodent models of obesity or type 2 diabetes are fraught with confounding metabolic factors such as elevated free fatty acids and proinflammatory cytokines, leptin resistance, and in some instances, hyperphagia. All of these factors may have different effects on the L cell, some of which still require further investigation. Reductions in GLP-1 levels may also indicate a decrease in the number of L cells in insulin resistance, obesity, or type 2 diabetes, but evidence for an impact on L cell number is conflicting (503, 504). The recent generation of transgenic mice that express an L cell-specific fluorescent protein (9) may permit a more quantitative examination of the impact of these conditions on L cell number. By developing these mice as models of type 2 diabetes, fluorescence-assisted cell sorting would allow for a more reliable method to quantify L cell number than immunohistological techniques. Finally, such studies may demonstrate if decreased L cell number directly contributes to alterations in GLP-1 levels and/or the development of diabetes.

4.4. **How does insulin resistance in the L cell affect secretagogue-stimulated GLP-1 secretion?**

In chapter 2, I determined that insulin resistance in the L cell attenuates GLP-1 secretion in response to insulin, GIP, PMA, and glucose. In GLUTag cells, insulin resistance decreased insulin receptor protein expression and activation (Figure 2-4), which subsequently leads to decreased activation of ERK1/2 and Akt, and impaired GLP-1 secretion. The mechanisms underlying decreased IR expression in the InsRes L cell are beyond the scope of this thesis, but
data from other groups suggest that this may be mediated through FOXO1- or ubiquitin-mediated pathways (371, 372). Furthermore, decreased IR phosphorylation could be due to increased expression and/or activity of phosphatases in insulin resistance (374). Taken together, these findings suggest that one of the key defects that reduces insulin-induced GLP-1 release from InsRes L cells is at a proximal step in the insulin signaling pathway. It is possible, however, that the defect in GLP-1 release occurs at distal step prior to exocytosis, thereby reducing GLP-1 secretion in response to insulin, GIP, PMA, and glucose. Hyperinsulinemic-insulin resistance may directly alter the expression of SNARE proteins, which is associated with decreased exocytosis (505, 506). Furthermore, results from chapter 3 demonstrate that the actin cytoskeleton functions as a barrier to regulate GLP-1 release and remodeling of the actin cytoskeleton is required for insulin- and PMA-induced GLP-1 release. It is currently not known if insulin resistance impairs actin remodeling in the L cell, but in vitro data from other cell models have demonstrated that this phenomenon can impair cellular function. Incubation of myotubes or adipocytes for 24 hr with high insulin and glucose, induces defects in insulin-stimulated actin reorganization, cell-surface expression of GLUT4, and glucose uptake (347, 507). Although it remains to be determined if GIP and glucose stimulate actin remodeling prior to GLP-1 release, one potential mechanism for decreased insulin-, PMA-, glucose-, and GIP-stimulated GLP-1 release from InsRes L cells is, therefore, defective actin remodeling to facilitate exocytosis.

In rodents, obesity-associated insulin resistance has been demonstrated to decrease the expression of the Cdc42-related GTPase, TC10 (508), and similar findings have been reported in insulin-resistant ob/ob mice (509). Since Cdc42 is a key Rho GTPase that mediates insulin-stimulated actin remodeling and GLP-1 secretion from the L cell, the impact of insulin resistance on expression of Cdc42 requires further investigation. Although the GEF protein responsible for Cdc42 activation in the L cell is also not known, reductions in the expression or activity of GEF
proteins could also prevent insulin-stimulated activation of Cdc42. Once such GEF is ARHGEF11, as mutations in ARHGEF11 are associated with defects in glucose utilization following an oral glucose challenge in individuals with type 2 diabetes, which suggests an impaired incretin response (510). Until the GEF protein involved in insulin-induced Cdc42 activation is identified, it is not possible to determine whether insulin resistance directly affects GEFs, but collectively, these findings demonstrate that numerous pathways can reduce the responsiveness of the L cell to insulin.

### 4.5. Candidate downstream effectors involved in insulin-stimulated GLP-1 secretion

In chapter 2, I established that activation of the MEK1/2-ERK1/2 pathway is critical for insulin-stimulated GLP-1 secretion; however, effector molecules downstream of ERK1/2 were not determined. It remains unclear as to whether ERK1/2 can directly modulate the exocytotic machinery (i.e. SNARE proteins) in the L cell. However, as the intestinal L cell shares numerous secretory mechanisms with pancreatic beta cell (138, 146-148), it is plausible that similar proteins involved in ERK1/2-dependent insulin secretion are present in the L cell. For example, Longuet et al. demonstrated that glucose-dependent insulin secretion in MIN6 clonal beta cells is associated with ERK1/2-mediated phosphorylation of synapsin I, a granule-associated protein, which upon phosphorylation, disassociates from the actin cytoskeleton and allows granules to undergo exocytosis (434). In murine GLUTag cells, I have identified the presence of both mRNA transcripts and protein for synapsin I (G.E.L., P.L.B., unpublished observations); however, further work is required to determine if synapsin I is downstream of ERK1/2 and involved in insulin-stimulated GLP-1 release from the L cell.

It is also still unclear as to how insulin activates the Rho GTPase Cdc42. SiRNA-mediated knockdown of Cdc42 and over-expression of the dominant-negative Cdc42 mutant demonstrates that this Rho GTPase is indispensable for insulin-stimulated GLP-1 secretion.
Activation of Cdc42 by insulin may be mediated by the canonical Cdc42 GEF, Dbl (511), or by the recently identified Intersectin-1L (512), but it is currently not known how insulin receptor activation signals to these proteins. Alternatively, in adipocytes, insulin-induced activation of Cdc42 is dependent on the activity of PI3K (351); however, this is not the case in the L cell, as inhibition of PI3K did not prevent insulin-induced GLP-1 secretion. These findings suggest that PI3K activity per se is not required for insulin-stimulated Cdc42 activation in the L cell. Rather, the PI3K regulatory subunit, p85α, may play a role in activating Cdc42. In hepatocytes, the p85α monomer is required for insulin-regulated Cdc42 activation (356), and it is postulated that p85α recruits a yet unidentified GEF protein to facilitate Cdc42 activation. Co-immunoprecipitation studies are required to determine whether a similar role for p85α exists in the L cell, although functional analysis of this interaction may be difficult to interpret as overexpression or knockdown of p85α levels can inhibit or augment insulin signaling, respectively (403, 404).

While the Cdc42-PAK1 axis influences insulin-stimulated activation of the MEK1/2-ERK1/2 pathway in the L cell, it is not known if there are any reciprocal actions of the MEK1/2-ERK1/2 pathway on Cdc42 activation. Cdc42 GTPase-activating protein (CdGAP) is a ubiquitously expressed GAP that enhances the GTPase activity of Cdc42 and interacts with ERK1/2 via SH3 domains. Phosphorylation of Thr776 within the C-terminal proline-rich domain by ERK1/2 inhibits the activity of CdGAP, and inhibition of ERK1/2 activation may promote the activity of this enzyme (513). This suggests that inhibition of the canonical ERK1/2 pathway, as demonstrated in chapter 2, may partially explain why insulin-stimulated GLP-1 release is attenuated in insulin resistance and highlights the complexity of insulin signaling in the L cell. The role of the MEK1/2-ERK1/2 pathway in actin remodeling is also not fully understood, but in the beta cell, ERK1/2 activity does not affect actin reorganization. In contrast, activation of the canonical ERK1/2 pathway is required for cytoskeletal reorganization in endometrial and
endothelial cells (514, 515). Further work is required to examine if insulin requires ERK1/2 to mediate its effects on actin remodeling in the L cell. Nevertheless, these findings suggest that activation of either pathway may be more complex than was originally proposed in chapter 3 (Figure 3-10).

4.6. Conclusions

Given the importance of GLP-1 to the regulation of glucose homeostasis and metabolism, it is understandable why pharmacological approaches to enhance the actions of GLP-1 are being used as a treatment for type 2 diabetes. However, GLP-1 is released in response to numerous secretagogues via distinct and overlapping signal transduction pathways, and one alternative approach to treat diabetes could be to target downstream effectors involved in GLP-1 secretion. As insulin resistance has deleterious effects on L cell function, future studies should examine if insulin-sensitizing agents are able to restore L cell function and GLP-1 secretion. While it appears that the Cdc42-PAK1 axis plays a critical role in regulating the activity of the canonical ERK1/2 pathway, actin reorganization, and GLP-1 secretion in the L cell, further studies are required to examine the roles of these proteins in vivo. Nonetheless, results from this thesis demonstrate, for the first time, that insulin is an endogenous GLP-1 secretagogue, and that insulin resistance may play a role in impairing GLP-1 secretion in conditions of obesity or type 2 diabetes.
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