Mechanisms underlying metformin-induced secretion of glucagon-like peptide-1 from the intestinal L-cell

by

Andrew James Mulherin

A thesis submitted in conformity with the requirements for the degree of Master’s of Science Graduate Department of Physiology University of Toronto

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Andrew Mulherin
Master of Science
Department of Physiology
University of Toronto
2011

General Abstract
The incretin hormone glucagon-like peptide-1 enhances glucose-dependent insulin secretion and is therefore a most attractive therapeutic approach for the treatment of Type 2 Diabetes Mellitus. The anti-diabetic drug, metformin, has previously been shown to increase circulating levels of GLP-1, although its mechanism of action is currently unknown. Neither metformin nor AICAR (activators of AMPK) directly stimulated GLP-1 secretion from the L-cell in vitro. However, oral treatment of rats with metformin enhanced plasma levels of active and total GLP-1, independent of GLP-1 degradation. Furthermore, pre-treatment with the general muscarinic antagonist, atropine, or the M3 antagonist, 4-DAMP, decreased metformin-induced GLP-1 secretion, while M1 and M2 antagonists did not. Chronic bilateral subdiaphragmatic vagotomy had no effect, while the GRP antagonist, RC-3095, reduced metformin-induced GLP-1 secretion. Therefore, I conclude that metformin-induced GLP-1 secretion occurs in part through the parasympathetic nervous system, the M3 and GRP receptors, but is independent of the vagus nerve.
Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Patricia Brubaker. It has been through her continuous guidance and support that I have gotten to where I am now. Her enthusiasm and dedication not only to her research but to the success of her students is inspiring; one could not ask for a more knowledgeable and motivating mentor. I have been fortunate to work under her direction for the past two years, and I have no doubt that her influence will help shape me into the clinician-scientist I hope to someday become.

To my parents I express the utmost gratitude for their constant support and encouragement throughout all of my studies in university, and most importantly, throughout my last two years as a graduate student. For the many telephone calls which have yielded words of wisdom and motivational speeches, I will forever be grateful for their love and support, and the confidence they have given me to push myself to new limits.

The amount of work I have accomplished during my Master’s would not have been possible without the help of many others; therefore, I would also like to thank the undergraduate students that I have had the opportunity to work with over the past two years. I am grateful to both Helena Kim, for her work on the metformin sensitization study, and Amy Oh, for her work on the FRIC secretion experiments. Additionally, I am also very appreciative of the efforts of Dr. Lina Lauffer and Anthony Grieco. Although I did not have the good fortune of working with them, their preliminary work on metformin and GLP-1 secretion in vitro provided the foundation for my master’s project and I am grateful for their contributions.

To those that have helped me with the various lab techniques and protocols I have used throughout this degree, I give my most sincere gratitude. I would especially like to thank our lab technician, Angelo Izzo, for teaching me many of the techniques that I have used to accomplish these experiments. I would also like to thank the staff of the Division of Comparative Medicine, especially Dr. Kate Banks, Frank Giuliano, Tracy McCook, Sara Johnson, and Rainer de Guzman for their assistance with many of the animal procedures performed.

To those that I have had the pleasure of working with in the Brubaker lab, I don’t think I could have been more fortunate than to end up with such great lab-mates. I would especially like to mention fellow graduate students Katie Rowland, Monika Poreba, Shivangi Trivedi, and Jasleen Chahal, postdoc Ganesh Sangle, and undergrad students Stuart Wiber, Charlotte Dong, and Erli Mingomataj; our time spent together both inside and outside of the lab has made for many fond memories. I am privileged to have worked alongside such great colleagues and friends.
I would like to separately thank another member of the Brubaker lab, Dr. Victor Wong. When I first joined the lab I had not counted on meeting such an amazing and sincere friend, mentor, and (somewhat unexpectedly) training partner. I have never seen someone so motivated to achieve success and I am grateful for the inspiration that he has instilled in me, and for the motivation he has given me to strive for perfection in my academic and athletic endeavours.

Finally, I would like to take an opportunity to thank the many other people that have influenced me and have made a positive impact in my life throughout the past two years. To my undergraduate professor, Dr. Peter Cashion, and project supervisor, Dr. Sara Eisler; their passion for teaching and research is what inspired me to pursue a graduate degree in physiology, and I will be forever grateful for their ‘push out the door’ that motivated me to leap out of my comfort zone and come to Toronto. I am also grateful to have a number of close friends, both in Toronto and in Fredericton, that have contributed greatly to my success during my two years in Toronto. Most importantly I would like to mention Dan Way, an admirable running adversary and a constant reminder that the hard work of grad school should be accompanied with lots of laughter, fun, and philosophical talks about life; and Trinda Hayden, to whom I am grateful for the many late nights spent talking and the numerous hours spent listening to practice presentations about metformin and GLP-1.

Thank you to everyone who has helped to make this possible.
# Table of Contents

General Abstract ii  
Acknowledgements iii  
Table of Contents v  
List of Figures vi  
List of Abbreviations vii

1 Introduction  
1.1 General rationale  
1.2 Type 2 diabetes mellitus  1  
1.2.1 Current treatment strategies for T2DM  3  
1.3 The physiology of glucagon-like peptide-1  4  
1.3.1 The incretin effect  4  
1.3.2 Production, circulation, and degradation of GLP-1  5  
1.3.3 Indirect regulation of GLP-1 secretion  8  
1.3.4 Direct regulation of GLP-1 Secretion  11  
1.3.4.1 Nutrient regulation  11  
1.3.4.2 Hormonal regulation  15  
1.3.4.3 Neural regulation  17  
1.3.5 Biological actions of GLP-1  18  
1.3.6 Incretin mimetics and GLP-1 therapy for T2DM  20  
1.4 Metformin  22  
1.4.1 Metformin as a treatment for T2DM  22  
1.4.2 Mechanism of action of metformin  23  
1.4.3 Other effects of metformin  24  
1.4.4 Metformin and GLP-1  27  
1.5 Hypothesis and aims  29

2 Methods  
2.1 In vitro experiments  30  
2.1.1 Cell culture  30  
2.1.1.1 GLUTag  30  
2.1.1.2 NCI-H716  30  
2.1.1.3 FRIC  31  
2.1.2 Secretion experiments  32  
2.1.3 Radioimmunoassay  33  
2.1.4 Western blot  34  
2.2 In Vivo Experiments  34  
2.2.1 Animals  34  
2.2.2 Secretion experiments  35  
2.2.3 Control experiments  35  
2.2.4 DPP-IV activity assay  36  
2.2.5 Bilateral subdiaphragmatic vagotomy  36  
2.3 Data analysis  38
3 Results

3.1 Assessment of metformin action in vivo 38
3.2 Assessment of metformin action in vitro 38
3.3 Role of the muscarinic receptors in mediating metformin-induced GLP-1 secretion 41
3.4 Role of the vagus nerve in mediating metformin-induced GLP-1 secretion 45
3.5 Role of the GRP receptor in mediating metformin-induced GLP-1 secretion 49

4 Discussion
4.1 Limitations and future studies 52

5 Reference List 62

List of Figures

Figure 1.1 – Differential processing of the proglucagon polypeptide in the pancreas and intestines 7
Figure 1.2 – Indirect regulation of GLP-1 secretion 10
Figure 3.1 – Metformin and AICAR stimulate GLP-1 secretion in rats 40
Figure 3.2 – Acute metformin treatment does not affect plasma DPP-IV activity in rats 40
Figure 3.3 – Metformin does not stimulate GLP-1 secretion in vitro 42-43
Figure 3.4 – Metformin does not sensitize the L-cell to GLP-1 secretagogues 44
Figure 3.5 – The M3 muscarinic receptor mediates metformin-induced GLP-1 secretion in vivo 46-47
Figure 3.6 – Metformin increases levels of active GLP-1 in vivo 48
Figure 3.7 – Bilateral subdiaphragmatic vagotomy does not affect metformin-induced GLP-1 secretion 50
Figure 3.8 – The GRP receptor mediates early metformin-induced GLP-1 Secretion 51
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>4-DAMP</td>
<td>1,1-dimethyl-4-diphenylacetoxypiperidinium</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-coenzyme A carboxylase</td>
</tr>
<tr>
<td>AICAR</td>
<td>aminomidazole carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CPT-1</td>
<td>carnitine parmitoyltransferase-1</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle's medium</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FRIC</td>
<td>fetal rat intestinal culture</td>
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<tr>
<td>G6ase</td>
<td>glucose-6-phosphatase</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GIP</td>
<td>glucose-dependent insulinotropic peptide</td>
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<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>gastrin-releasing peptide</td>
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<tr>
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<td>glicentin related polypeptide</td>
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<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<tr>
<td>IP</td>
<td>intervening peptide</td>
</tr>
<tr>
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<td>liver kinase B1</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MPGF</td>
<td>major proglucagon fragment</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
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<tr>
<td>Oct</td>
<td>organic cation transporter</td>
</tr>
<tr>
<td>PAM</td>
<td>peptidylglycine alpha-amidating monooxygenase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>prohormone convertase</td>
</tr>
<tr>
<td>PCOS</td>
<td>polycystic ovary syndrome</td>
</tr>
<tr>
<td>PEPCk</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>parasympathetic nervous system</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SGLT1</td>
<td>sodium-glucose co-transporter-1</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SU</td>
<td>sulfonylurea</td>
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<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
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<tr>
<td>TBS</td>
<td>trizma buffered saline</td>
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<tr>
<td>TEMED</td>
<td>tetramethylmethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinediones</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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**Prefixes and other abbreviations**

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<th>Prefix</th>
<th>Symbol</th>
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<tbody>
<tr>
<td>m</td>
<td>milli-</td>
<td>(x 10^{-3})</td>
</tr>
<tr>
<td>µ</td>
<td>micro-</td>
<td>(x 10^{-6})</td>
</tr>
<tr>
<td>p</td>
<td>pico-</td>
<td>(x 10^{-12})</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
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</tr>
<tr>
<td>ºC</td>
<td>degrees Celsius</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>l</td>
<td>litres</td>
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</tr>
<tr>
<td>M</td>
<td>moles per litre</td>
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</tr>
<tr>
<td>min</td>
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<tr>
<td>i.v.</td>
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</tr>
<tr>
<td>p.o.</td>
<td>per os</td>
<td></td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
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1 Introduction

1.1 General rationale

Type 2 Diabetes mellitus (T2DM) has become one of the greatest health problems in North America. Furthermore, with industrialization and spread of the Western diet and lifestyle to developing countries, the incidence of T2DM has increased dramatically all over the globe. The severity and prevalence of this disease highlights the importance of developing novel treatment options that are both effective and cost-efficient. Within the past decade, the incretin hormone glucagon-like peptide-1 (GLP-1) has gained considerable attention as a new drug for T2DM, proving as effective as conventional pharmacological therapies, and with fewer adverse effects. Therefore, there is a great interest in understanding the mechanisms involved in regulating the secretion, degradation, and actions of GLP-1. Interestingly, the commonly-prescribed T2DM drug, metformin, has been shown to increase levels of plasma GLP-1 in vivo, although the mechanism by which this occurs is still unknown. While some suggest that this effect is due to inhibited degradation of GLP-1, a growing consensus agrees that it is instead through increased secretion. A better understanding of the mechanism by which metformin enhances endogenous GLP-1 may lead to novel therapeutic avenues for the treatment of T2DM.

1.2 Type 2 diabetes mellitus

Diabetes mellitus can be classified into two categories: insulin-dependent diabetes mellitus (T1DM), which comprises only 5-10% of patients, and insulin-independent diabetes mellitus (T2DM) which makes up the remaining 90-95% (1). One common characteristic these two diseases share is the existence of insulin deficiency, but from here the pathophysiology of Type 1 and Type 2 diverge significantly.
T1DM is predominantly an autoimmune disease, and involves a selective destruction of the insulin-producing beta cells by the body’s immune system. Due to the etiology and pathology of T1DM, the treatment strategy is straightforward, as insulin is the only medication which can aid with glucose control.

On the other hand, T2DM is a much more complicated disease. Over 170 million people worldwide currently have T2DM, and this number is expected to increase to over 360 million within the next 20 years (2). Even more alarming is the prevalence of this disease among children and adolescents, which was once thought to be a disease of older generations (3). Simply, T2DM can be characterized by hyperglycemia due to insulin resistance and progressing insulin deficiency, however, in reality, it is a complex metabolic disorder resulting from a large number of underlying factors and conditions. Moreover, it is a progressive disease that often leads to further complications such as retinopathy, nephropathy, neuropathy, coronary artery disease, stroke, and peripheral arterial disease. In fact, T2DM is the leading cause of medically related disabilities in North America (4). Both the prevalence and severity of this disease have provided the motivation to further understand the pathophysiology of T2DM and to develop more effective treatments. Therefore, there is no single cause for this disease, and there is still controversy regarding the underlying factors leading to the development of T2DM (5). Regardless, fasting hyperglycemia remains the principle diagnostic for T2DM, and is primarily caused by the impaired uptake of glucose into the peripheral tissues (skeletal muscle and adipose tissue), as well as by increased glucose output by the liver (6).
1.2.1 Current treatment strategies for T2DM

In addition to insulin treatment itself, there are multiple classes of drugs currently available for the treatment of T2DM; the most common are discussed in brief below.

Metformin remains the gold standard for the treatment of T2DM. It is the most commonly prescribed T2DM drug, and has been available for over 50 years (7). Metformin is highly effective at reducing Hemoglobin A$_{1C}$ (HbA$_{1C}$) levels, with an average decrease of 1.0 to 2.0% (8). It has also been shown to have a low risk of serious side effects, although it has been implicated in cardiovascular risks and lactic acidosis. The primary reason for discontinuation is due to gastrointestinal discomfort, present in up to 50% of treated patients (7;9;10). A more detailed description of metformin’s effects will be discussed in section 1.4.

The thiazolidinediones (TZDs) are a class of PPAR activators. The TZDs bind to the nuclear receptor PPAR-$\gamma$, resulting in the upregulation of key genes involved in fatty acid storage and glucose metabolism; the overall result is increased insulin sensitivity and enhanced peripheral glucose utilization (4). TZDs are effective at reducing HbA$_{1C}$ levels on the order of 0.5 to 1.4% (8), and are additionally associated with improved health markers, such as decreased plasma lipid levels, decreased blood pressure, and improved endothelial function. Adverse effects of these drugs include increased risk of heart failure, and this is the reason for the controversy associated with the prescription of some TZDs (4).

The sulfonylureas (SUs) are a class of insulin secretagogues. These drugs bind selectively to the ATP-dependent potassium (K$_{ATP}$) channels of the beta cell. This inhibition leads to glucose-independent cell depolarization and influx of calcium, resulting in insulin secretion. These drugs have long been used to treat T2DM, and are effective in reducing HbA$_{1C}$ levels by
1.0 to 2.0% (8). Treatment with SUs has been shown to provoke weight gain in patients, and the most serious side effect remains the increased risk of hypoglycemia (4).

The alpha-glucosidase inhibitors are an oral class of drug used to slow the digestion, and consequent absorption, of dietary carbohydrates. These drugs bind competitively to the dissaccharidases present on the intestinal brush border that break down carbohydrate polymers. This inherently delays glucose uptake and thus reduces the overall glucose load commonly observed postprandial. These drugs are not as effective at lowering HbA1c levels as the previous therapies, and are associated with a reduction of 0.5 to 0.8% (8). The most common side effects of these therapies are gastrointestinal, due to the decreased absorption of nutrients.

Finally, incretin therapy has become a major approach to the treatment of T2DM within the past decade. These drugs are used to mimic the actions of GLP-1 and to increase the half-life of both GLP-1 and glucose-dependent insulinotropic peptide (GIP). The different classes of these drugs and their effectiveness with respect to GLP-1 therapy are further explained in section 1.3.7. These two hormones have been shown to increase glucose-dependent insulin secretion, in addition to numerous other beneficial effects. In fact, GLP-1 has gained considerable attention due to its overall favourable effects in patients with T2DM. The following section will discuss in more detail the physiology of the incretin, GLP-1.

1.3 The physiology of glucagon-like peptide-1

1.3.1 The incretin effect
The most notable and important action of GLP-1 is its effect as an incretin (11-13). The incretin effect itself was first identified in the early 1900s (14), although the key modulators involved were not determined until much later (15-17). The term ‘incretin effect’ refers specifically to the
augmentation of glucose-induced insulin secretion through the effects of oral nutrient-induced gut factors, known to be the two gastrointestinal hormones, GLP-1 and GIP (15;16). This effect can best be observed in individuals given an oral glucose load compared to individuals given an isoglycemic intravenous load; wherein oral administration results in a two or three-fold higher increase in insulin secretion compared to intravenous glucose (18;19). This effect is mediated by the secretion of GLP-1 and GIP in response to glucose ingestion (20;21). In addition to glucose-induced secretion, GLP-1 and GIP levels increase after a mixed meal, and this response plays an important role in mediating postprandial plasma insulin levels. Thus far, only GLP-1 and GIP have been identified as incretins (12), and their effects have been shown to be synergistic in stimulating insulin secretion (22). Furthermore, while individually both GLP-1 and GIP potentiate insulin secretion, GLP-1 is a more potent secretagogue than GIP at equivalent concentrations (13). However, GIP is found circulating the plasma at ten-times higher concentrations than GLP-1 and thus, physiologically, they appear to have equal insulin-stimulating potential (23;24). While both hormones are essential in mediating the incretin effect and regulating postprandial plasma glucose levels (20;21);(25-27), GLP-1 also exerts numerous other biological effects that have been shown to be anti-diabetic in nature. Therefore, this section will focus on the production, regulation, and secretion of GLP-1, as well as on its effects in vivo.

1.3.2 Production, circulation, and degradation of GLP-1

GLP-1 shares a significant sequence homology with glucagon, as well as with GLP-2 and, in fact, all three hormones are derived from the same gene, proglucagon. The proglucagon gene is expressed in the pancreatic alpha cells, and in the intestinal L-cells, hypothalamus, and brainstem (28;29). The proglucagon gene itself is transcribed and translated as a single 180 amino acid
polypeptide in each of these tissues (28), but it is the differential post-translational processing of glucagon that determines which hormones will be produced in which tissue (Fig 1.1). Hence, in the alpha cell, glucagon is produced by the actions of prohormone convertase (PC) 2, which also yields the peptides glicentin related polypeptide (GRPP), major proglucagon fragment (MPGF), and intervening peptide (IP) 1. On the other hand, in the intestinal L-cells, PC1/3 cleaves proglucagon to yield GLP-1 and GLP-2, in addition to glicentin, oxyntomodulin, and IP-2 (30;31). The GLP-1(1-37) fragment cleaved from proglucagon by PC1/3 is biologically inactive, and it undergoes further processing by the same enzyme before it is secreted (31). The active form of GLP-1 is present in two isoforms: GLP-1(7-37), and the further processed GLP-1(7-36NH$_2$). While these forms are both biologically active (32), the amidated form is found at higher concentrations in the plasma (33), and it is believed this processing by the enzyme peptidylglycine alpha-amidating monoxygenase (PAM) confers an enhanced half-life over the non-amidated form (34). The half-life of GLP-1 in vivo is approximately 2 minutes, due to its rapid degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV), otherwise known as CD26 (35). DPP-IV is a ubiquitous enzyme with a soluble, free floating form found in the circulation, and an anchored form that is expressed in numerous tissues throughout the body and on the surface of endothelial tissues, such as those that line blood vessels and, notably, the capillaries adjacent to L-cells where GLP-1 is secreted. As such, more than half of active GLP-1 has already been degraded to the inactive form before it reaches the systemic circulation (36). As a serine protease, this enzyme acts by cleaving dipeptides from the amino terminus of proteins containing an alanine or proline at position 2. Active GLP-1 contains an alanine at this position and thus is cleaved by DPP-IV between amino acids 8 and 9, producing the inactive forms GLP-1(9-37) and GLP-1(9-36NH$_2$) (37-39).
Figure 1.1 – Differential processing of the proglucagon polypeptide in the pancreas and intestines. In the alpha cell, prohormone convertase (PC) 2 cleaves the peptide into glicentin-related pancreatic peptide (GRPP), glucagon, intervening peptide-1 (IP1), and the major proglucagon fragment. In the L-cell, PC 1/3 cleaves proglucagon into glicentin, oxyntomodulin, GLP-1, IP2, and GLP-2. These peptides are also liberated in the brain, although PC 1/3 has not specifically been demonstrated to mediate this.

Despite the sequence homology of the three glucagon-related hormones, they have vastly different regulation, stimulation, and effects in the body. In the pancreatic alpha cells, proglucagon gene expression is upregulated primarily through fasting and hypoglycemia, and secreted glucagon acts opposite to the actions of insulin to increase plasma glucose levels through upregulation of hepatic glucose output (40). However, in the intestinal L-cell, proglucagon expression is upregulated in response to nutrient ingestion, and secreted GLP-1 acts to enhance the actions of insulin to reduce plasma glucose. In contrast, GLP-2 is a gut trophic hormone and oxyntomodulin is a satiety factor (40). GLP-1, GLP-2, and oxyntomodulin are secreted simultaneously from the intestinal L-cells (41), which are predominantly located in the distal ileum and colon (42).
1.3.3 Indirect regulation of GLP-1 secretion

Stimulation of the L-cell by nutrients and the resulting secretion of GLP-1 is complex and involves both indirect and direct mechanisms. Early studies looking at GLP-1 secretion in humans found that nutrient-induced GLP-1 secretion followed a biphasic pattern of secretion (43;44). Hence, an early spike in plasma GLP-1 levels was found within 10 to 15 minutes of food consumption, while a later, more prolonged rise occurred at approximately 60 to 90 minutes post-ingestion (43). The initial, profound spike in GLP-1 levels could not be explained by nutrients acting directly on the L-cell because 1) the majority of L-cells are located in the distal gut (42) where nutrients are unable to reach them in such a short time (45), and 2) while some (46) postulate that nutrients could be acting on proximally located L-cells in the duodenum, other studies show that there are too few L-cells in the proximal gut to account for the high levels of GLP-1 secreted in this early phase. This later point was part of an important set of experiments that demonstrated that in rats and humans, the concentration of L-cells increases from the proximal to the distal gut, and with highest numbers of these cells in the distal ileum and colon (42). Related studies showed that by removing the distal gut in anaesthetized rats, nutrient-induced GLP-1 secretion was abolished when fat was infused into the duodenum, indicating that distal L-cells were essential for this early secretion (47). Furthermore, since nutrients were not directly acting on these distal L-cells to induce secretion, it was proposed that an indirect loop must account for this early stimulation, and could be mediated through a number of hormonal and/or neural pathways (47). Experiments conducted in rats showed that ligation of the proximal gut to prevent nutrients from entering the ileum did not affect nutrient-induced GLP-1 secretion. Additionally, a bilateral subdiaphragmatic vagotomy completely abolished this increase, indicating that nutrients did indeed stimulate L-cells in the distal gut mediated through the vagus
nerve. A later study in humans had shown that the general muscarinic antagonist atropine reduced the early phase of nutrient-induced GLP-1 secretion (48).

Interestingly, studies by other groups have also established a role for the sympathetic nervous system in inhibiting GLP-1 secretion. Studies in pigs demonstrated that electrical stimulation of sympathetic fibres decreased electrically-stimulated GLP-1 secretion, an effect that was abolished with the alpha-adrenergic inhibitor, phentolamine (49). Further studies in rats confirmed these findings using selective agonists for alpha- and beta-adrenergic receptors, as well as selective receptor antagonists. These studies found both inhibitory and stimulatory roles for these receptors, whereby inhibiting the alpha-1 subtype resulted in enhanced GLP-1 secretion, consistent with Hansen et al. (49), and inhibition of the beta2- and alpha-2 subtypes resulted in decreased epinephrine-induced GLP-1 secretion, establishing an opposing stimulatory role (50).

Finally, in addition to the parasympathetic and sympathetic nervous systems, additional studies have also demonstrated roles for GIP (51) and gastrin-releasing peptide (GRP) (52) as intermediates in the proximal-distal loop. While it is not fully understood what role the proximal gut derived hormone, GIP, plays in mediating this indirect loop, studies have shown that it is a strong GLP-1 secretagogue. When fat is infused into the duodenum, GIP levels rise slightly before the rise in GLP-1 occurs (47). Moreover, physiological levels of GIP infused into rats results in an increase in GLP-1 secretion that is abolished by hepatic-branch vagotomy, while supra-physiological doses of GIP stimulate GLP-1 secretion that is not reduced through vagotomy. This indicates not only does GIP play a role in mediating the proximal-distal loop, but also that it can act directly on the L-cell under high enough concentrations to induce secretion (51). The distally-derived hormone, GRP, was also found to play a role in the indirect pathway.
Rats infused with supra-physiological doses of GRP showed increased levels of GLP-1 secretion, while rats treated with a GRP receptor-antagonist then infused duodenally with fat, showed deceased levels of secretion (52). Furthermore, nutrient-induced GLP-1 secretion is abrogated in GPR receptor null mice (53). Together these studies indicate the importance of GRP as an intermediate in this pathway.

Figure 1.2 shows a summary of this complex regulatory pathway, involving the indirect stimulation of GLP-1 secretion from nutrients in the distal gut, as well as the inhibitory regulation of the sympathetic nervous system.

Figure 1.2 – Indirect regulation of GLP-1 secretion. Nutrients entering the proximal gut activate the vagus nerve either through the secretion of GIP from K-cells, or through a yet to be determined alternative sensing method. In rodents vagal afferent stimulation occurs through the actions of GIP, while in humans less is known about this pathway. Stimulation of vagal afferent fibres leads to GLP-1 secretion, mediated through the vagal efferents and the muscarinic (M1 and M2) receptors, as well as through enteric GRP or cholinergic neurons. Furthermore, GIP may have a direct role in mediating L-cell stimulation. Finally, the sympathetic nervous system has been shown to play an inhibitory role in regulating GLP-1 secretion through activation of the adrenergic (specifically beta) receptors. Hashed lines represent currently unknown pathways or mechanisms.
1.3.4 Direct regulation of GLP-1 secretion

*In vivo* and *in vitro* experimentation has led an excellent understanding of the mechanisms by which nutrients and other effectors modulate GLP-1 secretion through direct interaction with the L-cell. Following is a brief look at known GLP-1 secretagogues, and their mechanisms of action.

1.3.4.1 Nutrient regulation

As mentioned above, the second phase of GLP-1 secretion is due to direct activation of the L-cell by luminal nutrients in the distal gut. While sugars, proteins, and fats have all been demonstrated *in vivo* and *in vitro* to stimulate GLP-1 secretion, it is believed that this second phase of secretion is primarily caused by fat, since the majority of sugars and proteins are readily absorbed within early sections of the gut and, under normal conditions, only fat reaches the distal ileum. Nonetheless, studies in rats have shown that direct perfusion of glucose (47;54), protein hydrolysate (55-57), or fat (47;54;55;58) into the distal ileum increases plasma GLP-1 levels. Furthermore, in humans with rapid gastric emptying (59), or in when glucose absorption in the proximal gut is reduced (60), resulting in glucose reaching the distal ileum, plasma GLP-1 levels are enhanced, indicating that distal L-cells are indeed sensitive to ingested glucose.

Glucose has been shown to be a strong stimulator of GLP-1 secretion from the L-cell *in vitro* (61-63). Most of what is known about how the L-cell senses and responds to glucose has been determined through use of the GLUTag L-cell line. These studies have demonstrated that glucose-induced GLP-1 secretion results primarily from membrane depolarization and an increase in intracellular calcium concentration (64), although the mechanism by which the L-cell senses these sugars and the pathway leading to secretion is not entirely known.

Electrophysiological studies in the GLUTag cells have shown that glucose elicits a closure of the
$\text{K}_{\text{ATP}}$ channels (63;65), consequently resulting in membrane depolarization and calcium influx via L-type voltage-gated calcium channels, leading to GLP-1 secretion (63-65). The localization of $\text{K}_{\text{ATP}}$ channels on the GLUTag L-cell and involvement in hormone secretion has been elaborately documented (63), but the role of these channels \textit{in vivo} remains controversial. For example, $\text{K}_{\text{ATP}}$ channel null mice display normal glucose-stimulated GLP-1 secretion (66), indicating the presence of an alternative sensing mechanism. It is therefore believed that alternate pathways exist, such as sodium influx through the actions of the sodium-glucose cotransporter-1 (SGLT1). These transporters are the primary means of transporting glucose from the intestinal lumen into epithelial cells, including the L-cell (65). In addition to importing glucose into the cell, these transporters also increase intracellular sodium, resulting in depolarization, thereby accounting for the $\text{K}_{\text{ATP}}$ channel-independent effects of glucose on GLP-1 secretion (65). In addition to the uptake of glucose from the intestinal lumen through the SGLTs, glucose is known to enter intestinal epithelial cells from the plasma through the GLUT2 transporter. Localization of these transporters on the L-cell has not yet been demonstrated, but studies in GLUT2 knockout mice show that these animals display impaired GLP-1 secretion to oral glucose (67).

Finally, a number of recent studies have suggested that glucose may also act on sweet taste receptors localized on the luminal border to induce GLP-1 secretion. The sweet taste receptors, gustducin and T1r2+T1r3, are expressed in enteroendocrine cells throughout the intestine (68) and studies have shown that the sweet-taste receptor antagonist lactisole reduces glucose-induced GLP-1 secretion both in humans (69) and \textit{in vitro} (70). Conversely, additional studies in humans and mice show that several artificial sweeteners and a non-sweet glucose analogue do not stimulate GLP-1 secretion, indicating that the activation of these receptors and subsequent
secretion of GLP-1 is based on more than their sweetness or structural analogy to glucose (71-73).

Fat has been found to stimulate GLP-1 through multiple receptors and pathways, depending on the type of fat. In fact, the magnitude of GLP-1 response to luminal fats has been found to be dependent on the degree of saturation and length of the fatty acid carbon chain (74-76). Monounsaturated fatty acids (MUFAs) stimulate GLP-1 secretion in vitro (61;62;74;77) and in vivo (55). Activation of the L-cell by MUFAs occurs via the G-protein coupled receptors GPR40 and GPR120, which are coupled to Goq subunits (78;79). Activation of these receptors leads to increased intracellular calcium through the phospholipase C (PLC) signalling pathway. Interestingly, it appears that GPR120 plays the dominant role in regulating MUFA-induced GLP-1 secretion (78). Although the exact mechanism of MUFA-induced GLP-1 secretion is unknown, previous studies in our lab have demonstrated a role for PKC zeta in mediating the effects of MUFAs and, more specifically, oleic acid (80;81). GPR119 is an additional receptor activated by the oleic acid analogue, oleoylethanolamide (82). GPR119 has been shown to be an important regulator of nutrient-induced GLP-1 secretion, as studies in GPR119R knockout mice have demonstrated reduced postprandial secretion of GLP-1 (83;84). Furthermore, activation of these receptors with a selective agonist leads to increased secretion. The cellular GPR119 signalling pathway is not fully understood, although the receptor is believed to be coupled to a Go subunit. Finally, bile acids have also been demonstrated to stimulate GLP-1 secretion through the TGR5 receptor, a GPCR also coupled to Go (85). In fact, TGR5 agonists have been shown to improve glucose homeostasis (86;87), and current provide a beneficial avenue for the development of GLP-1 secretagogues for the treatment of T2DM (88;89).
Protein is also a potent stimulator of GLP-1 secretion, although little is known about its mechanism of action. Studies in vivo have shown that whole protein does not stimulate GLP-1 secretion (57). On the other hand, numerous studies have shown that peptones and protein hydrolysates (small peptide fragments) induce secretion both in vivo (56;90) and in vitro (77;91). In fact, recent reports show that certain hydrolysates such as the corn protein, ZeinH, stimulate GLP-1 secretion not only by acting directly on the L-cell, but also through the indirect loop (56). Again, it is unknown how these peptide fragments stimulate direct or indirect secretion, whether there is a receptor specific for certain fragments, or if these fragments act non-specifically to activate receptors in the direct or indirect GLP-1 secretion pathway. A further study by Mochida et al. demonstrated that the ZeinH protein hydrolysate not only stimulated GLP-1 secretion in vivo, but also inhibited DPP-IV plasma activity, whereas a generic meat hydrolysate stimulated GLP-1 secretion to a lesser extent, and did not inhibit DPP-IV (92). This suggests that specific peptide fragments liberated by enzymatic digestion may act as agonists to existing receptors or as inhibitors to DPP-IV. Finally, amino acids such as glutamine, and to a lesser extent asparagine and alanine have been shown to stimulate GLP-1 secretion (44). The exact mechanism is still unknown, but it has been demonstrated that glutamine induces a cellular influx of sodium, membrane depolarization, calcium influx, and consequent hormone secretion (93). Overall, while much is still unknown about the mechanism by which protein stimulates GLP-1 secretion, the idea that these nutrients can enhance GLP-1 secretion without increasing plasma glucose levels provides a novel therapeutic potential for proteins as GLP-1 secretagogues.
1.3.4.2 Hormonal regulation

Considering that GLP-1 enhances postprandial insulin secretion (13), it is interesting that insulin may have reciprocal effects on the L-cell to induce a positive feed-forward loop on GLP-1 secretion. Insulin has recently been shown in our lab to enhance GLP-1 secretion in three *in vitro* cell models in a dose-dependent fashion (94). Furthermore, these studies showed that treatment of the L-cell with insulin resulted in the phosphorylation of Akt and ERK1/2, two established mediators of insulin action in other cell types. Additionally, insulin has also been shown to increase intestinal proglucagon gene expression and L-cell GLP-1 content *in vitro* (95). Overall, while the role of the mechanism of insulin-induced GLP-1 secretion remains uncertain, strong evidence exists in support of this pathway.

Leptin is secreted by white adipose tissue and acts on the hypothalamus to inhibit food intake (96). Obese individuals demonstrate not only an increased resistance to leptin, but also impaired postprandial GLP-1 levels (97;98), an effect that is reversed by weight loss (99). It was therefore hypothesized that leptin could play a regulatory role in GLP-1 secretion. However, leptin was unexpectedly found to increase GLP-1 secretion when administered to rodents *in vivo*, as well as *in vitro*, conclusive with the expression of the leptin receptor on the rodent and human L-cell (100). The cellular signalling mechanism by which leptin induces GLP-1 secretion is not fully known, but studies show that leptin increases STAT phosphorylation in the L-cell (100). Furthermore, leptin has been shown to exert hormone secretagogue activity in other endocrine cell lines, and these studies have shown a dependence on p44/42 and MAPK signalling (101); thus it is possible that leptin exerts its action on GLP-1 secretion through a similar pathway.

In addition to a role for GIP as an established intermediate in the proximal-distal loop as discussed earlier, it has also been shown to have direct effects on the L-cell. GIP is produced
predominantly in the proximal ileum by the K-cell (52;102), although it has also been shown to be present in the distal regions of the gut (103). GIP exerts its actions through activation of the GIP receptor, a GPCR in the glucagon receptor family which couples to a Ga_s subunit (104). Binding of GIP to its receptor leads to activation of adenylyl cyclase (AC) and increased intracellular cAMP, and consequently an influx of calcium resulting in GLP-1 secretion (105). While this pathway has been well demonstrated in the beta cell (106), the full mechanism underlying GIP-induced GLP-1 secretion in the L-cell has yet to be determined.

*In vivo* and *in vitro* studies have shown somatostatin to be a potent inhibitor of GLP-1 (61;107;108). Somatostatin is an inhibitory peptide produced and secreted in the intestine by enteric neurons and by D-cells, as the two isoforms (SS14 and SS28, respectively). The somatostatin receptor (sst) exists as 5 tissue specific isoforms, and it has been previously shown that the rat L-cell expresses one of these subtypes (sst5) (107). These GPCRs are known to couple to Ga_i subunits, and thus activation of these receptors leads to a decrease in cAMP and calcium levels and, consequently, inhibited GLP-1 secretion (66). Additionally, GLP-1 has been shown *in vitro* to increase the secretion of somatostatin, suggesting the presence of a negative feedback loop (107;109).

Finally, in addition to the aforementioned hormones which have been shown to increase GLP-1 secretion both *in vivo* and *in vitro*, there are a number of mediators that do not directly act on the L-cell, including cholecystokinin, glucagon, neurotensin, peptide YY, and vasoactive intestinal peptide (VIP). Intriguingly, the glucagon-like related peptides (GLP-1(7-37), GLP-1(1-37), and GLP-2) were also unable to stimulate secretion (61), suggesting to the absence of direct feedback regulation of the L-cell.
1.3.4.3 Neural regulation

GLP-1 is highly regulated by both the parasympathetic and sympathetic nervous systems. While acetylcholine acts through both the cholinergic muscarinic and nicotinic receptors, only the muscarinic receptors have been demonstrated to be present on the L-cell, and to be involved in L-cell secretion (110). Specifically, Anini et al. demonstrated in FRIC and primary rat ileum the presence of three of the muscarinic receptor subtypes (1, 2 and 3), and their involvement in regulating GLP-1 secretion both in vivo and in vitro (110;111).

Activation of the M1 receptor, which is linked to Gαq, leads to upregulation of PLC and IP3, which consequently increases intracellular calcium leading to GLP-1 secretion (112;113). Paradoxically, Anini et al. found that the M2 receptor, predominantly expressed in cardiac tissue and linked to Gαi (112;114), was also involved in GLP-1 secretion (110). The Gαi subunit, which leads to decreased intracellular cAMP through AC inhibition, is conventionally inhibitory in action, and increased cAMP is a known secretory pathway for GLP-1 (114). Finally, although the M3 receptor (also linked to a Gαq subunit) was shown to be present on the L-cell, Anini et al. demonstrated that it was not directly involved in regulating nutrient-induced GLP-1 secretion in vivo, or cholinergic agonist-induced secretion in vitro (110). Therefore, according to current research, while the L-cell expresses muscarinic receptors 1, 2 and 3, only subtypes 1 and 2 are actively involved in GLP-1 secretion.

Hansen et al. demonstrated the involvement of the sympathetic nervous system in regulating GLP-1 secretion in vivo (49). Additional studies have further established the role of the adrenergic receptors in mediating secretion. Interestingly, administration of a general adrenergic agonist in vivo led to an increased GLP-1 secretion in rats, while the use of a selective agonist for the alpha2-receptor receptor inhibited this secretion (50). It was further determined
that this increased secretion occurred via beta2-receptor occupation, which was verified by Plaisancie et al. (115). Furthermore, this inhibitory and stimulatory effect of the alpha and beta adrenergic receptors, respectively, has not yet been verified in vitro, and studies in our lab have demonstrated that the beta receptor specific agonist, isoproterenol, has no effect on GLP-1 secretion in GLUTag cells (62). This suggests an indirect regulation of the sympathetic nervous system that involves other mediators in vivo.

Finally, the neurotransmitter, gamma-aminobutyric acid (GABA), has been shown to stimulate GLP-1 secretion in vitro (116). GABA is secreted by GABAergic neurons of the enteric nervous system, and the GLUTag L-cell has been previously shown to express one of the three GABA receptor isoforms. Incubation with this chloride channel inhibitor expectedly results in an efflux of chlorine, followed by depolarization and calcium influx, resulting in GLP-1 secretion (116). Interestingly, these recent findings contradict earlier studies, demonstrating that GABA had no effect on GLP-1 secretion in vivo (117) in perfused rat ileum, or in vitro (62) in GLUTag cells. The reasons for these discrepancies are unknown, and could be due to experimental conditions, thus further studies are required to fully elucidate the mechanism of GABA-induced GLP-1 secretion.

1.3.5 Biological actions of GLP-1

In addition to the incretin effect, GLP-1 exerts numerous beneficial actions in vivo, in numerous organs and tissues. In the pancreas, the primarily and most recognized effect of GLP-1 is to stimulate glucose-dependent insulin secretion from the beta cell (13;16). GLP-1 acts together with glucose not only to increase insulin secretion, but also to increase insulin gene transcription, promote mRNA stability and translation, and consequently enhance intracellular concentrations
of insulin in the beta cell (118;119). Furthermore, GLP-1 has also been shown to improve beta cell function by increasing sensitivity to glucose (120), as GLP-1 upregulates the expression of key moderators in glucose sensing such as glucokinase (121;122), and the glucose transporter GLUT2 (123). Complementary to these actions, GLP-1 has also been found to have multiple longer-lasting benefits, including enhanced beta cell proliferation (124-126) and decreased apoptosis (125). Together, these beneficial effects demonstrate the importance of GLP-1 as a therapeutic for T2DM. In the alpha cell GLP-1 inhibits secretion of glucagon (127), although the mechanism by which this occurs is not entirely clear. A possible explanation is that GLP-1 acts indirectly through the stimulation of somatostatin secretion from the islet delta cells, which has a clear inhibitory effect on glucagon secretion. Consistent with this, studies in Type 1 diabetic patients have shown that GLP-1 exerts an inhibitory effect on glucagon secretion, independent of the effect of insulin (128). Furthermore, although the GLP-1 receptor (GLP-1R) has been localized on some alpha cells (129;130), expression has also been shown on the delta cells (129) thus GLP-1 most likely has a direct effect.

GLP-1 has also shown to have effects on the central nervous system to mediate satiety (131). Rodent studies have shown that GLP-1R agonists decrease food intake and body weight (131;132), at least in part through interactions with regulating neurons, particularly the vagus nerve. In rats, subdiaphragmatic vagotomy or brainstem transection eliminated the inhibitory effect of GLP-1 on food intake (133), illustrating that GLP-1 most likely exerts these anorectic effects through a peripheral neural pathway.

In addition to enhancing insulin secretion after a meal, GLP-1 also contributes to the reduction in postprandial hyperglycemia though its inhibitory actions on post-meal gastric emptying and
gastric acid secretion (134). Consequently, this slows the rate of nutrient absorption in the intestine, thus limiting the spike in postprandial blood glucose levels (135).

Multiple studies have also demonstrated the beneficial effects of GLP-1 in numerous other tissues. GLP-1R agonists increase arterial blood pressure and heart rate in addition to providing protective effects in models of cardiac injury or heart failure (136). In skeletal muscle, GLP-1 has been shown to increase glucose uptake and enhance glycogenesis, increase insulin-stimulated glucose metabolism and, in the liver, GLP-1 decreases hepatic glucose production. Finally, in adipocytes GLP-1 has been shown to have lipolytic and lipogenic effects (137;138) as well as to increase insulin-stimulated glucose metabolism.

### 1.3.6 Incretin mimetics and GLP-1 therapy for T2DM

As an incretin hormone, one of the most beneficial characteristics of GLP-1 for the treatment of T2DM is its ability to enhance glucose-stimulated insulin secretion (11;13). However, due to the high level of degradation of physiologically active GLP-1 by DPP-IV, several current therapies exploit the use of GLP-1 analogues that are resistant to this degradation. Exenatide, which is currently marketed under the trade name Byetta®, is one such example. The original compound, exendin-4, was isolated from the saliva of the Gila monster (*Heloderma suspectum*) and was shown to share 53% sequence homology with native GLP-1 (139). One critical difference in the amino acid sequence is the presence of a glycine at position 2 in exendin-4, instead of alanine at the same position in active GLP-1 (140). This renders it a poor substrate for DPP-IV, giving it not only a substantially longer half-life of 30 minutes, compared to 2 minutes for native GLP-1 (141) but, consequently, a more potent pharmacological activity than recombinant GLP-1 when administered in vivo (142;143). Additionally, even longer-acting GLP-1 analogues are currently
available and in clinical trials (11), and take advantage of a longer half-life than both exenatide and endogenous GLP-1. One such drug is liraglutide (Victoza®), which consists of native GLP-1 attached to a long carbon chain. This side group allows the analogue to bind to albumin, which slows glomerular filtration and extends the renal elimination half life (11;144). Liraglutide thus has an extended half-life of 12 hours and displays an effective biological activity paramount to exendin-4 (145).

A second class of GLP-1 oriented therapeutics are the DPP-IV inhibitors, which prolong the half-life of endogenous GLP-1 by inhibiting its degradation. Use of DPP-IV inhibitors has been shown to substantially increase levels of active GLP-1, as well as the related incretin GIP (146;147). The pharmacological effects of DPP-IV inhibitors are similar to the GLP-1 agonists: they increase glucose-dependent insulin secretion and decrease glucagon secretion. They do not, however, decrease gastric emptying or induce satiety (148). The longest used inhibitor, sitagliptin (Januvia®) has been shown to be effective as a monotherapy in reducing HbA1c levels, but not as effective as metformin alone (149). Therefore, sitagliptin and metformin are often co-prescribed, as they confer additive benefits (150).

A third class of drugs currently in development are the GLP-1 secretagogues, which enhance secretion directly from the L-cell. The difficulty associated with these drugs is finding an agonist that is selective to the L-cell. One such class of drugs are the GPR119 agonists, as discussed earlier. Although it is not exclusively selective for the L-cell, activation of GPR119 by these drugs has not been associated with any serious adverse effects (151;152). However, enhanced L-cell secretion is also associated with increased release of GLP-2 and oxyntomodulin, so possible side-effects cannot be discounted.
Interestingly, the long prescribed drug, metformin, has also recently been shown to enhance levels of GLP-1, through a mechanism that is now believed to occur through increased secretion. The following section will discuss the biguanide class drug, metformin, its mechanism of action, and the current research investigating metformin-induced increases in GLP-1.

1.4 Metformin

1.4.1 Metformin as a treatment for T2DM

Absorption of metformin occurs throughout the entire gut, with highest absorption occurring in the proximal regions (153). Absorption in the intestines is believed to occur through specific organic cation channels, while other forms of these channels regulate the uptake into specific organs. For example, the organic cation transporter-1 (Oct1) was found to be necessary for hepatic uptake of metformin, and plays a moderate role in intestinal uptake (154), while the transporter Oct2 is necessary for renal uptake and excretion (155). Absorption of metformin is dose-dependent, which suggests a saturation limit for these transporters (156). Once metformin is taken up into the plasma, it exists in a free form unbound to carrier proteins and, depending on the dose, has an overall bioavailability of 50 to 60% (157). Metformin is not metabolized by the liver, but is excreted unchanged in the urine with an elimination half-life of 4 to 8 hours (156). While the complete mechanisms by which metformin alleviates hyperglycemia in T2DM are not fully understood, a primary mode of action has long-been known to be inhibition of hepatic glucose output. In patients with T2DM, enhanced hepatic glucose production contributes substantially to their hyperglycemia; in fact it has been shown that hepatic production is increased two- to three-fold above normal (158;159). It is believed that metformin exerts many of its beneficial actions on hyperglycemia through the activation of AMPK, but more recent
studies have illustrated numerous AMPK-independent effects as well. The mechanisms underlying these AMPK-dependent and -independent effects will be discussed in further detail in the proceeding section. In addition to lowering hyperglycemia through decreased hepatic glucose output, metformin has also been shown to enhance insulin sensitivity in the skeletal muscle, leading to enhanced glucose uptake (158;160-163) and, more recently, to enhance incretin and other GPCR expression on the beta cell (164;165). Together, these effects have been shown to reduce hyperglycemia in patients with T2DM patients independent of sex, age, or weight (7;166;167).

1.4.2 **Mechanism of action of metformin**

Metformin leads to activation of AMPK in both liver and skeletal muscle. How biguanides activate this enzyme is still unknown, although there is evidence to suggest that this effect is dependent on the upstream protein-kinase, LKB1. Knockout of this kinase in the liver of mice leads to a loss of function of AMPK and increased gluconeogenic gene expression, resulting in hyperglycemia. The effectiveness of metformin in reducing hyperglycemia was also abolished (168). On the other hand, a later study proposed that neither LKB1 nor AMPK was a direct target of metformin in the muscle. This study in L6 skeletal muscle cells demonstrated that metformin activity was not affected by LKB1 knockdown, suggesting differential roles for this enzyme in liver versus skeletal muscle (169). They also demonstrated that metformin did not exert its actions either through inhibition of the mitochondrial respiratory chain (170-173), or through production of reactive nitrogen species (ROS) (174;175), which had been previously suggested. In contrast to muscle, in the liver, AMPK activation leads to reduction of activity of acetyl-Coenzyme A carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-
CoA, a component in fatty acid biosynthesis and inhibitor of carnitine parmitoyltransferase 1 (CPT-1), which imports fatty acids into the mitochondria for beta-oxidation. Inhibition of ACC leads to an increase in cellular acetyl-CoA and consequent decrease in malonyl-CoA, enabling CPT-1 to transport fatty acids into the mitochondria to drive beta oxidation.

Although it is well established that metformin decreases hyperglycemia through decreased hepatic glucose output, the mechanism involved remains controversial, as well as the relative contributions that decreased gluconeogenesis and glycogenolysis have in mediating this effect. Indeed, a number of studies have suggested both AMPK dependent (176) and independent (177) mechanisms leading to decreased gluconeogenesis, but the overall consensus is that metformin decreases hepatic glucose production through the down-regulation of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6ase). Notwithstanding, these glucose lowering effects in the liver and skeletal muscle help to decrease hyperglycemia in T2DM.

1.4.3 Other effects of metformin

In addition to its effects in T2DM, metformin has been found to exert many other beneficial actions in the improvement of numerous pathological conditions.

Polycystic ovary syndrome (PCOS) is a common affliction that affects between 6 to 10% percent of women of reproductive age, and is one of the leading causes of infertility (178). PCOS is highly correlated with obesity, insulin resistance, and diabetes (179). Patients with PCOS who are treated with metformin display a substantial improvement in ovulation and a decreased rate of miscarriage, in addition to the improvements in hyperglycemia, insulin resistance, and body weight that are predominantly associated with the disease (178). While the mechanism of action
remains unclear, it has been suggested that metformin could exert its actions indirectly, through improvements in insulin sensitivity, or through direct effects on the ovaries. Recent studies further indicate that these effects of metformin are AMPK and insulin dependent, and could act by enhancing insulin sensitivity and signalling (180;181).

Metformin has also recently been associated with positive outcomes in patients with cancer (182). In fact, the drug has shown significant potential to inhibit the growth of tumors in mouse models, and metformin in vitro has been shown to decrease the proliferation of a number of cancer cell models including breast, prostate, colon, endometrial, ovarian, and glioma (182). These beneficial effects can be attributed to both indirect and direct actions of metformin. Since insulin acts as a mitogenic agent to promote cell growth and survival (183), metformin indirectly decreases the prevalence of cancer in patients with T2DM through its effects to reduce blood glucose and, hence, insulin levels (184). Additionally, metformin has a direct effect on the inhibition of tumor growth through AMPK activation. These effects can be attributed to a reduction of mTOR activity, which is involved in the regulation of cell proliferation and survival. Dysregulation of this kinase has been determined to play a role in many instances of cancer (182). Activation of AMPK results in an inhibition of mTOR activity and reduction of a number of key downstream regulators involved in cell proliferation in tumor cell lines (185). Interestingly, a few recent studies have suggested that metformin may also exert some AMPK-independent inhibitory effects on some key tumor regulators (182;186).

Metformin has been demonstrated to reduce weight in patients with T2DM (187), and it has gained considerable interest as means to control weight in non-diabetic patients as well (188). These weight loss effects have been attributed to reduced carbohydrate absorption and metformin’s anorectic and lipolytic effects (189;190). Effects on satiety are not fully understood,
but could be due in part to leptin. Although studies in overweight subjects have shown that acute metformin does not enhance secretion of leptin (189), studies in obese, leptin resistant mice show that chronic metformin treatment increases sensitivity to leptin (191). Since leptin resistance is very common in the overweight, metformin could exert an anorectic effect through improved leptin sensitivity (187). Alternatively, metformin enhances GLP-1 secretion (discussed in section 1.4.4) which, as mentioned previously, also promotes satiety. Either of these mechanisms could explain the weight loss experienced with metformin treatment.

Metformin has also been shown to have numerous beneficial effects on cardiovascular health. In the UKPDS study, the risk of all cardiovascular-related events was decreased in patients treated with the drug (192). In fact, when compared to other therapies that resulted in equivalent decreases in HbA$_{1c}$ levels, patients treated with metformin had a lower incidence of cardiovascular mortality, suggestive of an effect of metformin independent of decreased hyperglycemia. Since insulin resistance and hyperinsulinemia has been associated with increased cardiovascular problems (193), by increasing insulin sensitivity and reducing baseline and glucose-stimulated insulin levels, metformin alleviates these problems. Furthermore, dyslipidemia has also been associated with increased cardiovascular risk, and as metformin has been shown to have a beneficial role in lipid metabolism by lowering levels of free fatty acids and triglycerides, while decreasing levels of low-density cholesterol and increasing levels of high-density cholesterol (193). Furthermore, a direct action of metformin on the heart has been found in studies looking at the cardioprotective role of AMPK. In animal studies, increased activation of AMPK with metformin results in reduced incidence of apoptosis in cardiomyocytes (194), and AMPK activation has been correlated with reduced cardiac ischaemia, arrhythmia and hypertrophy (195).
Finally, metformin has been found to increase plasma levels of GLP-1 in humans (146;189;196) and rodents (146;164;197;198). As discussed previously, GLP-1 exerts many beneficial, anti-diabetic effects \textit{in vivo}, and it was believed that some of these overlapping effects of metformin could be accounted for due to the actions of GLP-1. The ability of metformin to enhance L-cell secretion \textit{in vivo} was first suggested in 1980 by Malloy \textit{et al}. (199). Over thirty years later, metformin-induced increases in plasma GLP-1 levels are well established clinically, but the mechanism of action is still unknown. The following section will discuss in detail the current consensus regarding the mechanism of metformin-induced increases of GLP-1.

### 1.4.4 Metformin and GLP-1

Mannucci \textit{et al}. suggested in 2001 that increases in GLP-1 observed in T2DM patients treated with metformin could be due to inhibition of DPP-IV. They noted that these patients had enhanced levels of active GLP-1, which was consistent with what was seen in patients treated with DPP-IV inhibitors such as sitagliptin (149). Furthermore, plasma DPP-IV activity from these patients decreased in a dose-dependent fashion after chronic metformin treatment. Finally, \textit{in vitro} assay of purified porcine DPP-IV incubated with GLP-1(7-36NH$_2$) again showed a dose-dependent inhibition with increasing concentrations of metformin (196). This study was the first to demonstrate the potential for direct effects of metformin on DPP-IV. Consistent with these results, Lindsay \textit{et al}. later showed in T2DM patients that metformin decreased plasma DPP-IV activity after acute treatment \textit{in vivo}, as well as when isolated plasma from these individuals was incubated with metformin \textit{in vitro} (200). Interestingly, a comprehensive \textit{in vitro} study by Hinke \textit{et al}. refuted this claim by demonstrating that metformin did not directly inhibit DPP-IV activity in the test tube (201). Together, these experiments conclusively demonstrated that metformin
does not directly affect DPP-IV mediated degradation of GLP-1, at least in vitro. Moreover, a later study in diabetic rats confirmed these results by showing that plasma DPP-IV activity was not affected by incubation with metformin, despite an observed decrease in DPP-IV activity after in vivo metformin treatment (198). The authors concluded that metformin could therefore decrease secretion of soluble DPP-IV, rather than directly inhibit the enzyme. Two further studies in patients with T2DM (202) and in normal and ob/ob mice (203) also demonstrated a decrease in plasma DPP-IV activity after treatment with metformin in vivo, although in vitro testing was not performed in these studies to test direct inhibition. Therefore, these results also coincide with the findings of Hinke et al. and Lenhard et al. suggesting that metformin does not directly inhibit DPP-IV.

Yasuda et al. first demonstrated in 2002 that metformin-induced increases in GLP-1 were DPP-IV independent, by demonstrating that metformin and the other biguanides, buformin and phenformin, increased GLP-1 levels in DPP-IV deficient rats (197). Conclusive with previous studies (198;201), they also showed metformin did not inhibit enzymatic activity when incubated with porcine or rat DPP-IV in vitro. Finally, co-administration of metformin and a DPP-IV inhibitor enhanced levels of active GLP-1 in normal rats, while metformin alone had no effect (197). Together, these studies provide evidence both for and against an inhibitory role for metformin as a GLP-1 secretagogue. It has only been rather recently that a growing consensus has been made against the action of metformin as a DPP-IV inhibitor, and towards its role as a GLP-1 secretagogue. A 2010 study by Migoya et al. looked at the combined effect of metformin and sitagliptin on incretin levels, providing at last conclusive evidence that metformin enhances GLP-1 secretion, rather than inhibits degradation. In these individuals, treatment with metformin and sitagliptin together provided a synergistic effect on both active and total GLP-1 levels, while
treatment with metformin alone enhanced total but not active, indicative of enhanced secretion. Moreover, treatment of patients with sitagliptin enhanced active levels of both active GLP-1 and GIP, as expected, while treatment with metformin alone did not enhance levels of active GIP, providing further evidence that metformin does not inhibit DPP-IV. Metformin treatment also enhanced proglucagon gene expression and increased plasma levels of total GLP-1 in mice. Finally, DPP-IV activity measured in vitro and in vivo was not altered by metformin (146). Overall, therefore, there has been a shift from the idea that metformin acts as a DPP-IV inhibitor, to the understanding that metformin acts as a GLP-1 secretagogue. Nonetheless, how metformin enhances secretion of GLP-1 remains unknown, and its possible mechanism of action is the focus of this thesis.

1.5 Hypothesis and aims

Metformin increases plasma GLP-1 levels through a mechanism that has yet to be determined. I therefore hypothesize that metformin increases GLP-1 plasma levels through enhanced secretion from the L-cell. This will be tested using two distinct aims; first, to determine if metformin acts directly on the L-cell as a secretagogue or sensitizer to increase GLP-1 secretion; and second, to determine if metformin acts instead through an indirect pathway to stimulate GLP-1 secretion.
2 Methods

2.1 In vitro experiments

2.1.1 Cell culture

Cells were cultured as described previously for GLUTag (204), NCI-H716 (77;205), and FRIC (206-208). Following is a brief description of these methods.

2.1.1.1 GLUTag

Murine GLUTag cells were cultured in 10cm dishes in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL/Invitrogen, Burlington, Ont) containing high glucose (25mM) and supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co, St. Louis, Missouri), and were maintained in a humidified incubator at 37°C and 5% CO₂. Media was changed every 2-3 days and cells were split every 5 days, or once cells reached 80% confluence. Cells were washed with warm Hank’s buffered salt solution (HBSS; 5.0mM KCl; 0.4mM KH₂PO₄; 137mM NaCl; 4.0mM NaHCO₃; 0.4mM Na₂HPO₄; 5.6mM D-glucose; pH 7.4), trypsinized to facilitate removal of cells from plate and divided 1:3 in fresh medium. Two days before each experiment, cells were split 1:3 and seeded in a 24-well plate (for secretion experiments) or split 1:2 and seeded in a 6-well plate (for western blot experiments) in fresh medium. Plates were pre-coated with poly-D lysine (Sigma, St. Louis, Missouri) prior to splitting, to mediate cell adhesion to the plate.

2.1.1.2 NCI-H716

Human NCI-H716 cells were obtained from the American Type Culture Collection (Manassas, Virginia). Cells were grown in suspension in 10cm dishes in Roswell Park Memorial Institute (RPMI; Gibco BRL/Invitrogen) medium, containing high glucose (25mM) and 10% FBS, and maintained in a humidified incubator at 37°C and 5% CO₂. Cells were split 1:3 every 5 days and plated in fresh medium. Two days before each experiment, cells were split 1:3 and seeded in a
24-well plate (for secretion experiments) or split 1:2 and seeded in a 6-well plate (for western blot experiments) in fresh medium. Plates were pre-coated with Matrigel (Becton Dickinson and Co., Bedford, Massachusetts) prior to splitting to facilitate cell adhesion.

2.1.1.3 FRIC

Female Wistar rats at late gestation were purchased from Charles River Laboratories (St. Constant, Quebec). The procedure was approved by the Animal Care Committee of the University of Toronto. Tissue collection was performed on gestational days 19-20. Animals were anaesthetized with isofluorane and sacrificed by decapitation. A midline incision was made through the abdomen to expose the internal organs. The uterus was then opened and pups were individually removed from the amniotic sac and decapitated, then the fetal bodies were placed in 70% ethanol briefly, until intestinal tissues could be harvested. The abdomen of the pups was opened below the umbilical cord, exposing the intestines. The large and small intestines of each pup were removed and pooled together in ‘Culture Hanks’ solution (HBSS supplemented with 5% FBS, 40U/ml penicillin, and 40μg/ml streptomycin) then diced with scissors. To facilitate destruction of the extracellular matrix and cell dispersal, the intestines were incubated twice for 15 min at 37°C in Culture Hanks containing hyaluronidase (5mg/ml), deoxyribonuclease 1 (0.5mg/ml), and collagenase (4mg/ml) (Sigma Chemical Co). After each incubation, the supernatant from the digestion containing free floating cells was collected, washed in ‘Culture DMEM’, centrifuged and pooled together, then plated in standard 60mm dishes in ‘Culture DMEM’ (5% FBS, 40U/ml penicillin, and 40μg/ml streptomycin). The cells were incubated overnight at 37°C and 5% CO₂, and the experiment was conducted the following day.
2.1.2 Secretion experiments

On the day of the experiment, cells were washed twice with HBSS and incubated for 2h at 37°C with ‘Experimental DMEM’ with 25mM glucose, 0.5% FBS, plus vehicle (ethanol) for GLUTag and NCH-H716 experiments, or ‘Experimental DMEM’ 5.6mM glucose, 0.5% FBS, plus 40U/ml penicillin, 40μg/ml streptomycin, 20μU/ml insulin, and vehicle (ethanol) for FRIC, as the negative control; forskolin (adenylyl cyclase (AC) agonist) plus 3-isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor; 1 or 10μM each; Sigma, St. Louis, Missouri) as the positive control; metformin (5, 15, 45, 150, or 2000μM; Sigma Chemical Co); or aminoimidazole carboxamide ribonucleotide (AICAR; AMPK agonist; 100μM or 1000μM; Toronto Research Chemicals, Toronto, Ontario).

For metformin-sensitization experiments, GLUTag cells were washed with HBSS, pre-incubated with or without metformin (2000μM) in ‘Experimental DMEM’ and 0.5% FBS for 1h at 37°C, washed with warm HBSS and incubated for an additional 2h with forskolin and IBMX (10μM each), bethanechol (muscarinic agonist; 0.5 or 1mM; Sigma Chemical Co), bethanechol (0.5 or 1mM) plus metformin (2mM), or metformin alone (2mM).

After the incubation period for all experiments, cells were checked by microscopy to ensure there were no observable morphological differences between treatment groups. Media was then collected and centrifuged to remove free floating cells, and trifluoracetic acid (TFA; Thermo Scientific, Rockford, Illinois) was added to a final concentration of 0.2%. Cells were collected with a rubber cell-scaper and homogenized in cell lysis buffer (1N HCl, 5% formic acid, 1% TFA, and 0.1M NaCl). The collected samples were passed through a C_{18} Sep-pak cartridge (Waters, Milford, Massachusetts) for purification and stored in 80% isopropanol and 0.1% TFA at -20°C until the assay was conducted.
2.1.3 Radiommunoassay

Media and cell samples were dried and then incubated for 5 days at 4°C with anti-GLP-1(7-36NH₂) antibody (Enzo life Sciences, Plymouth Meeting, Pennsylvania), and ¹²⁵I-labelled GLP-1(7-36 NH₂) radioactive tracer (prepared in house) in assay buffer (56mM Barbitone (Medisca, Montreal, Quebec); 64mM EDTA, pH 8.0; 10% BSA). Following incubation, free peptide was adsorbed with charcoal and the fraction of free labelled peptide was extrapolated against a standard curve to determine GLP-1 content. Percent GLP-1 secretion was calculated as the concentration of GLP-1 in the media divided by the total GLP-1 (i.e. medium plus cell) content, multiplied by 100.

2.1.4 Western blot

GLUTag and NCI-H716 cells were washed with HBSS and incubated for 1h at 37°C with metformin (2000μM), AICAR (1000μM), or vehicle in ‘Experimental DMEM’ with 0.5% FBS for GLUTag, or RPMI with 0.5% FBS for NCI-H716. Cells were then quickly rinsed with cold HBSS and lysed with 100μl of cold RIPA lysis buffer (50mM β glycerol phosphate; 10mM HEPES (pH 7.4); 1% Triton X-100; 70mM NaCl; 2mM EGTA; 1mM activated sodium orthovanadate; 1mM NaF; and EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany)). Cells were then collected with a rubber scraper, homogenized, and stored at -20°C. Total protein content was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, California). Equal amounts of protein (100-150μg) in 20% loading buffer were heated at 100°C for 5min, then separated on 7.5% SDS-PAGE gel and transferred onto a PVDF membrane (Bio-Rad Laboratories). Membranes were incubated for 1h in blocking buffer (5%
non-fat powder milk in TBST (20mM Trizma base, 140mM NaCl, pH 7.6 plus 1% Tween-20). Membranes were washed 3x with TBST and then incubated overnight at 4°C in TBST plus 5% bovine serum albumin (BSA), with a rabbit anti-phosphorylated-AMPK antiserum (1:1000, Cell Signaling, Beverly, Massachusetts), which targets phosphorylated threonine 172 of the AMPK alpha subunit. After incubation, membranes were washed with TBST, incubated for 1h with horseradish peroxidase (HRP)-linked goat-anti-rabbit secondary antibody (1:2000, Cell Signaling) diluted in blocking buffer, washed in TBST, then visualized by electrochemical luminescence (ECL) detection system (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec). Membranes were subsequently incubated in stripping buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, and 100mM β-mercaptoethanol) at 50°C for 30min to remove antibodies. For the second round of blotting, membranes were blocked as before, then incubated overnight with a rabbit anti-AMPK antiserum (1:1000, Cell Signaling, Beverly, Massachusetts) and a rabbit anti-actin antiserum (1:1000, Sigma Chemical Co). Membranes were incubated with secondary antibody and imaged as above.

2.2 In vivo experiments

2.2.1 Animals

Male Wistar rats (250-350g) were purchased from Charles River Laboratories and housed in the Division of Comparative Medicine at the University of Toronto with a standard 12 hour light-dark cycle. Animals were housed for at least 2 weeks prior to the experiment, and handled daily for 3 to 5 days prior to the experiment to promote acclimation and reduce stress. Animals were fed regular chow, and fasted overnight (16 hours) before each experiment. All animal procedures were approved by the Animal Care Committee of the University of Toronto.
2.2.2 Secretion experiments

Rats were treated with metformin (300mg/kg, p.o.), AICAR (250mg/kg, s.c.), or an equal volume of vehicle (sterile saline, p.o. and s.c.). For the inhibitor studies, rats were administered sitagliptin (Januvia®, DPP-IV inhibitor; 5mg/kg, i.v.; Merck, Kirkland, Quebec), atropine (general muscarinic antagonist; 1mg/kg, i.v.; Sigma Chemical Co), pirenzepine (M1 antagonist; 0.5mg/kg, i.v.; Sigma Chemical Co), gallamine (M2 antagonist; 0.5mg/kg, i.v.; Sigma Chemical Co), 4-DAMP (M3 antagonist; 0.5mg/kg, i.v.; Tocris Bioscience, Ellisville, Missouri), RC-3095 (GRP receptor antagonist; 0.1mg/kg, s.c.; Sigma Chemical Co), or an equal volume of vehicle (sterile saline, i.v. or s.c.), 30 minutes prior to treatment with metformin or vehicle, as before. Blood samples (200μl) were collected from the lateral saphenous vein into heparinized glass capillary tubes, then immediately stored on ice; this was performed at regular intervals over a 2h period. An anti-coagulant and protease inhibitor solution (5000 KIU/ml aprotinin (Sigma, St. Louis, Missouri); 1mM diprotin A (DPP-IV inhibitor; Sigma, St. Louis, Missouri)) was added to a final concentration of 10% (vol/vol) to all samples except those used for the DPP-IV activity assay; as diprotin A was not added to these samples. Blood samples were centrifuged at 4°C for 10 minutes at 10,000 rpm, and plasma was collected and stored at -80°C. Plasma total and active GLP-1 were assayed using a kit from Meso Scale Discovery (MSD; Gaithersburg, Maryland).

2.2.3 Control experiments

As a positive control for successful inhibition of all of the muscarinic receptors, or of the M1 and M3 receptors specifically, salivary secretion was measured in animals treated with atropine, pirenzepine or 4-DAMP (209;210). In brief, saliva was collected with a Q-tip from the oral
cavity for 1 minute and weighed. Measurements were taken before and then at 30 and 120 minutes after administration of the drugs.

As a positive control for successful inhibition of the M2 muscarinic receptors, heart rate was measured via pulse oximeter in rats treated with gallamine (211). Rats were anesthetised with isofluorane (5% induction, 2.5% maintenance) and heart rate was measured in duplicate. Measurements were taken before and then at 30 and 120 minutes after administration of the drug.

2.2.4 DPP-IV activity assay
The DPP-IV synthetic substrate, Gly-Pro p-nitroanilide hydrochloride (gly-pro-pNA), and the colorimetric standard, p-nitroaniline, were purchased from Sigma Chemicals Co. The DPP-IV activity assay was followed according to the procedure provided. In brief, collected rat plasma samples (40μl per sample) were added to a reaction mixture of final concentration 100mM Tris (pH 8.0), 0.5mM Gly-Pro-pNA, and final volume of 200μl, in a standard 96 well plate. The reaction mixture was incubated at 37°C for 1 hour and spectrophotometer readings were taken at an absorbance of 405nm every 5 minutes. The molar amount of p-nitroaniline liberated from gly-pro-pNA was calculated from the standard curve using values from the linear range of the curve for each sample. DPP-IV activity was defined as: one unit equals the production of 1.0 μmole of p-nitroaniline from Gly-L-Pro p-nitroanilide per minute.

2.2.5 Bilateral subdiaphragmatic vagotomy
The surgical procedure was performed aseptically and all instruments were heat-sterilized before use. The animals were anaesthetized with isofluorane (5% induction, 2.5% maintenance) and surgically prepared by shaving the abdomen and wiping the area twice each with 70% ethanol
and betadine antiseptic solution. Prior to the first incision, animals were given a subcutaneous injection of buprenorphine (30μg/kg, s.c.) and ketoprofen (5mg/kg, s.c.) as analgesics.

The animals were placed in a supine position and a 2cm ventral midline skin incision was then made with its cranial terminus beginning at the xiphoid process, and ending caudal to the umbilical cord. A similar incision was made through the abdominal muscle wall, along the linea alba, exposing the internal organs. The liver was retracted cranially and the stomach caudally to expose the esophagus. Using curved mosquito forceps, the esophagus was retracted ventrally, so that the two branches of the vagus nerve (running along the anterior and posterior surface of the esophagus) could be visualized. An approximate 0.5cm segment of each branch was excised, along with any surrounding mesenteric tissue, to ensure that the branches were successfully dissected. For sham-operated animals, the previous steps were conducted so that the vagus nerve was visualized, but not severed. The muscle incision was closed with absorbable suture, and the skin with non-absorbable suture. Follow-up injections of the analgesics were given twice daily for two days following the operation. Post-surgery, the animals were doubly-housed and monitored daily for loose sutures and blood in the stool. Due to the possibility of gastric bloat (212), body weight was measured daily to look for an unusual (i.e. greater than 20%) decrease in body weight. After a one week recovery, the secretion experiment was conducted as before (section 2.2.2).

As a positive control for successful vagotomy, after an overnight fast animals were administered cholecystokinin (CCK; 10μg/kg, i.p.), or vehicle (PBS, i.p.) and food consumption was measured for 30 and 60 minutes, as it has been previously demonstrated that CCK’s ability to inhibit food intake is mediated through the vagus nerve (213).
After the experiment was complete, animals were sacrificed while anaesthetized via cardiac puncture and stomach weights were recorded and normalized to body weight as an indication of successful vagotomy, as it has been previously described that vagotomized rats exhibit increased stomach weight compared to sham (214).

2.3 Data analysis

*In vitro* GLP-1 secretion is expressed as a percentage of control secretion. AMPK phosphorylation is expressed as the fold of control. *In vivo* GLP-1 secretion is expressed either as absolute secretion or as the change (i.e. delta) from basal secretion. Delta area-under-curve (AUC) analysis for GLP-1 secretion was calculated from the delta secretion profiles using the trapezoidal rule. All data are expressed as the mean ± SEM. In some experiments, data were converted to log10 to normalize variance for statistical analysis. Statistical analyses were performed using Statistical Analysis System software (SAS; Cary, North Carolina). Statistical significance between experimental groups was determined by Student’s t-test, 1-way or 2-way ANOVA, as appropriate, followed by post hoc comparison using Student’s t-test, as appropriate. Statistical significance for these comparisons was assumed at P < 0.05.
3 Results

3.1 Assessment of metformin action in vivo

I first confirmed previous findings in normal Wistar rats, using a dose of metformin (300mg/kg) demonstrated to effectively enhance GLP-1 secretion (197). Since increases in active GLP-1 can be accredited to either increased secretion and/or decreased degradation, I measured total levels of GLP-1 (7-37, 7-36NH₂, 9-37, and 9-36NH₂), as this more closely reflects increased secretion, rather than decreased degradation. However, it cannot be dismissed that increases in total GLP-1 may also reflect decreased renal clearance. Metformin increased total GLP-1 compared to vehicle alone (Fig 3.1), with significant increases in secretion occurring after 60 minutes (212±73% versus vehicle, P < 0.05), consistent with Yasuda et al. (197). These increased levels of total GLP-1 were maintained for the remainder of the experiment, reaching 771±168% of vehicle (P < 0.001) 2h post treatment. To determine whether direct activation of AMPK can increase GLP-1 secretion, rats were treated with the AMPK agonist, AICAR. Pharmacological activation of AMPK led to a robust increase in GLP-1 secretion observable at 60 minutes (519±75% of vehicle, P < 0.001), but unlike the prolonged effects seen with metformin treatment, GLP-1 levels declined to just 354±96% of vehicle secretion after 2 hours (P < 0.05).

In contrast to its effects on GLP-1 levels, metformin had no effect on DPP-IV activity compared to vehicle (Fig 3.2A), as determined 2 hours after metformin treatment. Furthermore, DPP-IV activity was not affected by metformin treatment when measured at multiple time points over a 2 hour period (Fig 3.2B). As a positive control, DPP-IV activity was measured in the same assay for rats treated with sitagliptin with or without metformin (shown in Fig 3.6A).
Figure 3.1 – Metformin and AICAR increase GLP-1 plasma levels in rats. After an overnight fast, male Wistar rats were treated with metformin (300mg/kg, p.o.; diamonds), AICAR (250mg/kg, s.c.; squares), or vehicle (p.o. and s.c.; circles). n = 6, *P < 0.05, ***P < 0.001 compared to vehicle.

Figure 3.2 – Acute metformin treatment does not affect plasma DPP-IV activity in rats. After an overnight fast, male Wistar rats were treated with metformin (300mg/kg, p.o) or vehicle. Plasma DPP-IV activity was measured by enzyme assay from Sigma Chemical Co. A) Plasma DPP-IV activity in vehicle (black bars) or metformin (white bars) treated animals at 0 and 120 minutes. n = 6. B) Plasma DPP-IV activity in animals treated with metformin over 120 minutes. n = 5 – 6.
3.2 Assessment of metformin action in vitro

To establish whether metformin directly stimulated GLP-1 secretion from the L-cell in vitro, murine GLUTag, human NCI-H716, and rat FRIC cells were incubated with increasing concentrations of metformin. Lower concentrations (5 and 15μM) were used as they closely reflect clinically relevant plasma concentrations achieved in patients treated with metformin (215;216), in addition to supra-pharmacological concentrations (45, 150 and 2000μM). Cells were also incubated with AICAR (100 and 1000μM) as a positive control for AMPK stimulation. Neither metformin nor AICAR directly stimulated GLP-1 secretion, compared to vehicle, in any of the three models (Fig 3.3A-C). In GLUTag cells, basal (control) GLP-1 secretion was 4.2±0.6% of total cell content. Together, the AC activator, forskolin, and phosphodiesterase inhibitor, IBMX, increased GLP-1 secretion to 4.2±1.7 fold of control (P < 0.001). In NCI-H716 cells, forskolin and IMBX increased GLP-1 secretion to 2.5±0.3 fold of control secretion (3.6±0.6% of total cell content, P < 0.001). Finally, in FRIC cells, forskolin and IMBX increased GLP-1 secretion to 4.4±0.7 fold of control secretion (9.5±1.3% of total cell content, P < 0.001).

Furthermore, incubation of L-cells with metformin and AICAR led to AMPK phosphorylation in GLUTag cells (increased to 1.7±0.2, P < 0.01 and 1.2±0.2, P < 0.05 of control for metformin and AICAR, respectively) as well as in NCI-H716 cells to (2.1±0.3, P < 0.01 and 1.5±0.2, P < 0.05 fold for metformin and AICAR, respectively).

To determine whether metformin increases L-cell sensitization to physiological secretagogues, such as cholinergic agonists, GLUTag cells were pre-treated either with or without metformin (2000μM). As expected, forskolin and IBMX (10μM each) together produced a robust increase in GLP-1 secretion, compared to control, in cells pre-incubated with vehicle (to
Figure 3.3 - Cells were treated with vehicle (control), forskolin and IBMX (positive control; 1 or 10μM, as indicated), metformin (5, 15, 45,150, and 2000 μM), and AICAR (positive control; 100 and 1000 μM). Total GLP-1 by radioimmunoassay and phosphorylated AMPK was measured via immunoblot. A) Murine GLUTag cells (n = 8 – 23 for secretion, n = 8 for western blot), B) Human NCI-H716 cells (n = 6 – 25 for secretion, n = 8 for western blot), C) Fetal rat intestinal cells (n = 8 – 12 for secretion). *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.
5.2±0.5 fold of control, P < 0.001, Fig 3.4), while pre-incubation with metformin did not enhance this effect (to 5.9±1.0 fold of control, P < 0.001). Bethanechol (1000μM) increased secretion to 2.6±0.4 fold of control (6.6±1.1%, P < 0.001) in cells pre-incubated with vehicle, while a lower concentration of bethanechol (500μM) had no effect. Pre-incubation with metformin did not augment GLP-1 secretion in cells treated with either the low or high concentration of bethanechol. Furthermore, co-administration of bethanechol with metformin had no enhanced effect on GLP-1 secretion compared to bethanechol alone, with or without pre-incubation with metformin.

Figure 3.4 – Metformin does not sensitize the L-cell to GLP-1 secretagogues. GLUTag cells were pre-incubated for 1h with vehicle (black bars) or metformin (2000μM; white bars), followed by 2h incubation with metformin (2mM), forskolin and IBMX (10 μM each), bethanechol (0.5mM) with or without metformin (2mM), bethanechol (1mM) with or without metformin (2mM), or vehicle (control). Total GLP-1 was measured by RIA. n = 6 - 10, *P < 0.05 ***P < 0.001 compared to respective controls.
3.3 Role of the muscarinic receptors in mediating metformin-induced GLP-1 secretion

To elucidate whether the stimulatory effects of metformin on GLP-1 secretion *in vivo* involved muscarinic receptors and, hence, the parasympathetic nervous system, I administered the general muscarinic receptor antagonist, atropine, to rats 30 minutes prior to treatment with metformin. Atropine significantly reduced GLP-1 secretion in these animals (Fig 3.5A; showing delta secretion), at 30, 60, and 90 minutes (by 81±11% P < 0.01, 68±18% P < 0.05, and 42±14% compared to vehicle plus metformin, respectively) The delta area-under-curve (AUC) was also decreased by 55±11% P < 0.05 in these animals compared to vehicle plus metformin (Fig 3.5E). Pre-treatment with the M1 antagonist, pirenzepine, had no effect on GLP-1 secretion, or delta AUC (Fig 3.5B,E). The M2 antagonist, gallamine, also had no effect (Fig 3.5C,E). In contrast, pre-treatment with the M3 antagonist, 4-DAMP, resulted in a significant reduction in GLP-1 secretion at 30 and 60 minutes (60±12% P < 0.05, and 62±3% P < 0.05, respectively; Fig 3.5D). Delta AUC was also significantly decreased in 4-DAMP treated animals by 48±8% P < 0.05, compared to vehicle plus metformin (Fig 3.5E).

As a positive control for receptor inhibition, salivary secretion was measured in rats pre-treated with atropine, pirenzepine, and 4-DAMP. Salivary secretion was not affected by metformin treatment in animals pre-treated with vehicle (Fig 3.5F). Atropine significantly decreased salivary secretion 30 and 120 minutes post-administration, respectively, in both metformin -and vehicle- treated animals (Fig 3.5G). Pirenzepine effectively decreased salivary secretion by 51±14% P < 0.05, at 30 minutes, and 61±3% P < 0.01, at 120 minutes (Fig 3.5H). Finally, 4-DAMP also significantly decreased salivary secretion at both time points (Fig 3.5I). As an attempted positive control for M2 inhibition, we measured heart rate in animals pre-treated...
Fig. 2. 

A-D: Time course of delta tGLP-1 (pg/ml) in response to 4-DAMP, vehicle, atropine, pirenzepine, and gallamine. 

E: Bar graph showing delta AUC tGLP-1 (pg/min/ml) in response to the same treatments. 

* p < 0.05, ** p < 0.01 compared to vehicle.
Figure 3.5 – The M3 muscarinic receptor mediates metformin-induced increases in plasma GLP-1 in vivo. After an overnight fast, male Wistar rats were pre-treated with A) atropine (1000μg/kg, i.v.), B) pirenzepine (M1; 500μg/kg, i.v.), C) gallamine (M2; 500μg/kg, i.v.), D) 4-DAMP (M3; 500μg/kg, i.v.), or vehicle (i.v.), 30 min prior to treatment with metformin (300mg/kg, p.o.) or vehicle (p.o.). Diamonds: vehicle plus metformin; circles: inhibitor plus vehicle; squares: inhibitor plus metformin. Animals treated with vehicle plus metformin (diamonds) were randomized throughout the experiments and are shown in A, and replicated in B-C in grey for comparison. Data are expressed as delta total GLP-1. Basal GLP-1 secretion for metformin treated animals was 6.2±0.8pg/ml and was not changed by antagonist treatment. E) Delta Area-under-curve analysis for muscarinic inhibition. Black bars: vehicle; white bars: metformin. n = 6 – 9, *P < 0.05, **P < 0.01 compared to vehicle. Salivary secretion per minute was measured as a positive control for total, M1, and M3 inhibition. Rats were treated i.v. with F) vehicle, G) atropine, H) pirenzepine, or I) 4-DAMP, with or without metformin (p.o.). Data are expressed as absolute secretion (mg/min). Black bars: inhibitor (i.v.) plus vehicle (p.o.); white bars: inhibitor (i.v.) plus metformin (p.o.). n = 5 – 6, *P < 0.05 **P < 0.01, compared to vehicle.
with gallamine. While there was a trend to suggest decreased heart rate in animals treated with gallamine, this was not significant (data not shown).

Finally, in an initial pilot study looking at the effects of metformin on active GLP-1 levels, rats were treated with atropine, with or without metformin. Initial assays measuring active GLP-1 were unsuccessful due to the degradation of virtually all active GLP-1. Therefore, in the second study, rats were pre-treated with the DPP-IV inhibitor sitagliptin to negate the effects of degradation. Sitagliptin significantly reduced the activity of plasma DPP-IV (Fig 3.6A) in sitagliptin plus vehicle treated rats. Metformin did not have a synergistic effect on DPP-IV inhibition in animals treated with sitagliptin plus metformin. However, levels of active GLP-1 were significantly higher in rats treated with metformin than in those treated with vehicle alone.

Figure 3.6 – Metformin increases levels of plasma active GLP-1 in vivo. After an overnight fast, male Wistar rats were pre-treated with atropine (1000μg/kg, i.v.) and sitagliptin (DPP-IV inhibitor, 5000μg/kg, i.v.), 30 minutes before treatment with metformin (300mg/kg, p.o.) or vehicle. A) Plasma DPP-IV activity. Black bars: atropine, sitagliptin, plus vehicle; white bars: atropine, sitagliptin, plus metformin. n = 4 - 5, *P < 0.01. B) Plasma delta active GLP-1. Basal secretion was not significantly different between metformin and vehicle treated animals (3.9±1.2 versus 8.4±3.0). Black bars and circles: atropine, sitagliptin, plus vehicle; white bars and diamonds: atropine, sitagliptin, plus metformin. n = 4 – 5, *P < 0.05 **P < 0.01.
3.4 Role of the vagus nerve in mediating metformin-induced GLP-1 secretion

To determine whether the vagus nerve mediated metformin’s effects on GLP-1 secretion, I performed a chronic bilateral subdiaphragmatic vagotomy. Animal body weight decreased for up to one week post-surgery, although there were no significant differences in the overall change in body weight between vagotomized and sham-operated animals (data not shown), consistent with Mordes et al (217). GLP-1 secretion in vagotomized rats treated with metformin was not different compared to sham operated rats (Fig 3.7a), despite a significant decrease in basal levels of total GLP-1 (11.4±1.7pg/ml vs 7.1±0.7pg/ml, P < 0.05 compared to sham). To demonstrate the success of the vagotomy, a number of control tests were attempted. CCK, when administered locally in small doses, has been demonstrated to induce satiety through vagal nerve fibres (213). Thus, in vagotomized animals, we expected an abolishment of CCK’s inhibitory effect on food intake after an overnight fast. CCK (10ug/kg, i.p.) administered to vagotomized rats inhibited food intake as effectively as when administered to sham-operated animals (data not shown). As it has been previously shown that vagotomized rats display increased stomach weight compared to sham-operated animals (214), as a second control test, post-mortem stomach weight was measured. Although there was a trend towards increased stomach weight in vagotomized animals, it was also non-significant (data not shown).
Figure 3.7 – Bilateral subdiaphragmatic vagotomy does not affect metformin-induced increases in plasma GLP-1. Under anesthesia, male Wistar rats (250-300g) underwent bilateral subdiaphragmatic vagotomy or sham operation. After 1 week of recovery and an overnight fast, rats were treated with metformin (300mg/kg, p.o.). Basal total GLP-1 is shown in inset. Circles and black bars: sham-operation plus metformin; diamonds and white bars: vagotomy plus metformin. \( n = 8 - 10, *P < 0.05 \) compared to sham.

### 3.5 Role of the GRP receptor in mediating metformin-induced GLP-1 secretion

Finally, I evaluated the involvement of the enteric nervous system, as previous studies in have demonstrated the importance of the neuropeptide GRP in mediating GLP-1 secretion \textit{in vivo} (52;115) and \textit{in vitro} (61;77). I therefore pre-treated rats with the GRP receptor antagonist, RC-3095, before treatment with metformin or vehicle. RC-3095 pre-treatment did not alter basal GLP-1 levels (data not shown); however metformin-induced GLP-1 secretion was reduced by 52±6% (\( P < 0.05 \)) at 30 minutes, although not at later time points (Fig 3.8).
Figure 3.8 – The GRP receptor mediates early metformin-induced increases in plasma GLP-1. After an overnight fast, male Wistar rats were pre-treated with the GRP receptor antagonist RC-3095 (100μg/kg, s.c.) or vehicle (s.c.), 30 minutes prior to treatment with metformin (300mg/kg, p.o.) or vehicle (p.o.). A second injection of the inhibitor, or vehicle, was given 30 minutes after oral gavage. A) Plasma delta total GLP-1. Basal secretion for the vehicle plus metformin group was 1.1±0.4pg/ml and was not significantly different from basal secretion in animals pre-treated with antagonist. Diamonds: vehicle plus metformin; squares: inhibitor plus metformin; circles: inhibitor plus vehicle. B) Delta AUC of tGLP-1 secretion at 30 minutes. n = 6, *P < 0.05 compared to vehicle.
4 Discussion

T2DM is the one of the largest global health crises we face today, and the development of novel therapeutic approaches is vital to the resolution of this problem. Metformin has long been the gold standard in treatments for patients with T2DM. However, the relatively recent discovery that metformin enhances secretion of GLP-1 has made the understanding of its long-elusive mechanism of action that much more important.

Treatment with metformin is known to increase plasma levels of GLP-1 in humans (146;196;202), rats (197), and mice (146;164;218). Notably, many of these studies measured only active levels of GLP-1 (e.g. 7-37 and 7-36NH₂) to determine the effects of metformin (189;196-198;202;218). Therefore, I first demonstrated that orally-administered metformin increases levels of total GLP-1 in normal rats. Maximum levels of plasma GLP-1 were observed at 2 hours, while levels were not significantly different from vehicle at t = 15 and 30 minutes, consistent with the secretion profile of active GLP-1 in DPP-IV deficient rats measured by Yasuda et al (197) using the same dose of metformin. Interestingly, Maida et al. measured total GLP-1 secretion in mice treated with the same dose of metformin, yet peak levels of GLP-1 (comparable to the concentrations measured in our rats) were observed after just 10 minutes (219), suggesting species-specific differences in L-cell regulation and/or absorption and/or clearance of metformin. Active GLP-1 levels in rats treated with sitagliptin and atropine were also proportional to levels of total GLP-1 in the same rats, justifying the measurement of only total GLP-1 in the remaining experiments. AICAR treatment also significantly increased plasma GLP-1, again consistent with the results of the previous study (164); however, in mice, GLP-1 levels were approximately three-times higher than in rats treated with the same dose of metformin, indicating again species-specific differences. Nonetheless, these results indicate that
AMPK activation results in GLP-1 secretion. As metformin is known to exert many of its effects through an AMPK-dependent mechanism, this suggests a possible mechanism by which metformin enhances GLP-1 secretion.

Plasma DPP-IV activity was not affected by metformin treatment in vivo, consistent with previous studies (164). Furthermore, these results also ruled out the possibility that acute administration of metformin reduced secretion of soluble DPP-IV, contrary to what was suggested by Lenhard et al. (198), who demonstrated that metformin administered in vivo reduced DPP-IV plasma activity, while metformin incubated with DPP-IV in vitro did not affect enzyme activity. Furthermore, sitagliptin treatment significantly reduced plasma DPP-IV activity in my rats, an effect that was not enhanced by treatment with metformin, further demonstrating that metformin did not inhibit DPP-IV activity in my model. I therefore concluded that metformin enhanced plasma levels of both total and active GLP-1 in my studies through enhanced secretion, and not through DPP-IV inhibition. However, as this assay measures only the activity of plasma DPP-IV, independent of the anchored form, it cannot be dismissed that metformin may have an effect on this anchored form, especially in chronic treatment conditions, consistent with previous studies (189,200).

Three different in vitro models of the L-cell were used to determine if metformin directly stimulates GLP-1 secretion (aim 1). The first model, murine GLUTag cells, are a homogeneous immortalized L-cell line derived from a colonic tumor generated in a transgenic mouse expressing the simian virus 40 large T-antigen under the control of the proglucagon gene promoter (220). These cells have been extensively used for the determination of the molecular mechanisms leading to L-cell secretion (62;63;65;94;100;221;222), and have been shown to closely represent in vivo secretion data. The second model, the human NCI-H716 immortalized
cell line, was derived from an adenocarcinoma from the human cecum (223) and shown to display the characteristics of endocrine cells (205;224). These cells have been demonstrated to secrete GLP-1 in a regulated manner (77;94;100;111;222) consistent with in vivo data and, although a heterogeneous cell population, possess the obvious benefit of being of human origin.

The third and final model used was FRIC cells, a heterogeneous population of primary intestinal cells cultured from fetal rat intestine (206). While the use of a primary cell model can be beneficial for measuring L-cell function that closely reflects in vivo function, this model has an abundance of other cell types that can have unknown paracrine-mediated effects on L-cell function, as well as being derived from fetal rats in which gut cell differentiation may not be complete. Nonetheless, FRICs have been demonstrated to be an excellent model of L-cell secretion (61;74;94;100;208;225).

In each of the 3 cell models tested, neither metformin nor AICAR stimulated GLP-1 secretion, despite increasing AMPK phosphorylation (at least in the GLUTag and NCI-H716 cells). Therefore, although these agents do not directly induce secretion, they are biologically active in the L-cell. Moreover, AMPK is not involved in mediating GLP-1 secretion. This is especially interesting considering a recent study by Yu et al. (226), demonstrating the involvement of AMPK in mediating berberine-induced GLP-1 secretion from NCI-H716 cells. Berberine is an alkaloid that has been shown to stimulate AMPK phosphorylation and increase GLP-1 secretion in vivo and in vitro (226). In NCI-H716 cells, this stimulatory effect was reduced by treatment with Compound C, an AMPK antagonist. Nonetheless, it is curious that, while inhibition of AMPK decreases berberine-induced GLP-1 secretion, direct activation of AMPK with metformin or AICAR does not increase secretion; suggesting a more complex level of regulation within the L-cell.
Metformin has well-established actions as an insulin sensitizer (160;162;165) and a leptin sensitizer (191;227), in addition to more recent characterization as an incretin sensitizer in the beta cell (164), through both AMPK-dependent and –independent mechanisms. Thus, I also investigated the possible role of metformin in increasing L-cell sensitivity to an established in vivo L-cell stimulus, acetylcholine (110). Pre- and/or co-incubation of GLUTag cells with metformin did not enhance bethanechol-induced secretion of GLP-1, indicating that metformin does not increase L-cell sensitivity to this muscarinic receptor agonist. Furthermore, metformin did not increase L-cell sensitivity to the adenyl cyclase and cAMP-dependent pathway, demonstrated through stimulation with forskolin plus IBMX. However, while cholinergic activation of the L-cell has been demonstrated both in vitro and in vivo (110;111), and is a key regulator of GLP-1 secretion through the indirect-loop, there are other mediators that may also play a role in mediating these effects. Specifically, I have shown in the present study that, in vivo, GRP antagonists decrease metformin-induced GLP-1 secretion. Therefore, to fully rule out direct effects of metformin as an L-cell sensitizer, further studies will be required to look at increased responses to GRP. Furthermore, in the clinic, metformin may also enhance L-cell sensitivity to certain nutrient stimuli, whereby prolonged treatment with metformin enhances GLP-1 secretion after a standard mixed meal (146). Therefore further investigation into the effects of metformin on enhancing nutrient-induced secretion in vivo and in vitro is warranted.

Collectively, therefore, these results demonstrated that metformin does not directly act on the L-cell to induce GLP-1 secretion or enhance sensitivity and, hence, must act through an indirect pathway. As the cholinergic nervous system plays a dominant role in regulating nutrient-induced GLP-1 secretion (51;110), this was the first indirect pathway that was investigated (aim 2). Excitingly, administration of atropine decreased metformin-induced GLP-1 secretion,
implicating muscarinic receptors in this pathway. Selective involvement of the M3 receptor was then demonstrated through inhibition of metformin-induced GLP-1 release with 4-DAMP. The M1 and M2 receptors were both shown not to be involved in mediating metformin’s effects, an interesting finding considering the results of Anini et al. (110;111) showing that only the M1 receptor is involved in mediating duodenal nutrient-induced GLP-1 secretion in rats in vivo and that the M1 and M2 receptors are involved in mediating secretion in human and rats L-cells in vitro. In contrast to this, preliminary data in the murine L-cell shows that 4-DAMP suppresses bethanechol-induced GLP-1 secretion (data not shown), demonstrating that the M3 receptors are indeed actively involved in regulating secretion, at least in the murine GLUTag cell line. This suggests that cholinergic-induced GLP-1 secretion could be differentially regulated in the mouse as compared to the rat and human L-cell. However, due to the absence of an effective positive control for the M3 inhibition in these previous studies (110;111), it cannot be excluded that the M3 receptor is in fact also functional in mediating secretion in vivo and in vitro in the rat and human L-cell. Hence, by showing that M3 inhibition reduces metformin-induced GLP-1 secretion in vivo, it cannot be dismissed that this occurs at the level of the L-cell, although may also occur through an alternate pathway. As the importance of positive controls as been mentioned, this remains a limitation for the present study. The M2 muscarinic receptor is known to regulate heart rate (228); however, rats treated with gallamine did not display increased heart rates compared to control (data not shown). Thus, to definitively conclude that the M2 receptor is not involved in mediating metformin’s effects on GLP-1 secretion in vivo, further control studies will need to be performed with the specified dose of this M2 antagonist. Notwithstanding, the results of this study indicate that the M3, and not the M1 or M2, receptor is involved in the cholinergic-dependent effects of metformin on the L-cell. Interestingly, as shown in Figures
3.5A and D, neither atropine nor 4-DAMP provided a complete inhibition of metformin-induced GLP-1 secretion. While this could be due to the pharmacokinetics of the drugs (ineffective dose or short half-life), it is also possible that this is due to alternative metformin-dependent pathways mediating secretion.

The proximal-distal loop is mediated through the vagus nerve in rats (51) and it was therefore hypothesized that it may also mediate the actions of metformin. However, chronic bilateral subdiaphragmatic vagotomy did not abolish metformin-induced GLP-1 secretion, demonstrating that these effects were mediated through an alternate cholinergic pathway. Furthermore, the lack of an effect of vagotomy on metformin-induced GLP-1 secretion is also consistent with the finding of a role for the M3, as compared to the M1 ad M2 receptors in this pathway. However, interestingly, it was demonstrated that the vagotomized rats exhibited significantly lower basal GLP-1 secretion than sham-operated rats, consistent with the importance of the vagus nerve in regulating GLP-1 secretion. Moreover, this served as the positive control for successful vagotomy, as previously demonstrated measures such as increased stomach weight and the abolished inhibitory role of CCK on food intake were unsuccessful in indicating successful vagotomy (data not shown). The vagus nerve controls gastric emptying (212) and, consequently Wieczorek et al. (214) reported that the post-mortem stomach weights of vagotomized rats increased to over 4% of body weight, significantly higher than sham-operated rat stomach weights. In the present study, stomach weight in vagotomized animals was not significantly different than sham-operated animals; a factor that could be explained by the rats having undergone an overnight fast, which they were not subjected to in the referenced study. Furthermore, although it is well-established that CCK acts through the vagus nerve to inhibit food intake in rats (213;229), CCK has also been shown to exhibit its effects directly in
the brain at high enough concentrations. Therefore, due to the dose used for this control study, it is possible that CCK bypassed vagal afferents to act directly in the brain to inhibit food intake in vagotomized animals equivalently to the suppression found in sham-operated rats.

Finally, as the enteric nervous system neuropeptide, GRP, has been demonstrated to play a role in nutrient-induced GLP-1 secretion, this pathway was evaluated as a potential mediator of metformin-induced GLP-1 secretion by pre-treatment of rats at t = -30 min with the GRP receptor antagonist, RC-3095. Administration of RC-3095 reduced the effects of metformin briefly, but significantly. This reduction was only observed at 30 minutes after the administration of metformin, although it is unknown whether this loss of effect is due to properties of the drug. It has been reported that the effects of RC-3095 on the inhibition of gastrin secretion in rats decline after 60 minutes (230), despite findings that the plasma elimination half-life (albeit in humans) is substantially longer (231). Therefore, the waning effects of GRP receptor antagonism observed in the present study could be the result of the low effective half-life of this drug, requiring further studies that employ a longer-acting inhibitor.

Overall, therefore, I have shown that metformin acts at least in part through the muscarinic M3 and GRP receptors, independent of the vagus nerve, to stimulate GLP-1 secretion in the rat. Nonetheless, the exact pathway by which this occurs remains unknown, and may be involve a number of additional modulators; hormonal, neural, or both. For example, metformin has been demonstrated to inhibit sympathetic fibres (232;233). As the sympathetic nervous system has been implicated as a negative regulator of the L-cell (49), such an effect of metformin would lead to increased GLP-1 secretion. Metformin has also been demonstrated to cross the blood brain barrier (234) and, thus, it is entirely possible that metformin exerts its actions in the brain, acting on sympathetic fibres which may then interact with the enteric nervous system.
Additionally, while it is possible that metformin acts directly to activate cholinergic fibres, there is currently little research to support such an action.

4.1 Limitations and future studies

While the current study has provided evidence that metformin acts through cholinergic pathways to stimulate GLP-1 secretion, much is left unknown about its mechanism of action. A number of additional studies were attempted to elucidate the role of the enteric nervous system in mediating these effects. These unsuccessful experiments included elucidation of the role of the submucous plexus in mediating the effects of metformin, as well as the demonstration of the metformin-induced activation of GRP-positive neurons in the submucous plexus. The first of these experiments involved perfusing lidocaine, a local anaesthetic, through the small and large intestine of anaesthetized rats to freeze the sub-epithelial neurons. This technique has been previously used to assess involvement of the submucous plexus to regulate hormone secretion, but only with small isolated segments of intestine at a time (219). Due to the presence of L-cells at varying densities throughout the entire gut, it was therefore necessary to apply this anaesthetic throughout. For reasons unknown, this procedure was associated with a high mortality rate and, thus an alternative method for investigating the involvement of the submucous plexus must be devised. The second experiment involved an immunohistochemical approach to identify activated GRP-positive neurons in the submucous plexus of rats treated with metformin. To demonstrate that GRP directly mediated the effects of metformin on the L-cell, whole mount preparations of submucous plexus from the distal gut were labelled with primary antibodies for GRP and the immediate-early gene product, C-FOS. However, labelling for these neurons was unsuccessful and, thus, remains as another potential approach for future studies.
The AMPK knockout mouse has been well established as a model to investigate glucose homeostasis and insulin sensitivity (235), but it may be used as a model to assess the actions of metformin on GLP-1 secretion. Numerous studies have indicated AMPK-independent mechanisms of action of metformin (164;236;237) and, therefore, it will be important to determine whether the effects of metformin on GLP-1 secretion are mediated through an AMPK-dependent or –independent pathway. It is interesting to note that while AICAR stimulated GLP-1 secretion in vivo, and Yu et al. demonstrated AMPK-dependent effects of berberine on GLP-1 secretion in vitro (226), it is not necessarily true that metformin also acts through AMPK on its target cell in this pathway. Therefore, the use of an AMPK knockout mouse would further elucidate the mechanism of action.

As mentioned previously, the sympathetic nervous system has been shown to play an inhibitory role in GLP-1 secretion (49) and, therefore, as a sympathetic inhibitor (232;233), metformin may act simultaneously through the PNS to activate and through the SNS to alleviate inhibition from the L-cell. Future studies should employ the use of general and selective alpha- and beta- adrenergic receptor agonists alone and in combination with muscarinic receptor antagonists to determine a role for this system as well as potential synergistic effects between the two systems in regulating the L-cell. Finally, the M3 muscarinic receptor may be active on the L-cell after all, and thus may act in parallel to the GRP receptor to mediate GLP-1 secretion. Thus, similar studies should be performed using M3 and GRP receptor antagonists together to determine possible additive effects.

While it is possible that metformin-induced increases in GLP-1 may be due to decreased renal clearance of the peptide, this seems unlikely. I have demonstrated that the M3 receptor is involved in mediating the actions of metformin, and previous studies have shown that the rat
kidney does not express the M3 receptor (238). Therefore, this remains a possible avenue to investigate. Finally, while the present study has clearly demonstrated that metformin increases plasma levels of GLP-1 in acute conditions, it must be recognized that that this does not reflect the clinical setting, as patients are exposed to metformin for longer periods of chronic treatment. Mannucci et al. (189) demonstrated that chronic metformin treatment enhanced glucose-induced GLP-1 secretion in both healthy and diabetic subjects. Therefore, while metformin does in fact increase secretion in acute conditions, future studies should investigate the mechanisms involved in the enhancement of nutrient-induced GLP-1 secretion demonstrated with chronic metformin treatment.

Together, GLP-1 and metformin exert a multitude of beneficial effects in patients with T2DM; these effects have not gone unnoticed, as novel combinations of metformin and GLP-1 agonists and DPP-IV inhibitors continue to enter the clinic. Now that the GLP-1 agonists and DPP-IV inhibitors have become well-established, the next new wave is anticipated to be GLP-1 secretagogues. By elucidating the mechanism through which metformin enhances GLP-1 secretion, the results of the present study will not only provide a more comprehensive understanding of the benefits of co-administration of metformin and incretin therapy, but also a more thorough understanding of the pathways regulating GLP-1 secretion, opening the door to potential therapeutic avenues in the future.
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