Gut hormone secretion by enteroendocrine cells in human ileum and colon

by

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Abstract

Background and purpose

Type 2 diabetes (T2D), a metabolic disease strongly associated with obesity, represents a tremendous burden on healthcare systems worldwide. The curative effect of bariatric surgery indicates T2D pathogenesis has a significant gastrointestinal component. Enteroendocrine cells (EECs) are specialized endocrine cells dispersed throughout the gut epithelium and collectively constitute the largest endocrine organ in the body. Although gut hormones are implicated in maintaining glucose homeostasis and energy balance, our understanding of the mechanisms governing their secretion in humans remains largely incomplete.

This thesis aimed to: (a) develop a platform to study the secretory response of human enteroendocrine L cells, a subtype of EEC that secrete glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), (b) determine whether various reported pathways that govern GLP-1 and PYY secretion in rodents exist in humans, and (c) establish the classical hyperglycaemic pancreatic hormone, glucagon, as a gut hormone, following recent findings of extrapancreatic glucagon in pancreatectomized patients.

Experimental approach

Attempts to use a Percoll density gradient to enrich human L cells from primary mixed intestinal epithelial cell cultures were met with limited success. An ex vivo secretion platform using gut mucosae obtained from surgical specimens was therefore developed to study the secretory response of human L cells upon stimulation by a variety of compounds acting through a number of different pathways.

Key results

1) High glucose concentrations resembling postprandial luminal concentrations potently stimulated GLP-1 release in human duodenum and ileum, but not colon. This response is primarily driven by the electrogenic activity of the sodium glucose co-transporter 1, the facilitated transport of glucose by GLUT2, K_{ATP} channel closure and mitochondrial metabolism.
2) Acute exposure to the first-line anti-diabetes drug, metformin, potently triggers L cell secretion from human colon and ileum tissue. Baseline GLP-1 and PYY, and metformin-induced release, were unchanged across BMI and in subjects with type 2 diabetes. GLP-1 and PYY co-release was tightly correlated. AMPK inhibition blocked the L cell response to

metformin, as did antagonists of membrane transporters associated with metformin internalisation.

3) Acute exposure to the endogenous melanocortin 4 receptor agonist, α-Melanocyte stimulating hormone (α-MSH), and its more potent analogue, [Nle4,D-Phe7]-α-MSH (NDP-α-MSH), significantly triggered PYY and GLP-1 secretion from ileal and colonic mucosae. The stimulatory effect of NDP-α-MSH was attenuated by the endogenous melanocortin 4 receptor antagonist, Agouti-related peptide (AgRP).
4) The human gut epithelia release GLP-1 upon exposure to the myokine interleukin 6 (IL-6). This stimulatory response is also observed with IC7, a rationally-designed IL-6 receptor agonist with a lower inflammatory potency than IL-6.
5) The classical endocytotic protein, dynamin, is implicated in controlling L cell exocytosis.
6) The human gut epithelium is a source of fully-processed glucagon. The release of gut-derived glucagon is triggered by exposure to the amino acid arginine and levels of glucose resembling those seen postprandially in the gut lumen. The mechanism underlying glucose-induced glucagon release from the gut is different to that governing glucose-induced GLP-1 release.

Conclusions

Collectively, these results show that many pathways govern L cell secretion exist in humans, although some major differences were also observed to those observed in rodents. Additionally, the presence of gut-derived glucagon represents a new potential therapeutic target for treating T2D.

Declaration

I, Emily Wai-Ling Sun, certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Acknowledgement

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Abbreviations

5-HT	Serotonin
AgRP	Agouti-related peptide
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DIO	Diet-induced obesity
DJB	Duodenal-jejunal bypass
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPP4	Dipeptidyl peptidase 4
EC	Enterochromaffin
EDTA	Ethylenediaminetetraacetic acid
EEC	Enteroendocrince cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FRIC	Foetal rat intestinal culture
GCGR	Glucagon receptor
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GIPR	Glucose-dependent insulinotropic peptide receptor
GLP	Glucagon-like peptide
GLP-1R	Glucagon-like peptide 1 receptor

GLP-1RA	Glucagon-like receptor agonist
GLUT	Facilitative glucose transporter
GPCR, GPR	G-protein coupled receptor
GSIS	Glucose-stimulated insulin secretion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HPLC	High-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
ICV	Intracerebroventricular
IL	Interleukin
K_{ATP} channel	ATP-sensitive potassium channel
LAGB	Laparoscopic adjustable gastric banding
LC	Liquid chromatography
LPS	Lipopolysaccharide
MC4R	Melanocortin-4 receptor
MS	Mass spectrometry
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OXM	Oxyntomodulin
PBS	Phosphate-buffered saline
PC	Prohormone convertase
PGDP	Proglucagon-derived peptide
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY
RIA	Radioimmunoassay
RYGB	Roux-en-Y gastric bypass
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SGLT	Sodium glucose co-transporter

SNARE	soluble *N*-ethyl-maleimide-sensitive fusion protein attachment protein receptor
SST	Somatostatin
SSTR	Somatostatin receptor
STZ	Streptozotocin
T2D	Type 2 diabetes
TNF	Tumour necrosis factor
TPH	Tryptophan hydroxylase
VSG	Vertical sleeve gastrectomy
α-MSH	Alpha-melanocyte stimulating hormone

1 The role of the enteroendocrine system in diabetes pathogenesis

1.1 Introduction

Diabetes is a disease of disrupted glucose homeostasis. The World Health Organization (WHO) defines diabetes as "fasting blood glucose of 7.0 mM or greater; or on medications for raised blood glucose". It is estimated the global prevalence of diabetes was more than 9% among adults aged 18 years and over in 2014[1]. In the most recent status report on non-communicable diseases compiled by the WHO, there was an estimated 1.5 million deaths caused directly by diabetes globally and the WHO projected diabetes will be the 7th leading cause of death in 2030. With approximately 1 million Australians being diagnosed with diabetes, the total annual cost for Australians with the disease is $6 billion and is expected to increase substantially if the disease prevalence continues to rise at its current rate[2,3]. Microvascular complications of diabetes such as retinopathy are the leading causes of blindness in the developed world, while diabetic neuropathy and nephropathy are the main causes for amputations and dialysis, respectively[4]. Furthermore, diabetic macrovascular complications often develop in the form of atherosclerosis, which significantly increases the risk of myocardial infarction and stroke[5]. While there is an array of aetiologies for diabetes, type 2 diabetes mellitus (T2D) is by far the most common type and accounts for up to 90-95% of diabetes diagnosis[1].

Blood glucose levels are regulated by the interplay between two pancreatic hormones, glucagon and insulin, which are produced by α and β cells in the endocrine pancreas, respectively (Figure 1.1). Glucagon is a catabolic, glucose-mobilizing hormone that promotes the release of glucose from the liver through glycogenolysis[6] while insulin is the anabolic, glucose-disposing hormone that encourages glucose uptake into insulin-sensitive tissues in the periphery. During fasting, the glucagon-to-insulin ratio markedly increases to allow glucagon's action to dominate over that of insulin[7], thereby maintaining blood glucose levels within the optimal physiological range of 4 – 6 mM in humans[8]. Hypoglycaemia is defined as blood glucose levels below 3.9 mM[9] and can cause a range of neurological symptoms such as headache, loss of consciousness and seizures. If left untreated, hypoglycaemia could result in severe brain damage[10]. Conversely, insulin action dominates in postprandial periods. It acts at insulin receptors on peripheral organs such as the liver, skeletal muscles and adipose tissue to encourage the uptake and storage of glucose into these organs, via glycogeneogenesis and lipogenesis, essentially clearing "excess" glucose from the circulation to maintain glucose homeostasis. Insulin further lowers blood glucose by inhibiting hepatic glucose

output, acting directly on the liver and indirectly on neighbouring α cells by suppressing glucagon secretion. Postprandial glucose disposal is crucial in maintaining glucose homeostasis as excess glucose in circulation (hyperglycaemia) causes oxidative stress in nerves, the kidneys and the endothelial cells of the vasculature, which are implicated in the development of diabetic microvascular complications.

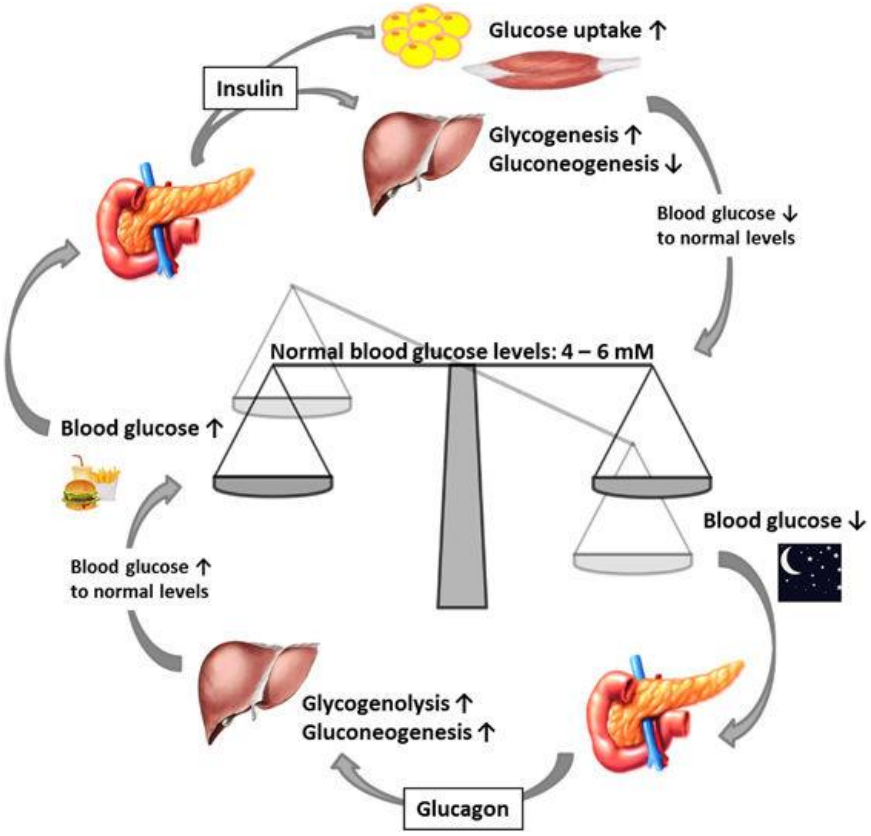


Figure 1.1.1 Glucose homeostasis is maintained by the interplay between the hypoglycaemic actions of insulin and hyperglycaemic actions of glucagon. Reprinted from reference [11].

1.2 Type 2 diabetes and its aetiology

T2D is characterized by insulin resistance coupled with varying degrees of insulin insufficiency, which combine to attenuate insulin action. As such, glucose cannot be effectively cleared from the circulation by insulin-sensitive tissues such as the liver, skeletal muscles and adipose tissue, rendering the individual hyperglycaemic (Figure 1.2.1). In the face of hyperglycaemia, β cells of the endocrine pancreas increase insulin output as a compensatory mechanism. This is typically characterized by an initial hyperinsulinaemic phase, which prevents the individual from becoming chronically hyperglycaemic[12]. However, worsening insulin resistance increases the demands on β cells to increase insulin output. It is not uncommon for individuals with marked insulin resistance to not develop diabetes, provided their endocrine pancreas can compensate for the increased insulin demand[13].

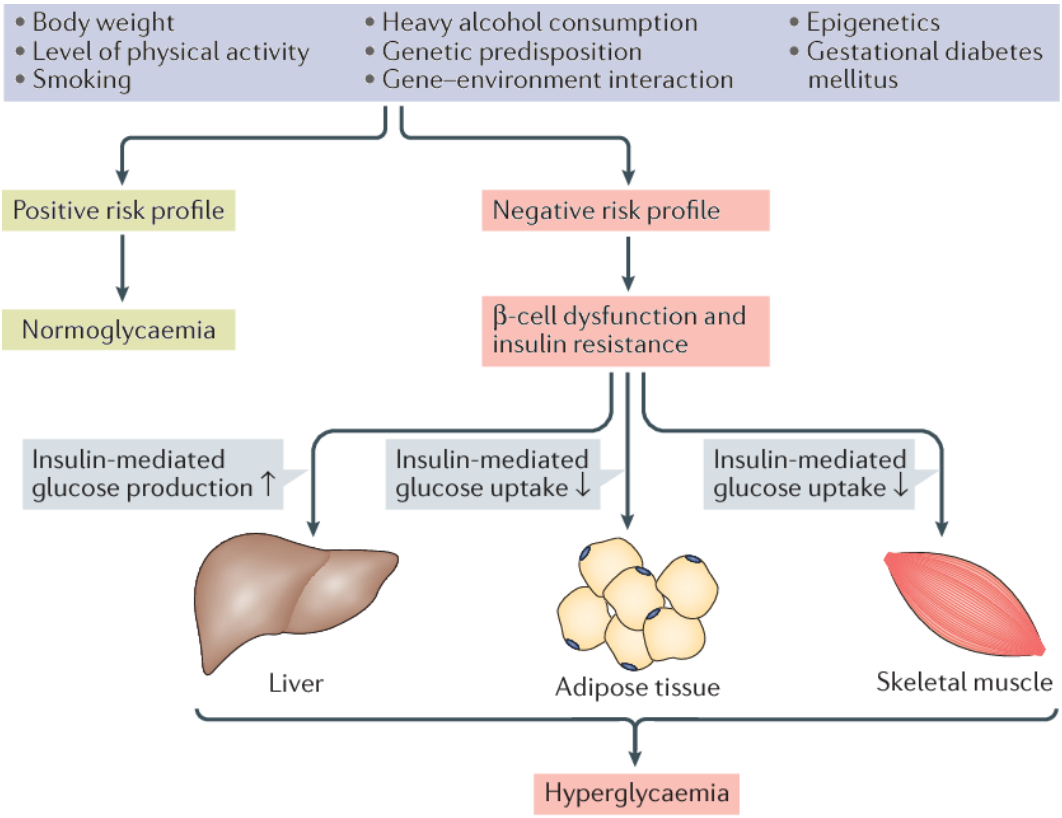


Figure 1.2.1 The risk factors and aetiology of type 2 diabetes. T2D develops as a result of reduced insulin action in insulin-sensitive tissues coupled with impaired insulin secretion by pancreatic β cells. Reprinted from reference [14].

Diabetes typically progresses from a "pre-diabetes" state, at which stage an individual either has impaired fasting glucose and/or impaired glucose tolerance. Impaired fasting glucose is defined as blood glucose levels between 6.1 – 7 mM after an overnight fast while impaired glucose tolerance is defined as blood glucose levels between 7.8 - 11 mM 2 hours after a 75 g oral glucose load[15]. Since β cells are prone to glucotoxicity[16-21], hyperglycaemia secondary to attenuated insulin action further exacerbates deterioration in β cell health. This creates a cycle that can ultimately result in loss of β cell mass and function, rendering the endocrine pancreas no longer being able to produce sufficient insulin to clear glucose and the subsequent diagnosis of diabetes.

T2D is a complex, multifactorial, polygenic disease[22]. Genetic predisposition remains the most prominent risk factor with first-degree relatives of T2D patients having a 3.5 times higher risk of developing T2D than the general population[23]. T2D is highly concordant between identical twins[24-27]. Aging[28,29], polycystic ovarian syndrome[30,31] and the use of atypical antipsychotics[32,33] are also risk factors for developing T2D. However, the most important modifiable risk factor for developing T2D is obesity, which is profoundly implicated in the development of insulin resistance[34,35] and impaired β cell function[36]. Insulin resistance is positively correlated with visceral fat deposits[37,38] and central obesity[39,40]. Hyperlipidaemia secondary to obesity is a prominent cause for β cell dysfunction due to

increased lipid deposition within the pancreas[41,42]. The importance of excess weight in T2D development is evident by the fact that one of the most reliable ways to induce T2D phenotypes in laboratory animals is to put the animals on an obesogenic diet, or to select animals with an obesogenic genetic background[43]. Therefore, it is of no surprise that weight loss, especially the reduction of visceral fat deposit is highly relevant in the prevention and treatment of T2D[44].

For many years, diabetes research and clinical management has adopted a mostly insulino-centric view[45]. Enhancing insulin action, either through exogenous insulin supplementation, increasing insulin secretion (sulfonylureas, GLP-1 receptor (GLP-1R) agonists or dipeptidyl peptidase 4 (DPP4) inhibitors) or increasing peripheral insulin sensitivity (biguanides and thiazolidinediones), forms the basis of most clinically available anti-diabetic therapeutics[46]. Despite more than half a century of research, the optimal control of blood glucose in diabetic patients has not been achieved with these predominantly insulino-centric approaches. More recent approaches to combat hyperglycaemia involve reducing intestinal glucose absorption, promoting urinal glucose excretion and reducing glucagon secretion[47].

1.2.1 Potential gastrointestinal components to diabetes pathogenesis: lessons from bariatric surgeries

As a significant portion of T2D patients have already experienced considerable impairment in β cell function by the time of diagnosis[12], T2D has been overwhelmingly treated as a disease of the endocrine pancreas of impaired insulin function. Whilst insulino-centric approaches are effective in reducing glycaemia and therefore significantly reduce the risks of the development of micro-and macrovascular complications, none have curative effects. Despite relentless research, T2D remained a disease with no definite cure until the emergence of bariatric surgeries. Multiple studies in the 1970s showed that small bowel bypass operation, then a treatment for obesity, came with the unanticipated outcome of marked improvement in glycaemic control[48,49]. Laparoscopic adjustable gastric banding (LAGB), Vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB) are the three most commonly performed bariatric procedures (Figure 1.2.2). However, the efficacy of bariatric produces involving anatomical manipulations of the gastrointestinal (GI) tract, namely VSG and RYGB, in improving glycaemic profiles, is far superior to that of LAGB, a purely restrictive procedure[50]. In many cases, complete remission of T2D is achieved, enabling the removal of anti-diabetic medications to maintain adequate glycaemic control[51]. Although originally thought to be a weight-dependent effect, it was later established that weight loss through caloric restriction and exercise could not achieve T2D remission in such a dramatic way and T2D is resolved within days after bariatric surgeries in many cases, long before any significant weight loss was achieved[52].

Importantly, long-term follow up studies report that many post-bariatric patients remain in diabetes remission for decades[53-56].

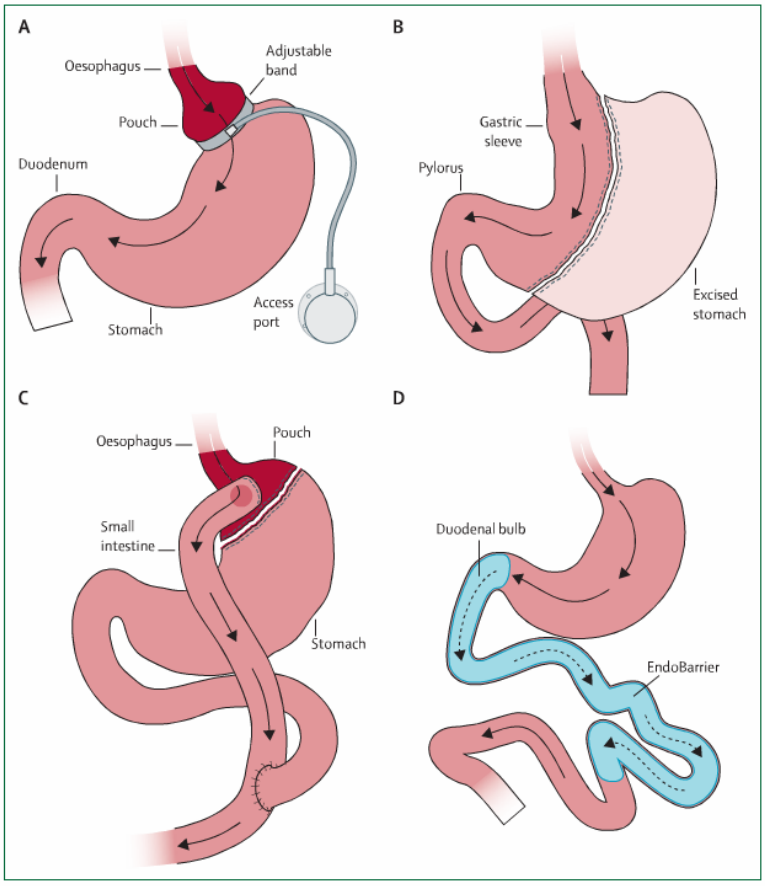


Figure 1.2.2 Schematic representation of the GI anatomy after different bariatric procedures. (A) Laparoscopic adjustable gastric banding (LAGB), is a purely restrictive procedure. An adjustable silicone band is place around the top part of the stomach to create a small gastric pouch. Food intake in patients is reduced as a result of reduce capacity of the gastric pouch to accommodate ingested food. The nutrient flow after LAGB is not altered. (B) Vertical sleeve gastrectomy (VSG), 70-80% of the stomach is excised and the remainder is stapled to form the gastric sleeve. Ingested nutrient bypasses the excised gastric fundus but flows normally distal to the pylorus. (C) Roux-en-Y gastric bypass (RYGB), a large part of the stomach is excised and stapled, forming a small pouch is at the top part of the stomach, which is anastomosed to jejunum. After RYGB, ingested nutrients flow from the gastric pouch directly into the jejunum, bypassing the excised stomach and the entire length of the duodenum. (D) EndoBarrier, a gastrointestinal liner that is endoscopically placed in the duodenum to devoid nutrient exposure to the duodenum. Reprinted from reference [50].

The fact that many T2D cases could be "cured" by anatomical rearrangements of the GI tract suggests the disease itself may have a significant, yet underappreciated, gastrointestinal component to its underlying pathogenesis[57-59]. This view is further supported by the impressive glucose-lowering efficacy of the less invasive EndoBarrier®, which is essentially an endoscopically placed liner to prevent contact between ingested nutrients and the lumen of the proximal small intestine[60-62]. In addition, experimental bariatric procedures such as ileal transposition and duodenal-jejunal bypass

(DJB) surgeries are highly efficacious in improving glycaemia in diabetic animal models[63-65]. Intriguingly, DJB improves glucose tolerance in insulin-deficient animals, suggesting glucose homeostasis could be improved via an insulin-independent pathway[63,66]. Therefore the GI tract is highly important for T2D treatment[67] and if such, the gut could also be implicated in the pathogenesis of the disease[68-70].

With the global diabetes drug market estimated to reach USD $116 billion by 2023[71], it is not surprising that there is an ongoing race within the pharmaceutical industry to develop efficacious "bariatric mimetic" pharmacotherapies[72-74]. However, the progress is significantly hindered by the lack of thorough understanding of the mechanisms underlying the anti-diabetic actions of bariatric surgeries. Although many hypotheses have been proposed[50,75-86], the exact mechanism(s) of the anti-diabetic action of bariatric surgery is still a subject of ongoing debate. One of the most profound changes that occurs after gastric bypass surgery is an individual's postprandial gut hormone profile. Bariatric surgeries such as RYGB and VSG typically result in a postprandial surge of gut hormones such as the insulinotropic GLP-1 and the anorectic PYY. The levels of these hormones are typically a few fold higher than in un-operated individuals[87], likely due to the increased exposure of the distal gut to nutrients and the higher number of GLP-1 and PYY-positive cells post-surgery[88,89]. Whether the postprandial GLP-1 and PYY surges are coincidental or an essential component of the underlying mechanism of RYGB remains a controversial topic[77,90-92]. Nonetheless, the endocrine system of the GI tract is still a very attractive target for developing anti-diabetic treatments. I will provide an overview of the enteroendocrine system in the following section and discuss how it is implicated in T2D pathogenesis.

1.3 The enteroendocrine system

Although commonly underappreciated, the 1% of specialized endocrine cells sparsely scattered throughout the gut epithelium constitute the largest endocrine organ in the body by mass[93]. Collectively referred to as the enteroendocrine system, these endocrine cells "sample" luminal contents on the apical/brush border membrane and release more than 20 different hormones on their basolateral sides in response to different stimulants. Moreover, mechanical stimulations, neural inputs from the autonomic and enteric nervous systems[94], and pro- and anti-inflammatory signals from immune cells[95,96] are also known stimulants of gut hormone secretion from enteroendocrine cells (EECs) (Figure 1.3.1). These hormones are implicated in a wide range of physiological functions including GI motility, appetite control and glucose homeostasis[97].

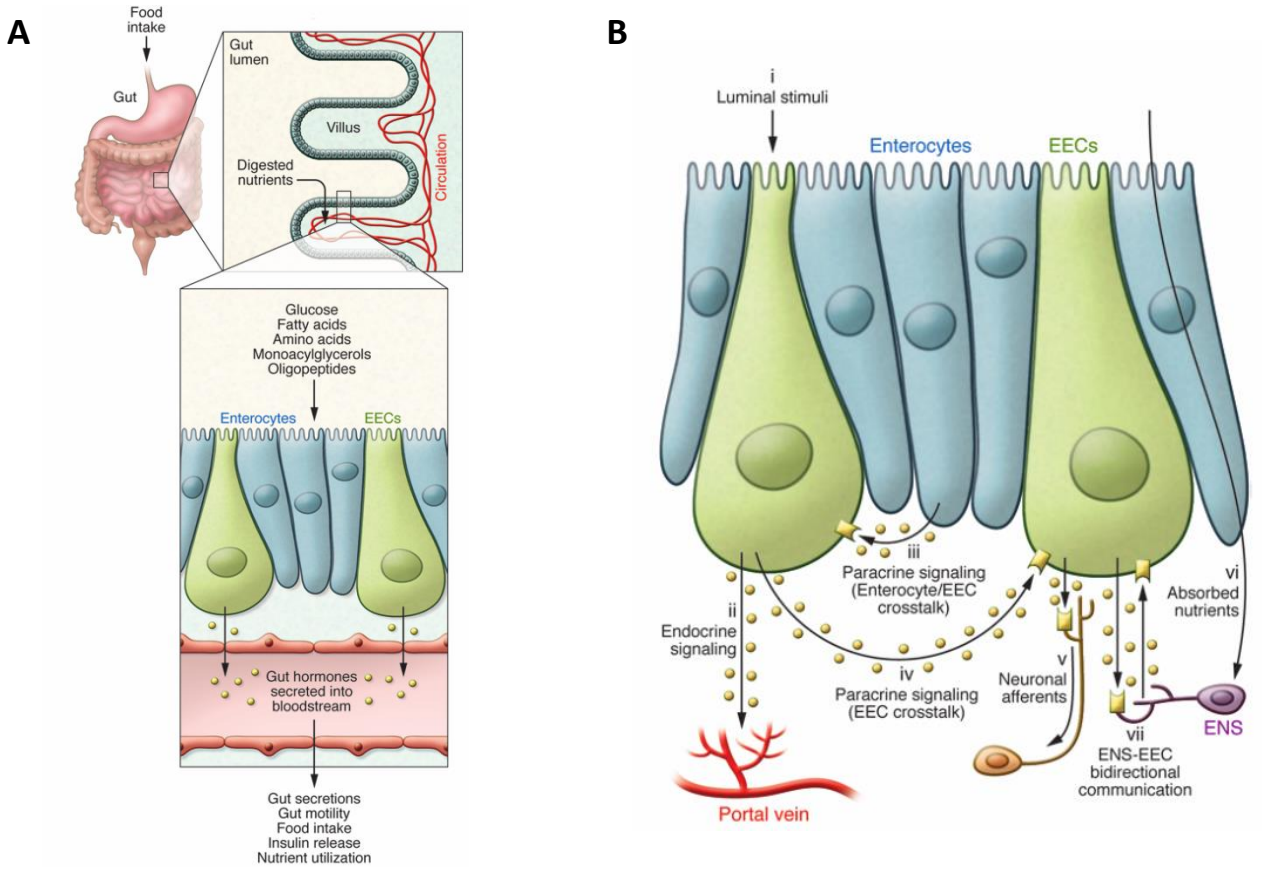


Figure 1.3.1 (A) Luminal nutrient exposure stimulates enteroendocrine cells (EECs) and they release different hormones on the basolateral side in response to the stimulation. These hormones are involved in regulating a wide range of physiological functions. (B) Signalling pathways to and from EECs: (i) luminal stimuli such as nutrients directly trigger the release of many hormones from EECs. (ii) The hormones released by EECs enter the circulation through the portal vein. EECs receive inputs from neighbouring (iii) enterocytes and (iv) other EECs via paracrine signalling. (v) Hormonal cues from EECs are relayed to the CNS via neuronal afferents of the vagus nerve. (vi) Nutrients can also regulate EEC secretion indirectly by acting on the enteric nervous system (ENS) as there are considerable bi-directional communications between EECs and the ENS. Reprinted from reference [98].

Unlike other endocrine cells elsewhere in the body that are found in clusters, EECs are dispersed throughout the mucosal epithelium of the GI tract[99], as a result of Notch-mediated differentiation of pluripotent stem cells of the intestinal epithelia[100,101]. While all the epithelial cells (enterocytes, Goblet cells, Paneth cells and EECs) of the intestinal mucosa originate from this pool of pluripotent stem cells, once a stem cell is committed to differentiate into a secretory cell, it inhibits neighbouring cells from adopting the same fate via Notch-signalling. The cell further differentiates into an endocrine cell under the control of the transcription factors Neurogenin3 and NeuroD (Figure 1.3.2). Owing to this lateral inhibition, it is rare to locate two EECs adjacent to each other along the intestine.

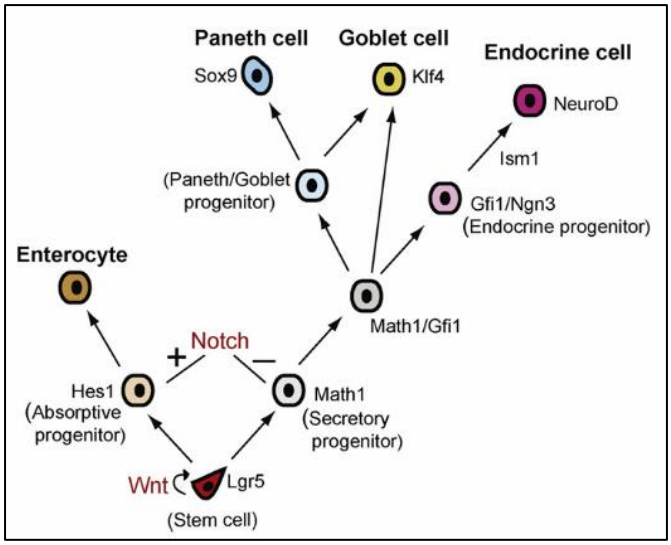
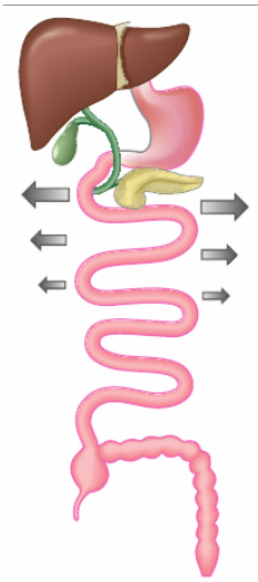


Figure 1.3.2 The differentiation of enteroendocrine cells from pluripotent stem cells (marked by Lgr5) in the crypts of the intestinal epithelia. Expression of Math1 or Hes1 differentiates the secretory lineage from the absorptive lineage (enterocytes). Notch signalling in Math1-expressing cells prevents neighbouring cells from developing into the same cell type. Endocrine progenitors express Neurogenin3 (Ngn3) and subsequently NeuroD, allowing them to differentiate into EECs. Reprinted from reference [100].

The EEC population constitutes different cell types, each of which has its own specialized functions, usually characterized by its secretory products. It is now accepted that there are vast overlaps in the secretory profiles of EECs[102] and the "one cell type, one hormone" dogma is widely rejected. Studies using transgenic mice expressing fluorescent reporter proteins driven by promoters of different gut hormones revealed that multiple hormones can be simultaneously expressed by an individual EEC[103,104]. Immunohistochemical analysis of these fluorescent-reporter protein-tagged EECs reveals that a substantial population of GLP-1 secreting L cells in the upper small intestine also express CCK or GIP[104] and vice versa[103]. The fact that ablating diphtheria toxin receptor-expressing GLP-1-secreting L cells simultaneously reduced CCK- and GIP-expressing cells further validates these findings[103]. High-resolution microscopy shows that these different hormones are packaged into separate vesicles within the EEC[105-107], which could enable potential differential exocytosis depending on the stimulus[108]. Expression of EEC hormones are also regionally distinct, as many gut hormones are confined to specific regions of the gut, while a subset, such as serotonin and somatostatin, are present throughout the GI tract[109,110](Table 1.3.1). Emerging evidence suggests that it is perhaps more appropriate to characterize EECs based on their anatomical location along the GI tract rather than their secretory profiles. Microarray analysis of fluorescent protein-tagged EECs using fluorescent-assisted cell sorting (FACS) revealed GLP-1-L cells in the small intestine are more similar to GIP-secreting K cells in the same region than are to GLP-1 secreting L cells in the colon[104]. [102,103] In the following sections, I will discuss the role of several of these gut hormones in the

context of energy balance and glucose homeostasis and importantly, how they are implicated in obesity and T2D pathogenesis.



Gut region	Intestinal processes	Luminal stimuli of EECs	Principal gut hormones
Stomach	Acid secretion Mechanical disruption	Acid Digested protein	SST, histamine, 5-HT, ghrelin, gastrin
Duodenum Jejunum Proximal ileum	Release of bile acids, pancreatic and intestinal enzymes, bicarbonate Digestion Absorption (➡)	Monosaccharides Free fatty acids Monoacylglycerols Amino acids Di/tripeptides Bile acids	Duodenum: GIP, ghrelin, CCK, 5-HT, SST Jejunum, ileum: GLP-1, GLP-2, PYY, 5-HT, Nts
Terminal ileum	Bile acid reabsorption	Bile acids Unabsorbed nutrients	GLP-1, GLP-2, PYY, Nts, 5-HT
Colon Rectum	Bacterial metabolism	Short-chain fatty acids Indole Secondary bile acids	GLP-1, GLP-2, PYY, Nts, Insl5, 5-HT

Table 1.3.1 The hormonal profiles of the different regions of the gut. 5-HT (serotonin), GIP (glucose-dependent insulinotropic peptide), CCK (cholecystokinin), SST (somatostatin), GLP-1 and GLP-2 (Glucagon-like peptide 1 & 2), PYY (peptide YY), Nts (neurotensin), Insl5 (insulin-like peptide 5). Reprinted from [110].

1.3.1 Serotonin (5-HT)

Serotonin (or 5-Hydroxytryptamine, 5-HT) is produced by enterochromaffin (EC) cells, which constitute approximately 50 % of the total EEC population and are scattered throughout the length of the gut, from the stomach to the distal colon[102,109]. Although better-known for its central actions, more than 90 % of circulating 5-HT is synthesized by EC cells and stored in platelets[111,112]. Tryptophan hydroxylase 1 (TPH1) is the rate-limiting enzyme of 5-HT synthesis in EC cells and its expression in the gut mucosa is limited to EC cells. EC cells have the capacity to sense[113,114] and secrete 5-HT in response to, a wide range of nutrients present in the gut lumen such as glucose and fructose[115,116], the medium chain fatty acid, lauric acid[117], various tastants and olfactants[114]. 5-HT secretion from EC cells is also regulated by neural and endocrine inputs such as adrenergic stimulation and GABA and somatostatin inhibition[118].

Although traditionally regarded as a regulator of gastric motility[119] and more recently, a mediator in the pathogenesis of inflammatory intestinal disorders[111,120], gut-derived 5-HT is now also recognised as an important player in energy balance and glucose homeostasis[121-124] (Figure 1.3.3). In contrast to its action in the central nervous system, in which it favours negative energy balance and promotes weight loss[125], peripheral 5-HT is a potent driver of obesity and perturbed glucose homeostasis. This notion stemmed from observations that diet-induced obese (DIO) rodents had higher circulating and

intestinal 5-HT when compared with lean counterparts[126]. Importantly, *TPH1* expression is significantly elevated in obese humans[127,128] and circulating 5-HT levels are significantly elevated in T2D patients[129-131]. The causative role of elevated gut-derived 5-HT in driving these phenotypes was later established by studies showing inhibition of intestinal TPH1 in mice, either through tissue-specific ablation or pharmacological inhibition, protected mice from glucose intolerance and weight gain secondary to a high fat diet[132-134]. Multiple metabolically important organs are potential targets for the obesogenic and diabetogenic effects of peripheral 5-HT via an array of 5-HT receptors. Gut-derived 5-HT markedly increases hepatic glucose output, a main driver of hyperglycaemia, by increasing hepatic gluconeogenesis and glycogenolysis[135] and inhibiting glucose uptake and glycogen synthesis in the liver[132]. In addition, gut-derived 5-HT promotes lipolysis in adipocytes, thereby liberating free fatty acids (FFAs) and glycerol[132], which are important substrates for hepatic gluconeogenesis and thus further enhancing hepatic glucose output. Moreover, gut-derived serotonin promotes weight gain through attenuating thermogenesis in brown adipose tissue and inhibiting browning of white adipose tissue[133,134], thus reducing energy expenditure.

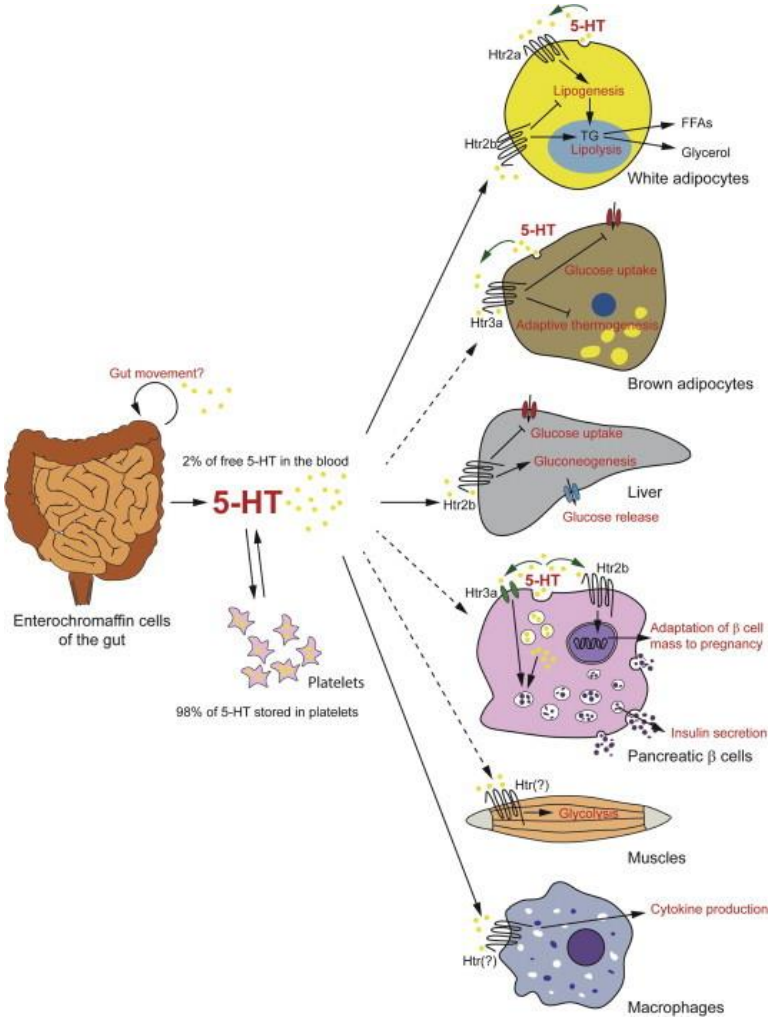


Figure 1.3.3 The metabolic effects of 5-HT derived from enterochromaffin (EC) cells from the gut epithelium. 5-HT promotes lipolysis in white adipocytes and inhibits thermogenesis in brown adipocytes. It also stimulates hepatic glucose output and may promote glycolysis in skeletal muscles. 5-HT receptors (Htrs). Reprinted from reference [124].

5-HT also promotes β-cell mass expansion, enhances glucose-stimulated insulin secretion[136,137] and inhibits glucagon secretion[138]. Such effects appear to be contrary to the aforementioned diabetogenic effects of 5-HT. However, it is worth noting that it is unlikely for gut-derived 5-HT to be mediating these effects as β-cells produce and secrete serotonin endogenously, which signals in an autocrine[139] and paracrine fashion[138] within pancreatic islets. Indeed, eliminating gut-derived 5-HT did not significantly affect glucose-induced insulin secretion *in vivo*[132], supporting the notion that islet function is not directly regulated by gut-derived 5-HT.

An obesogenic Western diet significantly increases *Tph1* mRNA and EC cell number in rodent small intestine[128,140] and recent work from our laboratory and collaborators clearly demonstrated that obese humans have increased EC density in the duodenum[127]. Thus, there is clear evidence showing EC cell-derived 5-HT negatively impacts energy balance and glucose homeostasis with the underlying causes of elevated 5-HT levels remaining unclear[121].

1.3.2 Somatostatin (SST)

Somatostatin is an inhibitory hormone in a number of endocrine systems throughout the body such as the endocrine pancreas and the hypothalamus. SST is also synthesized and secreted by enteroendocrine D cells, which are most abundant in the gastric epithelium and present throughout the entire length of the intestinal epithelium[141]. Somatostatin is the gene product of *SST*, which encodes for a 120-amino acid peptide precursor, preprosomatostatin. Post-translational cleavage of the precursor gives rise to two bioactive forms that are found in the circulation, the 14-amino acid SST-14 and the 28-amino acid SST-28, which is a 14-amino acid extension of the N-terminus of SST-14. It is now widely accepted that the SST-28 is the predominant form produced by enteroendocrine D cells while SST-14 is mainly produced within the CNS. The secretion of somatostatin from the gut can be triggered by various stimuli such as lipids or decreased luminal pH[142-144]. In addition, intestinal SST secretion is also under the control of the enteric[145,146] and parasympathetic[130] nervous systems.

Gut-derived SST inhibits gastric acid production by suppressing secretions from gastric chief cells and parietal cells. Moreover, SST inhibits nutrient absorption[147] and negatively regulates the secretion of virtually all other gut hormones[148]. The inhibitory actions of intestinal somatostatin is also implicated in anti-inflammatory responses within the intestinal epithelia by inhibiting pro-inflammatory cytokine secretion by local immune cells[149,150]. These actions are mediated by a family of five different somatostatin receptors (SSTRs), all of which are GPCRs that are negatively coupled to

adenylyl cyclase[151]. The role of gut-derived somatostatin in metabolism proved to be difficult to elucidate given that *SST* is expressed by various endocrine cell types throughout the body. Although administration of SST and its analogues are beneficial in relieving hyperglycaemia during insulinopaenia by attenuating glucagon action, it is unlikely that gut-derived somatostatin is directly implicated in the regulation of pancreatic islet hormone secretion. Rather, this is regulated by SST-derived from pancreatic δ cells[152]. A gut-specific somatostatin knockout animal model has not yet been reported and until such an animal model is phenotypically characterized, the role of intestinal somatostatin in metabolism will remain unclear.

1.3.3 Cholecystokinin (CCK)

Cholecystokinin (CCK) is a neuropeptide produced by enteroendocrine I cells predominantly found in the proximal duodenum. CCK-8, an octapeptide cleaved from the C-terminus of the 95 amino acid prohormone, is the shortest bioactive CCK peptide[153]. As the bioactivity of CCK resides in the C-terminus of the prohormone, all other bioactive CCK peptides such as CCK-22, CCK-33 and CCK-58, are N-terminus extensions of CCK-8[154,155]. CCK is also produced by enteric neurons and is highly expressed in the CNS. Two types of CCK receptors have been identified. CCK_1R is predominantly found in the periphery and CCK_2R is localized to the CNS. CCK_1R is highly selective for sulphated CCK-peptides while CCK_2R binds equally to both sulphated and unsulphated forms. I cell CCK secretion is triggered by nutrient ingestion. Fats are the most potent of the macronutrients to trigger CCK release while carbohydrates are the weakest. The role of CCK in regulating digestive function has long been appreciated as it is a potent stimulator of gallbladder contraction and exocrine pancreatic secretion, which aid digestion and absorption by the release of bile and pancreatic enzymes into the intestinal lumen, respectively. CCK also inhibits gastric emptying and colonic motility[156,157].

The importance of CCK in maintaining energy balance was first recognized when its potent satiating action was described[158-160]. Postprandial release of CCK acts on vagal afferent CCK_1R[161,162], which signals to POMC neurons in the nucleus tractus soliatrius (NTS) in the brainstem to induce acute satiation[163,164]. Subsequent studies revealed that while CCK reduces meal size, this is typically compensated by increasing meal frequency and thus, has limited impact on cumulative food intake in an acute setting[165,166]. Nonetheless, the importance of CCK-induced satiation in energy balance and glucose homeostasis became evident when the phenotype of OLETF rats, which lack functional CCK_1R[167], was described[168]. These rats display profound hyperphagia from birth and subsequently become obese and diabetic later in life, which closely resembles the development of T2D in humans secondary to obesity[169,170]. Interestingly, pair-feeding these rats to control rats with functional CCK_1R completely prevented the development of the obese and diabetic phenotype[171,172], which indicates increased food intake is the primary driver of the phenotype. However, it is important to note that

there are major species differences in the actions of CCK. Although ablation of *Cck$_1$r* in mice resulted in increased meal size, it had no significant impact on cumulative food intake in these mice[173] and *Cck$_1$r*-deficient mice have normal bodyweight and glycaemia[173,174], contrasting the spontaneously obese and diabetic phenotype of OLEFT rats. It was later shown that neuropeptide Y (NPY) neurons in the mouse dorsomedial hypothalamus do not normally express CCK_1R[174], a pathway that partly mediates CCK-induced satiation in rats[171,175]. It remains to be determined whether this pathway exists in humans. Nonetheless, recent studies showed that twice-daily injection of a CCK-8 analogue caused significant weight loss and improved glycaemia in diet-induced obese (DIO) and *ob/ob* mice after one month[176,177], suggesting exogenous CCK could play a role in maintaining energy homeostasis in the face of metabolic challenges such as a high fat diet.

There is also evidence supporting a role of CCK in maintaining glucose homeostasis independent of its satiating effects. Although data regarding CCK's effect on insulin secretion is somewhat conflicting[178-182], it is clear that CCK significantly reduces postprandial glucose excursions in humans through its inhibitory effect on gastric emptying[183], a major determinant of postprandial glycaemia[184]. In addition, CCK administration significantly increases the secretion of the incretin, GLP-1[185], which has potent insulinotropic and glucagonostatic actions[69](see section 1.3.5.1.1). This effect likely occurs through the direct stimulation of enteroendocrine L cells by bile acids[186] released from CCK-induced gallbladder contraction[185]. Moreover, CCK could be implicated in regulating hepatic glucose output as intraduodenal administration of CCK-8 in rats significantly reduced hepatic glucose production in a vagally-mediated manner[172].

While there is no doubt CCK is an important regulator of energy and glucose homeostasis, it remains difficult to determine the contribution by I cell-derived CCK as the neuropeptide is also released by a subset of enteric neurons[187] that could potentially act on vagal afferent CCK_1R to mediate many of the physiological actions of CCK discussed above. In addition, CCK is expressed in other peripheral tissue such as pancreatic islets[188,189], cardiomyocytes[190] and the anterior pituitary[191], which could confound findings from loss-of-function animal models such as *Cck$_1$r*-deficient OLETF rats and *Cck* knockout mice[192]. Thus, until a model of gut-specific CCK ablation is developed, the physiological effects ascribed to CCK derived from I cells will have to be interpreted with great caution.

1.3.4 Glucose-dependent Insulinotropic Peptide (GIP)

Glucose-dependent Inuslinotropic Peptide (GIP) is a 42-amino acid peptide hormone produced by a subset of enteroendocrine cells called K cells that are located in the proximal intestine, predominantly in the duodenum and jejunum[193,194]. GIP-secreting cells were recently shown to produce other hormones such as CCK and GLP-1[103]. GIP secretion is stimulated upon nutrient

ingestion with glucose[195], amino acids[196] and lipids[197] all being potent stimulants. GIP exerts its actions by binding to the GIP receptor (GIPR), a class II GPCR that is expressed by pancreatic islet cells[198], adipocytes[199,200] and CNS neurons[201]. GIPR activation results in increased cAMP production and increased intracellular calcium[202]. Secreted GIP is rapidly degraded by dipeptidyl peptidase IV (DPP4), a serine protease that is widely expressed throughout the body, especially in endothelial cells lining blood vessels[68]. Originally termed Gastric-inhibitory Peptide due its inhibitory effects on gastric secretion at pharmacological doses[203]**,** GIP was subsequently shown to have marked insulinotropic potency and has since been renamed to reflect this property. Together with GLP-1, the two hormones account for more than 70 % of postprandial insulin secretion[204]. In addition to its insulinotropic effect, GIP increases insulin biosynthesis[68] and is trophic to pancreatic β cells by promoting proliferation while also inhibiting apoptosis. It is therefore not surprising that mice deficient in *Gipr* display impaired oral glucose intolerance[205,206]. The insulinotropic effects of GIP are dramatically attenuated in T2D patients[207,208], which is believed to be a major contributing factor to impaired postprandial insulin secretion in these patients. While the mechanism underlying the diminished insulin response to GIP has not been fully elucidated, β cell *GIPR* expression is significantly reduced in T2D human pancreatic islets when compared with non-diabetic donors[209], in line with findings in rodent models of T2D[210-212]. Since the binding of GIP to GIPR results in receptor internalization as part of the signal transduction cascade[213,214], it is possible that chronic hyperglycaemia hinders effective receptor recycling. Receptor desensitization after prolonged GIPR signalling has been reported[215], which is supported by findings demonstrating comparable glycaemic and insulin levels in wildtype controls and transgenic mice overexpressing *Gip*[216]. This indicates β cells are refractory to chronically elevated GIP levels. Notably, the insulinotropic potency of GIP is markedly reduced in non-diabetic first-degree relatives of T2D patients, which is likely to be one of the many genetic factors that predisposes these individuals to develop T2D later in life[217]. Moreover, GIP is glucagonotropic[218-221] and thus could worsen hyperglycaemia in the face of attenuated insulin action.

Several studies have reported elevated GIP levels in obese humans[222,223]. The lack of a reliable GIPR antagonist suitable for human studies[224,225] means elucidation of the physiological roles of GIP predominantly relies on transgenic mouse models of *Gip* and *Gipr* knockouts and of *Gip* overexpression. Potential obesogenic effects of GIP were first suggested when the phenotype of *Gipr* knockout mice was first reported. Not only were these animals protected from diet-induced obesity, *Gipr*-deficiency also protected leptin-deficient *ob/ob* mice from age-related weight gain and worsening glucose tolerance[205]. The obesogenic role of GIP has been largely attributed to its lipogenic effects. GIP promotes lipid uptake and inhibits lipolysis in adipocytes[226], closely resembling

the anabolic actions of insulin. Elevated GIP levels and duodenal K cell hyperplasia[227] occur in DIO mice, likely as an adaptive response to prevent excess fat from depositing in other metabolically active organs such as the liver and skeletal muscles. However, GIP induces osteopontin expression in adipocytes[228]. This adipokine is associated with obesity-related systemic low grade inflammation[229,230], which has a well-established role in the development of insulin resistance[231-234]. Such adaptive responses could have indirect deleterious effects on glucose homeostasis. This notion is supported by recent findings that showed adipocyte-specific *Gipr* ablation protected mice from DIO-induced insulin resistance and hepatic steatosis, potentially by reducing circulating levels of pro-inflammatory cytokines[235]. *Gipr* deficiency has also been demonstrated to protect mice from ovariectomy-induced weight gain[236]. The obesogenic effects of GIP are only apparent in the face of nutrient excess, as chow-fed *Gipr* and *Gip* knockout animals are not leaner than their wildtype counterparts[206]. The role of GIP in energy balance is further complicated by paradoxical findings that showed transgenic mice overexpressing *Gip* were leaner than wildtype controls when fed a standard-chow or high-fat diet[237] and that GIPR signalling can enhance GLP-1-induced weight loss[238].

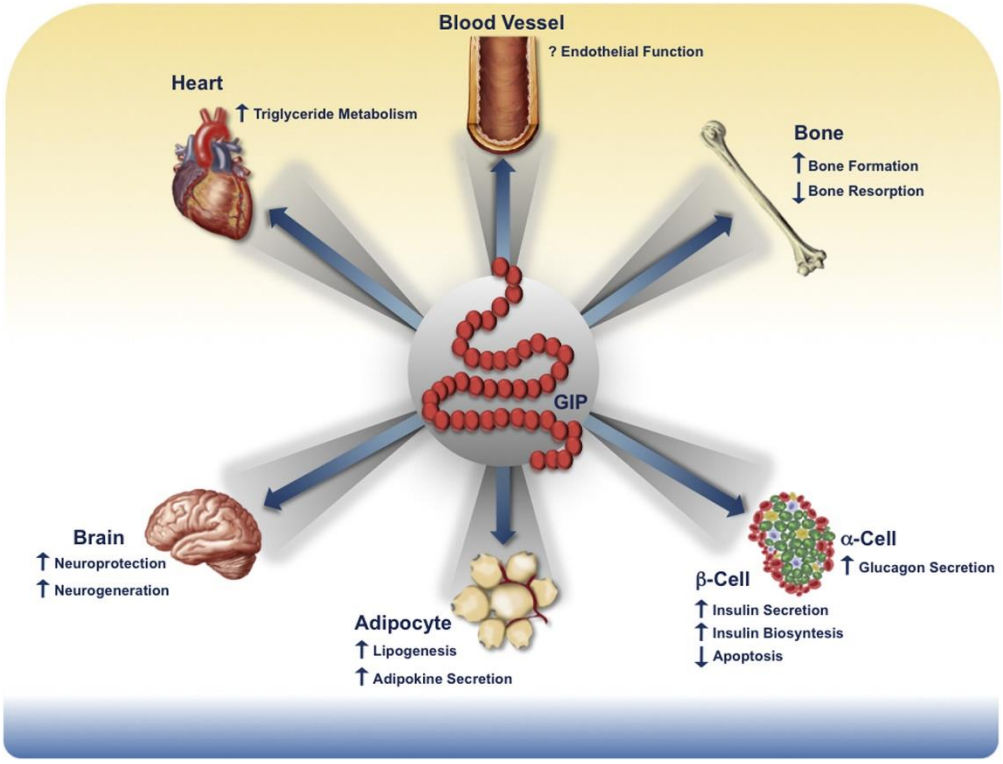


Figure 1.3.4 The biological actions of GIP. GIP stimulates secretion from both pancreatic α and β cells. It exerts trophic effects on β cells and is also lipogenic. Reprinted from reference 70.

1.3.5 Proglucagon-derived Peptides (PGDPs)

The glucagon gene (*GCG*) encodes for the 180-amino acid preproglucagon peptide, which is differentially processed by the three known cell types that express *GCG*, pancreatic α cells,

enteroendocrine L cells and a subset of neurons in the NTS of the brainstem. Following translation from *GCG* mRNA, the signal peptide, which consists of the first twenty amino acids in the N-terminus is cleaved to give rise to a prohormone, proglucagon. In pancreatic α cells, the proglucagon peptide is predominantly processed by prohormone convertase (PC) 2, which gives rise to glucagon, glicentin-related pancreatic peptide and major proglucagon fragment. In the enteroendocrine L cells and NTS neurons, and to a lesser extent in α cells[239], proglucagon is processed by PC1/3, which gives rise to four different proglucagon-derived peptides, glucagon-like peptide (GLP)-1, GLP-2, oxyntomodulin (OXM) and glicentin. The differential post-translational processing of the proglucagon peptide is depicted in Figure 1.3.5.

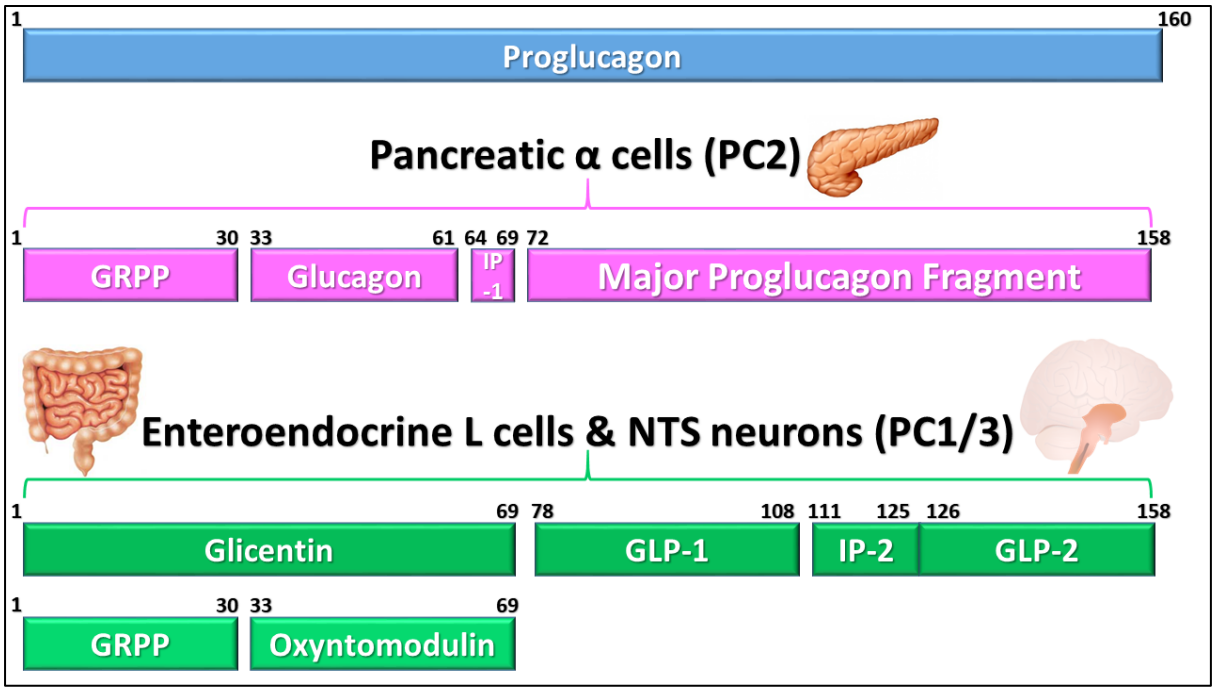


Figure 1.3.5 Post-translational processing of the proglucagon peptide. In enteroendocrine L cells and in a specific population of NTS neurons, proglucagon is processed by PC1/3, giving rise to glicentin, oxyntomodulin, GLP-1 and GLP-2. In pancreatic α cells, proglucagon is processed predominantly by PC2 to produce glucagon, although low level of GLP-1 is also produced PC1/3 in α cells.

1.3.5.1 Glucagon-like Peptide 1 (GLP-1)

GLP-1 is a 30 amino acid peptide derived from the proglucagon peptide. Post-translational processing by PC1/3 produces two biologically active forms, GLP-$1_{7\text{-}36}$-amide (GLP-$1_{7\text{-}36NH2}$) and GLP-$1_{7\text{-}37}$, with the former being the predominant form produced by enteroendocrine L cells in humans[240] and rodents[241]. Considering both forms of GLP-1 peptides have identical biological activity to GLP-$1_{1\text{-}37}$[242], the term GLP-1 will be used to refer to all biologically active forms of GLP-1 peptides. Secreted GLP-1 binds to the GLP-1 receptor (GLP-1R), a class B G_s-coupled GPCR, which results in adenylyl cyclase activation and subsequent increase in intracellular cAMP and PKA activation[69] (Figure 1.3.6).

GLP-1R is widely expressed throughout the body, including pancreatic islets, brain, heart, kidneys[69]. Many of the physiological actions of gut-derived GLP-1 are mediated by GLP-1R present on vagal afferents[243,244]. Endogenous GLP-1 has a short half-life of under two minutes as it undergoes rapid enzymatic degradation, primarily by DPP4, which also degrades GIP[69]. There is also evidence supporting the role of neutral endopeptidase 24.11 in GLP-1 degradation *in vivo*[245,246].

GLP-1 is secreted postprandially by enteroendocrine L cells upon nutrient exposure, typically within 10-15 minutes after nutrient ingestion[69]. Since the majority of L cells in humans resides in the distal part of the small intestine and colon, the immediate postprandial GLP-1 surge was originally thought to be vagally-mediated [247]. However, the fact that the postprandial GLP-1 response in vagotomized patients is exaggerated, rather than attenuated, refutes this notion[236,237]. Moreover, although L cells express muscarinic receptors[248,249] and acetylcholine triggers GLP-1 secretion in an *ex vivo* porcine ileal perfusion preparation[250], direct vagal stimulation did not trigger the secretion of GLP-1, or other hormones that are co-secreted by L cells[250]. Therefore, GLP-1 secretion is likely to be regulated by cholinergic neurons within the enteric nervous system, rather than the parasympathetic nervous system[250]. It is now established that a considerable, albeit smaller, population of GLP-1 secreting L cells resides in the duodenum in humans[251] and can be activated upon glucose exposure[252], which is likely to account for the rapid postprandial GLP-1 surge observed *in vivo*.

One of the most well-recognized physiological functions of GLP-1 is its incretin effect[253]. Aforementioned, GLP-1 and GIP account for up to 70 % of insulin secreted upon ingestion of oral glucose[254]. The desirable glucose-dependent hypoglycaemic effects of GLP-1 have been widely exploited by the pharmaceutical industry as effective anti-diabetic agents. The two major classes of GLP-1 based therapies that are currently clinically available are the GLP-1R agonists and DPP4 inhibitors. The biological actions of GLP-1 are depicted in Figure 1.3.6. The physiological functions of GLP-1 in the context of glucose and energy homeostasis will be discussed in detail in the following sections.

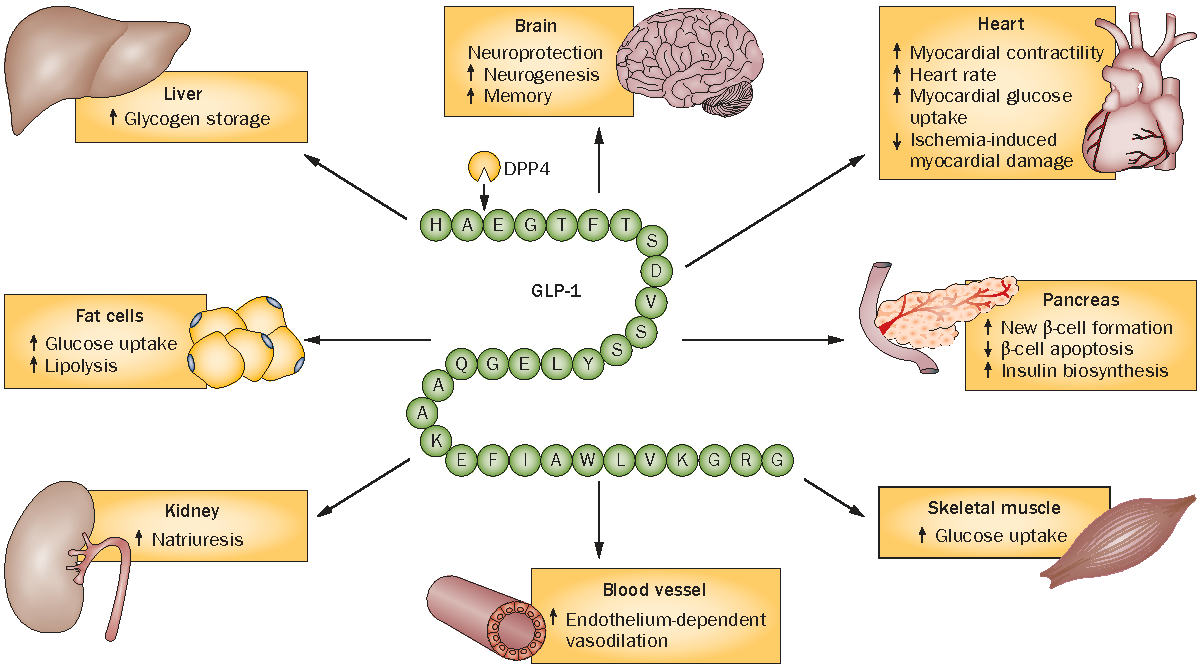


Figure 1.3.6 The biological actions of GLP-1. Reprinted from reference [255].

1.3.5.1.1 GLP-1 and glucose homeostasis

The curative effects of bariatric surgery on diabetic individuals have been attributed to significantly elevated circulating GLP-1 levels post-surgery[79,256], which is evident by the marked improvement of glycaemic control independent of weight loss[257-260]. Although this view remains controversial[261,262], it is widely accepted that GLP-1 is an important regulator of glucose homeostasis through its action on multiple targets.

1.3.5.1.1.1 GLP-1 and β cells

GLP-1 is an incretin that enhances glucose-stimulated insulin secretion (GSIS) from β cells[253]. Unlike sulfonylureas, which trigger insulin secretion from β cells independent of glucose concentration and thus, puts an individual at risk of hypoglycaemia, insulin secretion stimulated by postprandial increases in GLP-1 only occurs at glucose concentrations above 6 mM[263], thereby markedly reducing the risk of hypoglycaemia upon the administration of exogenous GLP-1. GLP-1 promotes β cell exocytosis through GLP-1R, which is highly expressed on the surface of β cells[264,265]. As depicted in Figure 1.3.7, binding of GLP-1 to the $G_{\alpha s}$-coupled GLP-1R leads to adenylyl cyclase activation and subsequently, increased intracellular cAMP and the downstream activation of PKA and Epac2. This increases intracellular calcium concentrations, either through opening of L-type calcium channels or by mobilizing intracellular calcium stores[266], to trigger exocytosis of docked insulin-containing vesicles (termed the readily releasable pool). PKC is implicated in enhanced insulin secretion by GLP-1[263] as GLP-1R can also be G_q-coupled [267]. Although it is generally accepted that GLP-1 retains its insulinotropic potency in T2D individuals[268], especially considering the anti-diabetic efficacy of GLP-1-

based therapy, its incretin effect can be temporarily impaired in healthy individuals with acutely disturbed glucose homeostasis[269]. Indeed, reduced incretin potency of GLP-1, or so-called "GLP-1 resistance", has been reported in T2D patients, albeit to a lesser extent to that of GIP[270-274]. Chronic hyperglycaemia[275] and hyperlipidaemia[276] downregulate GLP-1R expression and impairs the downstream signalling pathways governing GSIS in β cells, which could underlie the reduced insulinotropic effects of GLP-1 in T2D[277,278]. Importantly, the insulinotropic potency of GLP-1 can be restored by correcting hyperglycaemia in many T2D patients[273], except in individuals that are genetically predisposed to reduced GLP-1-stimulated insulin response[209,279,280].

In addition to its acute insulinotropic action, GLP-1 is a trophic factor for β cells and a positive regulator of β cell mass[281,282]. Prolonged GLP-1 treatment prevents β cell apoptosis[283-286], and promotes β cells neogenesis[287-289] and self-replication[288,290-292]. These mitogenic effects of GLP-1 are likely mediated through transactivation of the epidermal growth factor receptor[293], the inhibition of the transcription factor FoxO1[294] and increased *Pdx1* expression in β cells[295,296] (Figure 1.3.7). The latter also contributes to the improved β cell function by GLP-1[284,297-299] as PDX1 is an important transcription factor that regulates the expression of many key β cell genes central to β cell function, such as *INS* (insulin), *GCK* (glucokinase) and *SLC2A2* (GLUT2)[300], which are indispensable in insulin biosynthesis[301] and glucose-sensing[302,303].

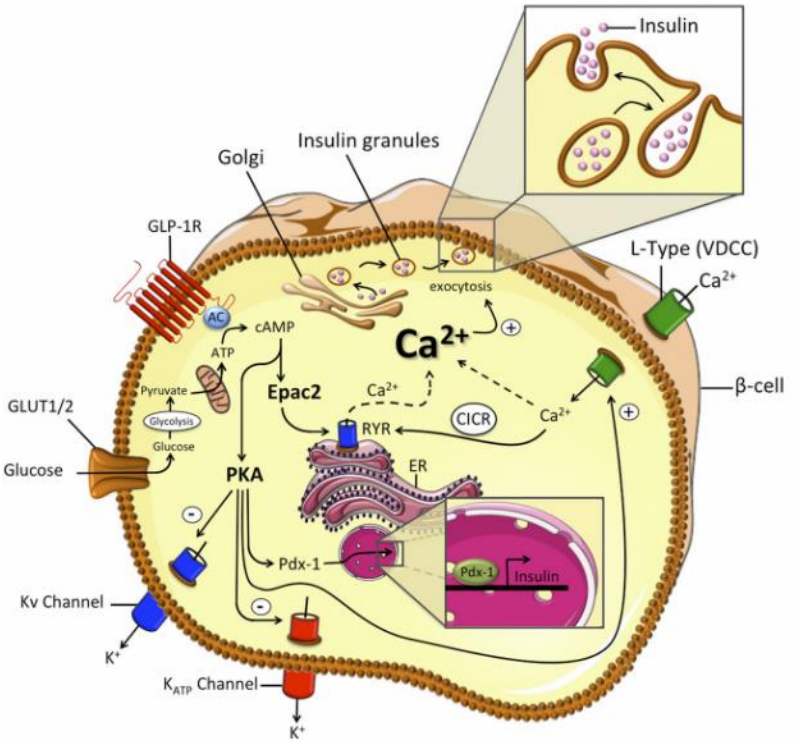


Figure 1.3.7 Mechanisms underlying the insulinotropic and trophic effects of GLP-1 on pancreatic β cells. GLP-1 binds to GLP-1R, which leads to activation of adenylyl cyclase, elevating cAMP level, thereby activating PKA and Epac2 to increase intracellular Ca^{2+} levels, subsequently resulting in the

release of insulin-containing vesicles from the readily releasable pool. PKA activation also leads increased insulin biosynthesis by increasing the expression of Pdx1. RYR: ryanodine calcium channel; CICR: calcium-induced calcium release. Reprinted from reference [304].

Although enteroendocrine L cells secrete GLP-1 upon meal ingestion, the magnitude of its postprandial surge is much smaller than that of the other incretin, GIP[68]. Moreover, only 10 % of secreted GLP-1 actually reaches the systemic circulation[305] to exert any endocrine effects on pancreatic β cells, as up to 70 % of intestinally-derived GLP-1 is degraded by local DPP4 before reaching the portal circulation[306], where it is subjected to further degradation in the liver[307]. These observations have led to increased questioning of the role of gut-derived GLP-1 as a true incretin that augments β cell GSIS in an endocrine fashion[308,309]. Indeed, several recent studies using tissue-specific *Glp1r* and *Gcg* knockdown and reactivating mice models demonstrated while β cell GLP-1Rs are crucial in maintaining glucose homeostasis[310,311], it is pancreatic α cell-derived, not enteroendocrine L cell-derived GLP-1, that mediates its insulinotropic effects[312]. Nevertheless, given postprandial GLP-1 response is typically increased by up to 30-fold in post-RYGB and VSG patients[313], it is generally accepted that intestinally-derived GLP-1 does indeed act as a true incretin hormone to improve β cell insulin response in these settings[309,314].

1.3.5.1.1.2 GLP-1 and α cells

Glucagon is a potent hyperglycaemic hormone secreted by pancreatic α cells in response to hypoglycaemia[315]. Its diabetogenic effect is discussed in detail in section 1.3.5.5. GLP-1 has a potent glucagonostatic effect *in vitro*[316,317], *ex vivo*[318-320] and *in vivo*[321-327]. The glucagonostatic effect of GLP-1 contributes equally to its glucose-lowering action as its insulinotropic effect[323] and this inhibitory effect is well-preserved in T2D patients[321-323,327,328]. However, the mechanisms underlying its inhibitory effect on pancreatic α cells remain debated as it is unclear if α cells actually express functional GLP-1R. Several studies have provided immunohistochemical evidence that rodent α cells do express GLP-1R[316,329], although results from these studies should be interpreted with great caution as the validity of most commercially available antibodies directed against GLP-1R has been questioned[330,331]. To overcome the issue of antibody specificity, a transgenic mouse model that express a fluorescent protein under the *Glp1r* promoter has been described and the authors reported that only a very small population of α cells express GLP-1R[332]. The use of α cell-specific *Glp1r* knockout animal models will thus provide valuable insights as to whether the lowly expressed GLP-1R has any physiological relevance in α cell physiology, although such model has not been reported in the literature. To date, evidence to support GLP-1R expression in human α cells is lacking[264,333]. Thus, the inhibitory effect of GLP-1 on glucagon secretion is likely to be indirect, potentially via the paracrine effects of insulin and somatostatin[152,315]. Indeed, GLP-1R is highly expressed in pancreatic β cells and to a smaller extent, in δ cells[332], which secrete insulin and

somatostatin, respectively, upon GLP-1 exposure[334,335]. However, the fact that the glucagonostatic effect of GLP-1 is preserved in C-peptide negative T1D patients[325,336] indicates that insulin, or β cell secretory products, are not required for the glucagonostatic effect of GLP-1. On the other hand, rodent pancreas perfusion studies demonstrated that GLP-1 induced glucagon suppression is completely reverted in the presence of a somatostatin 2 receptor antagonist[318,320], which implies somatostatin mediates the glucagonostatic effect of GLP-1, although results contradicting this notion have also been reported[316]. Regardless of the underlying mechanisms, it should be noted the glucagonostatic effect of GLP-1 accounts for much of its anti-diabetic efficacy[323], a notion that is often under appreciated.

1.3.5.1.1.3 GLP-1 and the liver

While impaired intraperitoneal glucose intolerance induced by *Glp1r* knockdown in mice can be fully rescued by selective expression of *Glp1r* in β cells[310,311], oral glucose tolerance is not restored by β cell-specific *Glp1r* re-expression[311]. This indicates that extra-pancreatic GLP-1R is involved. The liver is one of the most important sites of glucose homeostasis as it accounts for up to 50% of postprandial glucose disposal and up to 80 % of endogenous glucose output in the fasted state[337]. Portal glucose signalling is an integral part of postprandial glucose clearance[338] and there is ample evidence to support the role of GLP-1 in mediating this effect. The hypoglycaemic effect of portal glucose delivery[339] is completely abolished in *Glp1r*$^{-/-}$ mice[340]. Moreover, *Glp1r* knockdown significantly increased endogenous glucose production in insulinopaenic, glucagon receptor (GCGR)-deficient mice[341], further supporting the notion that GLP-1 exerts some of its glucose-lowering effects independent of the pancreatic islets by acting on the liver[342]. Intraportal delivery of GLP-1, either through the hepatic portal vein or the hepatic artery, at concentrations that resemble postprandial intra-portal GLP-1 levels, increased net hepatic glucose uptake in canines under hyperinsulinaemic-hyperglycaemic clamp conditions[343]. Direct infusion of GLP-1 during a pancreatic clamp, where the effects of GLP-1 on insulin and glucagon secretion were abolished by somatostatin infusion, significantly decreased endogenous glucose production in humans[344]. Importantly, the authors from this study cited a markedly higher insulin replacement dose as the reason why a similar study[242] did not observe any inhibitory effect of GLP-1 effect on endogenous glucose output during a pancreatic clamp. In addition to its inhibitory effect on hepatic glucose production, GLP-1 and its analogues have hepatoprotective effects such as reducing hepatic steatosis[345,346] and circulating liver enzyme levels[347] in various DIO rodent models. Such protective effects are of high relevance as there are links between liver diseases and the development of hepatic insulin resistance, which is a strong risk factor of developing T2D[348,349].

How GLP-1 exerts these hepatic effects remains a subject of ongoing investigation. In stark contrast to insulin and glucagon, whose receptors are highly expressed in hepatocytes and count the liver as their major target organ for their metabolic effects, there is no direct evidence showing the liver expresses GLP-1R. Immunohistochemical staining with a monoclonal antibody against GLP-1R[333] and the use of mouse model expressing a fluorescent-tagged protein under the *Glp1r* promoter[332] both failed to detect GLP-1R expression in hepatic tissue. Although it is generally accepted that the hepatic effects of GLP-1 are mediated by GLP-1R expressed by vagal afferents[350], the precise mechanisms underlying these effects are still unclear. It has been suggested that GLP-1 exerts its effect on the liver by activating vagal afferents innervating the hepatic portal vein[351]. However, portal delivery of GLP-1, either through the hepatic artery or the hepatic portal vein, increased net hepatic glucose uptake to a similar extent[343]. In addition, while hepatic portal vein denervation impairs oral glucose tolerance, it did not affect the glycaemic effect of the GLP-1R agonist exenatide[352].

1.3.5.1.1.4 GLP-1 and skeletal muscles

Skeletal muscles account for up to 30% of postprandial glucose disposal[337]. Immunoblot evidence supports the expression of GLP-1R in human skeletal muscles[353]. GLP-1 facilitates glucose uptake and glycogen synthesis in skeletal muscles in a GLP-1R-dependent manner *in vitro*[353-355] while other studies reported that GLP-1 had no significant effects[356]. It is worth noting that the concentrations of GLP-1 used in these *in vitro* experiments were at high nanomolar range, several orders of magnitude higher than what is deemed physiologically relevant, even in post-gastric bypass patients. Moreover, *Glp1r* knockout mice do not display defects in glucose uptake in their skeletal muscles[357,358], which casts considerable doubt over the physiological relevance of these *in vitro* findings.

1.3.5.1.1.5 GLP-1 and gut motility

Gut motility is a major determinant of postprandial glycaemia[359-361] as it controls the rate of nutrients entering the small intestine from the stomach, which in turns governs the rate of nutrient exposure and absorption by the small intestine and thus, postprandial glucose excursion and gut hormone release. Gastric emptying takes place when peristaltic contractions of the stomach force the release of chyme through the pyloric sphincter into the duodenum for absorption[362]. Despite being under the tight control of the autonomic and enteric nervous systems, inter-individual gastric emptying rates are highly variable and the rate of nutrient entry into the duodenum ranges from 1-4 kCal/min in healthy individuals[363]. This could account for the 35% variance in peak postprandial plasma glucose level observed in these healthy individuals[364]. GLP-1 inhibits gastric emptying[365-367] in a vagus-dependent manner[368], as evidenced by the lack of anti-motility effect of GLP-1 in vagotomised patients[369]. There is growing appreciation for the notion that a considerable portion of

glucose-lowering effect of GLP-1 is underscored by its inhibitory effect on gastric motility[367,370,371], which is preserved in obese and T2D patients[362]. The glucose-lowering effect of exogenous GLP-1 is markedly reduced, although not completely abolished, when its decelerating effect on gastric emptying is antagonized by intravenous erythromycin[372]. Moreover, GLP-1R activation inhibits small intestinal motility[373], which markedly slows the rate of glucose absorption and thus, reduces postprandial glucose excursions[374].

1.3.5.1.2 GLP-1 and energy balance

In addition to its multifaceted glucose-lowering effect, GLP-1 plays a role in regulating energy balance. This role is likely to be redundant, as whole body[375,376] and various tissue-specific[311,377,378] *Glp1r* knockout mice all have normal bodyweight. GLP-1R is widely expressed in brainstem and hypothalamic regions[379] that are implicated in appetite control. Indeed, the anorectic effect of GLP-1 in humans was noted not long after the discovery of its glucose-lowering effects[380,381]. Intracerebroventricular (ICV) injection of GLP-1R agonists into these specific brain regions markedly reduces food intake in rodents[382], and while acute administration of the GLP-1R antagonist exendin 9-39 did not consistently elicit acute hyperphagic responses[383-386], chronic treatment with the antagonist did produce profound hyperphagia and fat mass accumulation[387]. Peripheral administration of GLP-1R agonists can also reduce food intake[386,388] . The GLP-1R agonist, liraglutide, is now clinically used as a weight-loss remedy in conjunction with lifestyle modifications in obese/overweight individuals[389]. In addition, exaggerated postprandial GLP-1 release is believed to contribute to the increased satiety reported by many gastric-bypass surgery patients[50,390,391].

There are several ways in which GLP-1 exerts its anorectic effect (Figure 1.3.8). In humans, acute administration of pharmacological doses of GLP-1 significantly induce satiety and reduce food intake[380,392,393], an effect that is abolished in truncally vagotomised humans[369]. Similar effects are also observed in rodents, in which acute peripheral administration of GLP-1 or its analogues significantly reduce meal size and meal frequency in a vagus-dependent manner[244,394]. The nodose ganglia express GLP-1R[244,332,395] and considering the short half-life of endogenous GLP-1, it is proposed that GLP-1 released by enteroendocrine L cells following nutrient exposure activates vagal afferent GLP-1R signalling in a paracrine fashion[396,397]. Indeed, peripheral administration of GLP-1 activates vagal afferents[243]. The fact that peripheral administration of a high molecular weight, albumin-conjugated GLP-1R agonist was effective in reducing acute food intake in rodents[398,399] lends support to the notion that GLP-1 acts peripherally to induce satiety. Part of this acute anorectic effect is attributed to the potent inhibitory effect of GLP-1 on gastric emptying, thereby delaying the relief from meal-induced gastric distension and reduces appetite[400]. This vagal signal is also relayed to appetite control centres, namely the NTS in the brainstem to reduce food intake[401]. Peripherally administered

GLP-1 activates neurons in the area postrema, caudual nucleus tractus solitaries, the lateral parabrachial nucleus and the central nucleus of the amygdala, as evidenced by increased c-Fos immunoreactivity in these brain regions upon intraperitoneal GLP-1 administration[388,402]. However, weight-loss achieved with chronic administration of the GLP-1R agonist, liraglutide, is independent of vagal signalling[377,403]. The chronic anorectic effect of liraglutide is preserved in autonomic nerve-specific *Glp1r* knockout mice and the cumulative food intake over 14 days of these mice was no different from that of wildtype mice[377]. It is well-established that tachyphylaxis quickly develops towards the decelerating effect of GLP-1 on gastric emptying[404], which is vagally-mediated[368]. Therefore, it is possible that other vagally-mediated anorectic signals are relatively short-lived and hence, not implicated in long-term energy balance. Indeed, the inhibitory effect of liraglutide on gastric emptying was markedly attenuated in rats after twice-daily administration over two weeks despite the treatment significantly reducing bodyweight of the treated animals[405]. Nonetheless, under normal physiological settings, GLP-1 levels are only elevated transiently and thus, this pathway should be considered physiologically relevant in inhibiting short-term food intake.

In addition to acting on peripheral targets, there is now strong evidence showing that peripherally administered GLP-1 and GLP-1R agonists have considerable access to circumventricular organs and some hypothalamic regions behind the blood brain barrier, likely through the fenestrated capillaries that are highly abundant in these areas[403,406,407]. The weight loss effect of peripherally administered liraglutide is dependent on the arcuate nucleus (ARC)[403], a hypothalamic nucleus that is pivotal in long-term appetite control[408]. Although mice with POMC-neuron-specific *Glp1r* knockdown did not differ in body weight or acute food intake from their wildtype counterparts on a standard chow diet[378], they did gain more weight and fat mass after high-fat feeding[403]. Such findings suggest that GLP-1R in the ARC could play a role in maintaining energy homeostasis under metabolic stress such as DIO. Liraglutide activates the anorexigenic CART/POMC neurons in the ARC by directly binding to GLP-1R on these neurons, which then act on other hypothalamic targets such as the paraventricular nucleus of the hypothalamus and the lateral hypothalamic area to induce satiety[408,409]. Moreover, liraglutide reduces food intake by decreasing feeding drive by indirectly inhibiting the firing of orexigenic NPY/AgRP neurons in the ARC. Although these neurons do not express GLP-1R, liraglutide-activated CART/POMC neurons inhibit NPY/AgRP neurons through GABAergic interneurons in the ARC[403]. Mice pair-fed to their liraglutide-treated counterparts had higher levels of *Npy/Agrp* expression, which implies GLP-1R activation in the ARC significantly induces satiety without increasing hunger secondary to reduced food intake. Moreover, GLP-1 is implicated in regulating hedonic eating through brainstem GLP-1Rs[410,411]. Direct activation of GLP-1R in the NTS of rats significantly reduced acute intake of palatable food[410] but not normal chow[412]. This is

complemented by a recent report showing selective knockdown of *Glp1r* in NTS neurons in rats markedly increased the motivation to work for palatable food in operant responses experiments[413]. Given GLP-1 has a very short half-life, these central actions are likely to be more relevant in pharmacological settings, such as the chronic use of DPP4-resistant GLP-1R analogues or in post-gastric bypass surgeries, in which postprandial GLP-1 response are profoundly exaggerated such that the levels reaching these central targets are sufficient to elicit anorectic responses.

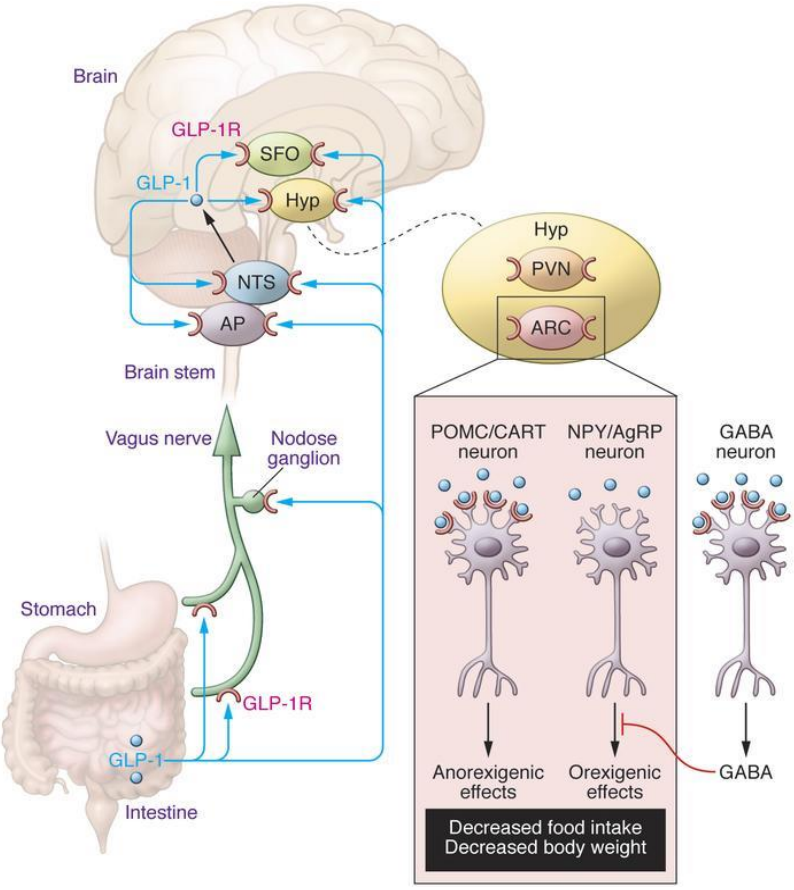


Figure 1.3.8 GLP-1 contributes to negative energy balance by reducing food intake. Acute anorectic effects are likely mediated by vagal afferent GLP-1R while chronic anorectic effect is mediated by arcuate nucleus (ARC) neurons in the hypothalamus (hyp): GLP-1 directly activates the anorexic POMC/CART neurons and indirectly inhibits the orexigenic NPY/AgRP neurons by acting on GABA interneurons. GLP-1 also modulates hedonic eating behaviours by acting of NTS neurons in the brainstem. SFO: subfornical organ; PVN: paraventricular nuclues; AP: area postrema. Reprinted from reference [414].

Several groups reported that GLP-1R agonist-treated mice lost considerably more weight than their pair-fed littermates[403,415,416], indicating that the treatment could also increase energy expenditure[415-417]. However, the underlying mechanisms that can account for this difference have not been fully elucidated. While there is some evidence that GLP-1R agonists increase diet-induced thermogenesis[416], its effect on energy expenditure is so far largely inconclusive as others have found

GLP-1R agonists had no significant influence[403] and one study showed that whole body *Glp1r*$^{-/-}$ mice had higher energy expenditure than wildtype mice[418]. The latter finding is consistent with a recent study that showed pan-hypothalamic, but not POMC- or Sim1-neuron-specific *Glp1r* knockdown (Sim1 is a crucial transcription factor for the development of PVN neurons[419]), increased energy expenditure in mice, although this was accompanied by significantly higher food intake, which resulted in a lack of any net effects on body weight[378]. To date, most evidence supports the notion that peripheral GLP-1 contributes to, but is not crucial in, maintaining energy homeostasis, in contrast to its pivotal role in maintaining glucose homeostasis.

1.3.5.2 Glucagon-like Peptide 2 (GLP-2)

GLP-2 is a 33 amino acid peptide that is co-secreted with GLP-1 by L cells in an equimolar ratio upon nutrient ingestion[420]. GLP-2 is subjected to the same enzymatic degradation as GLP-1, although its 7-minute half-life is considerably longer than the half-life of GLP-1[421]. GLP-2 acts on GLP-2 receptors (GLP-2R) to exert trophic effects on intestinal epithelia by enhancing crypt cell proliferation and inhibiting apoptosis. As such, GLP-2 plays a beneficial role in adaptive intestinal growth, the maintenance of the integrity of the intestinal mucosa and the regulation of nutrient digestion and absorption[422,423]. The GLP-2R agonist teduglutide is used clinically to improve intestinal function in patients with small bowel syndrome[424]. Despite the structural similarity with GLP-1, GLP-2 has no known insulinotropic effect[425]. Although pancreatic α cells express functional GLP-2R[426] and despite its considerable glucagonotropic effect at supraphysiological doses, exogenous GLP-2 infusion had no significant effects on plasma glucose levels in humans[427]. Some rodent studies have argued for a role of GLP-2 in maintaining glucose homeostasis and energy balance but this has not been investigated in humans[428].

1.3.5.3 Oxyntomodulin (OXM)

Oxyntomodulin was once referred to as "gut glucagon"[429] or "enteroglucagon"[430], owing to its glucagon-like immunoreactivity[431] and its ability to stimulate cAMP production in hepatocytes in a similar fashion to glucagon[429]. It was later deduced that OXM is a 37-amino acid peptide that contains the entire amino acid sequence of glucagon[430] (Figure 1.3.1) and is co-secreted with GLP-1 by enteroendocrine L cells at an equimolar ratio[432]. Therefore, stimulants that trigger the release of GLP-1 would also trigger the release of an equal amount of OXM. While OXM stimulates the fundic glands of rat stomachs[433] and inhibit gastric acid secretion, from which its name was derived ("modulator of the oxyntic glands of the stomach")[433], a receptor specific for OXM has not yet been identified[434]. Despite its weak agonist activity at both GLP-1R[435] and GCGR[436], the functional significance of OXM under normal physiological conditions remains unclear. The potencies of OXM at GLP-1R and GCGR are in the nanomolar range[434,437,438] but circulating levels of OXM are typically 0

- 30 pM, although this can increase by up to 10-fold in post-RYGB patients[432]. While postprandial levels of OXM are significantly lower in obese or T2D patients when compared with healthy control subjects[432], the physiological relevance of such difference remains uncertain. Nevertheless, pharmacological levels of OXM (sufficient to activate GLP-1R and GCGR) have anti-obesity effect in humans as it significantly reduced appetite[439,440] and increased energy expenditure[441]. In addition, OXM treatment improved glucose tolerance in high-fat fed mice by potentiating GSIS[442] in a glucose-dependent manner[443] and has anti-apoptotic effects on β cells[443]. OXM infusion significantly reduced glycaemic excursions by augmenting glucose-dependent insulin secretion in obese subjects with or without T2D[444]. Together, these observations prompted the investigation into the potential metabolic benefits of the co-activation of GLP-1R and GCGR[445,446], which led to the subsequent development of GLP-1R/GCGR co-agonists[238,447], and later, GIPR/GLP-1R/GCGR tri-agonists[448] (discussed in detail in section 1.3.5.5). The anti-obesity effect of some of these agonists are currently being evaluated in clinical trials[449].

1.3.5.4 Glicentin and glicentin-related pancreatic polypeptide (GRPP)

Contrasting the extensive amount of research about other PGDPs, only a very limited body of published literature describes the functions of glicentin and its cleavage product, glicentin-related pancreatic polypeptide (GRPP). Similar to oxyntomodulin, no receptors specific for the two peptides have yet been identified. Glicentin inhibits gastric acid secretion in rodents and may play a role in controlling GI motility but appears to have no significant effect on glucose homeostasis or energy balance[450]. However, glicentin$_{1\text{-}61}$, a cleavage product of the parent peptide glicentin$_{1\text{-}69,}$ is a weak agonist at GCGR and has significant insulinotropic effects, although it is suggested that this cleavage product is likely to be of pancreatic origin[451]. GRPP, on the other hand, has no agonist or antagonist activity on GCGR and GLP-1R and inhibits GSIS in isolated rat pancreas, although this effect was not observed in isolated rat islets[452]. Therefore, the physiological significance of glicentin and GRPP remains to be investigated.

1.3.5.5 Glucagon

Glucagon is a 29-amino acid peptide hormone and one of the many products of the glucagon (*GCG*) gene. Secreted glucagon acts on the G_s-coupled glucagon receptor (GCGR) to activate adenylyl cyclase, to then increase cAMP and intracellular Ca^{2+} concentrations[453]. GCGRs are highly expressed in the liver and the kidneys while smaller amount of GCGR mRNA is present in the heart, adipocytes, brain, retina and the endocrine pancreas[454,455]. Although glucagon is primarily produced by pancreatic α cells, it is not the body's only source of glucagon. Early studies demonstrated that unlike insulin and C-peptide, circulating glucagon-like immunoreactivity remained detectable in humans that had undergone total pancreatectomy [456,457], a surgery that would have theoretically

eliminated the body's only source of glucagon [458]. Such findings were complemented by evidence that the gut epithelium was a source of extra-pancreatic glucagon [457,459-462]. This notion is strongly supported by results from a recent study that showed the release of extra-pancreatic glucagon in pancreatectomized patients was triggered by enteral, but not parenteral glucose[463]. Figure 1.3.9 summarizes the physiological actions of glucagon. The following sections focus on the metabolic effects of glucagon.

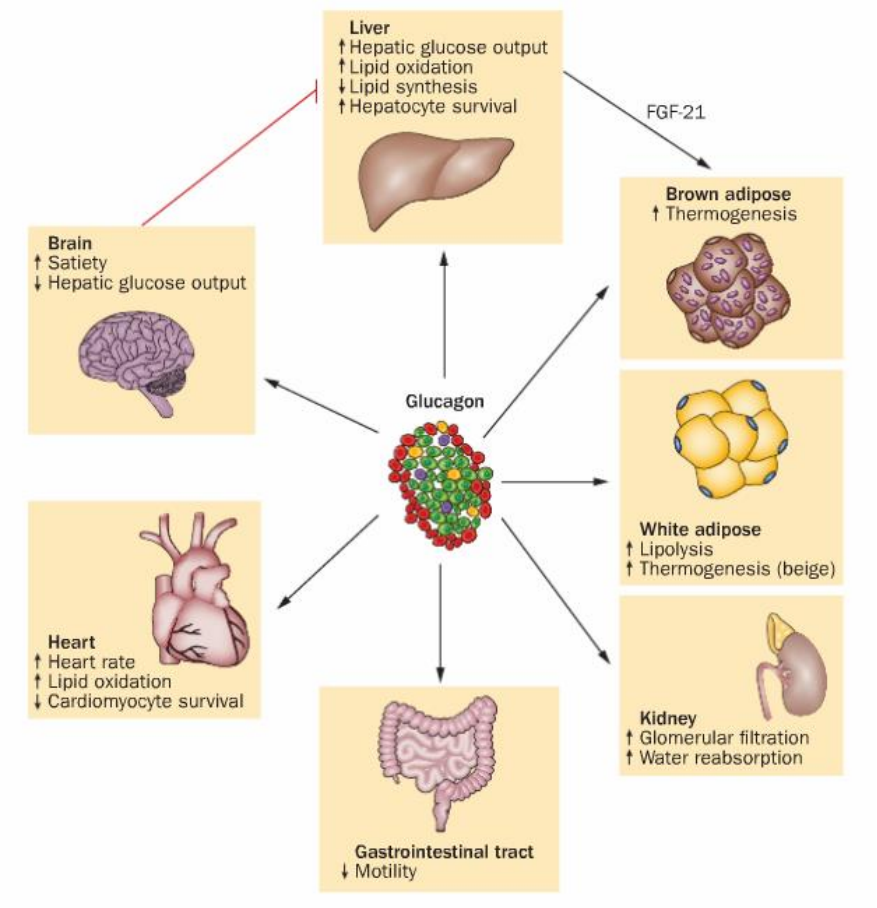


Figure 1.3.9 The physiological actions of glucagon. Glucagon increases hepatic glucose output by acting directly on the liver and indirectly via central mechanisms. It is a positive chronotrope and increases glomerular filtration in the kidneys. Glucagon promotes negative energy balance by inducing satiety in the brain and by acting on adipocytes to promote thermogenesis. Reprinted from reference [464].

1.3.5.5.1 Glucagon and glucose homeostasis

Glucagon is one of the major counter-regulatory hormones and its release is potently triggered by hypoglycaemia[465-467]. Glucagon is catabolic and promotes the mobilization of glucose from the liver[468,469]. During fasting, the glucagon-to-insulin ratio increases to allow glucagon's action to dominate over insulin, in order to avoid hypoglycaemia and meet the fuel demands of the body[7]. Glucagon acts on hepatic GCGRs to increase circulating glucose by facilitating hepatic glycogenolysis and gluconeogenesis[6], and may also facilitate gluconeogenesis in renal and intestinal tissue during

fasting[470]. Moreover, glucagon is an important regulator of amino acid turnover, which can further influence glycaemia by controlling the availability of glucogenic amino acids[471].

1.3.5.5.1.1 Glycaemic implications of hyperglucagonaemia

T2D patients and individuals with impaired glucose tolerance have fasting and in particular, postprandial hyperglucagonaemia, relative to healthy individuals, despite the fact that they are typically hyperinsulinaemic[321,472-485]. One should not underestimate the significance of postprandial hyperglucagonaemia as a major driver of exaggerated postprandial glucose excursions, and therefore postprandial hyperglycaemia, in these patients[479,486-488]. Epidemiological evidence supports the notion that postprandial hyperglycaemia is a standalone risk factor for cardiovascular disease in diabetes patients, independent of fasting hyperglycaemia[489,490]. Thus, it is likely that postprandial hyperglucagonaemia, together with the loss of incretin effects, plays a significant role in the development of impaired oral glucose tolerance in patients with type 2 diabetes and in increasing the risk of associated co-morbidities. Conversely, increased fasting levels of glucagon are often associated with lower insulin sensitivity and impaired glucose tolerance[472,491], which are strong predictors of the development of type 2 diabetes. There is also evidence to support a role of augmented glucagon secretion in the impaired glucose tolerance arising secondary to the use of atypical antipsychotics[32,492-495], which is a well-recognized side-effect associated with these drugs[496,497].

While glucagon is required for normal β cell function and augments glucose-stimulated insulin secretion (GSIS)[498-500], there is evidence that hyperglucagonaemia precedes the development, and may be a significant driver, of β cell dysfunction[501]. Indeed, a two-week continuous infusion of a stable glucagon analogue significantly impaired *in vivo* and *ex vivo* GSIS capacity in β cells, despite the expansion of β cell mass in mice[502]. Hyperglycaemia secondary to hyperglucagonaemia is likely to be a significant contributor to glucotoxicity in β cells, which is known to dramatically diminish β cell function[17-21] and to attenuate insulin-mediated glucose disposal[485,503]. Longstanding hyperglycaemia can also cause glucotoxicity in pancreatic β cells, leading to progressively attenuated insulin secretion[17-20,485,504-507], progressive disinhibition of glucagon secretion by α cells, and a cycle of perpetually worsening hyperglycaemia. It is therefore unsurprising that declining β cell function in newly diagnosed type 1 diabetic children is closely associated with increased plasma glucagon levels[508], and that type 1 diabetes patients are characterized by pronounced concomitant insulinopaenia and hyperglucagonaemia[321,472].

The notion of hyperglucagonaemia as a main driver of diabetic hyperglycaemia[460,509] and potentially life-threatening diabetic ketoacidosis[510-512], is strongly supported by studies showing that

somatostatin, a potent inhibitor of both α cell and β cell secretion, attenuated severe hyperglycaemia in insulinopaenic patients in the absence of exogenous insulin supplementations. In addition, somatostatin infusion experiments demonstrated that postprandial hyperglucagonaemia, as opposed to insulin insufficiency alone, was a significant determinant of postprandial hyperglycaemia observed in both type 1[476,488] and type 2 diabetes patients[486].

Whilst it is without doubt that glucagon is a major driver of hyperglycaemia, it is important to acknowledge that glucagon's potent hyperglycaemic effect in normoglycaemic individuals is relatively short-lived[513-515]. The acute hyperglycaemic action of glucagon primarily depends on its ability to stimulate hepatic glycogenolysis and thus, is limited by the availability of hepatic glycogen stores[516,517]. This is evident in studies showing that although somatostatin infusion transiently reduces blood glucose levels due to attenuation of glucagon secretion, prolonged infusion induces hyperglycaemia in non-insulin-depleted subjects. This is due to the potent inhibitory effect of somatostatin on endogenous insulin secretion and the waning hypoglycaemic effects of glucagon suppression once hepatic glycogen stores are depleted[515,516,518]. This is also evident in patients with glucagonoma, a rare neuroendocrine tumour disease characterized by the hypersecretion of glucagon, resulting in plasma glucagon levels more than ten-fold higher than normal[519,520]. Although diagnosis of diabetes are often (but not always) made in these patients prior to diagnosis of the α cell tumour (diagnosis is delayed by an average of three years due to the rarity of the disease[521]), many of these patients are only mildly glucose-intolerant despite marked hyperglucagonaemia[522,523]. Indeed, adequate glycaemic control in these patients is often achieved with sulfonylureas or exogenous insulin supplementations[524-526]. To date, only several isolated cases of overt diabetic ketoacidosis secondary to glucagonoma have been reported[527-530]. However, the glycaemic status of patients with glucagonomas should be interpreted with caution as such neuroendocrine tumours are often poorly differentiated and hypersecrete a myriad of other hormones[526], including GLP-1[531-534]. Plasma levels of other hormones are seldom reported, especially in early clinical case reports. Moreover, there have been reports of concurrent diagnosis of insulinomas in patients with glucagonomas[526,535-538], which further complicates the clinical picture. Nonetheless, such observations strongly support the role of insulin in maintaining glucose homeostasis in the face of profound hyperglucagonaemia. However, the fact that hyperglycaemia could be ameliorated by suppressing glucagon secretion, in the absence of insulin, emphasises that diabetes should be considered as a bi-hormonal disease[539], characterized by insufficient insulin activity and reciprocal glucagon hypersecretion[45].

1.3.5.5.1.2 GCGR signalling as a therapeutic target for diabetes treatment

Early clinical studies showed that suppressing glucagon secretion in insulinopaenic patients with somatostatin infusion powerfully attenuated the severe hyperglycaemia attributed to insulin insufficiency[460,509]. Although somatostatin is highly effective in alleviating acute diabetic ketoacidosis[540-542] and has been proposed as a potential therapy in diabetes management[543], the fact that it also inhibits secretion of insulin[544] and a myriad of extra-pancreatic hormones[545-548], prohibits the use of it, and its analogues, as viable anti-diabetic remedies[549,550]. Nonetheless, these experiments did provide strong evidence in support of glucagon as a viable therapeutic target for anti-diabetic drugs.

1.3.5.5.1.2.1 Lessons learnt from various GCGR knockdown models

Rodent studies have provided powerful evidence supporting glucagon receptor signalling as a critical component driving the development of diabetes. It was first demonstrated that hyperglycaemia in streptozotocin (STZ, a rodent β cell toxin)-treated rats could be rescued by the use of a GCGR antagonist[551] or a neutralizing antibody against GCGR alone, without supplementation of exogenous insulin[552], confirming a primary role of GCGR signalling in this form of hyperglycaemia. It was later shown that reducing *Gcgr* expression by the use of an antisense oligonucleotide in diabetic, obese leptin receptor-deficient *db/db* mice significantly improved glucose tolerance and insulin sensitivity without affecting body weight[553]. These findings were later extended to other diabetic models, including the leptin-deficient *ob/ob* mice and Zucker Diabetic Fatty rats[554]. Similar observations were reported in DIO mice treated with a GCGR antagonist, in which glucose tolerance was markedly improved without significant impact on body weight[555]. Interestingly, *Gcgr* knockout mice of various strains all display markedly improved glucose tolerance and insulin sensitivity in comparison to their wildtype littermates, despite the presence of significant hyperglucagonaemia secondary to α cell hyperplasia[556,557]. Further investigations in *Gcgr* knockout mice revealed that disruption of GCGR signalling conferred resistance to HFD-induced obesity and glucose intolerance, as well as HFD-induced hepatic steatosis[558]. Intriguingly, *Gcgr* knockout mice were resistant to STZ-induced hyperglycaemia and partly protected from STZ-induced β cell destruction[558], in accordance with earlier findings that showed pharmacological blockade of GCGR alone could alleviate hyperglycaemia in this model of insulin insufficiency[551,552]. It was subsequently shown that the dose of STZ required to fully ablate β cells in *Gcgr* knockout mice was double that required in wildtype animals. Even then, *Gcgr* knockout mice were still protected from severe hyperglycaemia secondary to absolute insulin deficiency[559]. Other means to disrupt GCGR signalling by near-complete α cell ablation[560] or a GCGR antagonizing antibody[561] were both effective in suppressing STZ-induced diabetes in mice, without the need for exogenous insulin supplementation. Indeed, normalization of

HbA1c in STZ-treated mice was achieved after 12 weekly injections of the GCGR-antibody[561]. However, it should be noted that hyperglycaemia in STZ-treated mice did not significantly improve upon acute disruption of GCGR signalling using a GCGR antagonist or a neutralizing anti-glucagon monoclonal antibody[562], demonstrating that the glucose-normalizing benefits of GCGR antagonism in the face of total β cell ablation occur over a longer timeframe. Nonetheless, such results strongly support the diabetogenic nature of GCGR signalling during insulin deficiency.

1.3.5.5.1.2.2 The liver is the major target of the hyperglycaemic effect of glucagon

Increased hepatic glucose output is one of the main drivers of hyperglycaemia in patients with diabetes[563,564]. Hyperglucagonaemia is a major contributing factor[474] as glucagon is a potent stimulant of hepatic glucose production[6,565]. The relevance of the liver-islet α cell axis is underscored by the fact that mice with hepatocyte-specific *Gcgr* ablation displayed a similar improvement in glucose tolerance to their full body knockout counterparts[566]. GCGR activation in hepatocytes results in upregulation of key glucogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase[567]. Unsurprisingly, the glycaemic benefits conferred by GCGR antagonism are believed to be primarily driven by a corresponding reduction in hepatic glucose output[341,554,560]. Hepatic gluconeogenic genes were significantly downregulated while hepatic glycogen content was higher in *Gcgr* antisense oligonucleotide-treated rats[554], in mice with α cell ablation[560] and in *Gcgr* knockout mice[559], compared with their corresponding controls. GCGR antagonists may also improve glucose tolerance independent of hepatic glucose outputs, with evidence that hepatic GCGR activation increases the secretion of the neuropeptide Kisspeptin 1, from the liver, which acts via the Kisspeptin 1 receptor on β cells to inhibit cAMP synthesis and attenuate GSIS[568].

1.3.5.5.1.2.3 Other mechanisms underlying metabolic benefits of GCGR knockdown

Plasma GLP-1 levels increase substantially in patients treated with a GCGR antagonist[569], consistent with the higher GLP-1 levels observed in different animal models of disrupted GCGR signalling[341,554,557,558,566,570]. Indeed, elevated GLP-1 levels in these models are partly responsible for improved glucose tolerance[69], as pharmacological or genetic disruption of GLP-1R signalling reverses some, but not all, of the glycaemic benefits conferred by *Gcgr* ablation alone[341]. A recent study demonstrated it is specifically the increased levels of islet-derived GLP-1, rather than intestine-derived GLP-1, that contributes to the improved oral and intraperitoneal glucose tolerance of mice with disrupted GCGR signalling[312]. However, despite the absence of GLP-1, whole body *Gcg* knockout mice display superior glucose tolerance compared to their wildtype counterparts[312,571-574]. The phenotype of whole body *Gcg* knockout mice closely resemble that of *Gcgr* knockout mice[312,575], which implies glycaemic benefits conferred by attenuated glucagon action are largely independent of enhanced GLP-1 action. It is possible that the lean phenotype conferred by genetic ablation of

Gcgr[556,557,559] may contribute to improved insulin sensitivity and glucose tolerance. It should be noted that other methods of GCGR inhibition were equally effective in improving glucose tolerance without affecting bodyweight[553-555,561].

Based on these pre-clinical results, several orally-available GCGR antagonists have entered clinical trials and demonstrated impressive glucose-lowering efficacy in T2D patients[569,576,577]. However, GCGR antagonists are not without undesirable side effects. Safety concerns related to off-target effects such as elevated blood pressure[578] and LDL cholesterol[579] have been reported. Concerns over potential liver toxicity[579] have been raised as GCGR signalling is heavily implicated in hepatocyte survival[580]. Indeed, increased liver fat disposition and liver enzyme levels were reported in patients treated with GCGR antagonists[579].

1.3.5.5.2 Glucagon and energy homeostasis

1.3.5.5.2.1 Glucagon induces satiety

Peripherally administered glucagon significantly reduces food intake in humans[439,581-583] while neutralization of glucagon with antibodies directed at the peptide potently increase acute food intake in rats[584]. Such an effect is likely mediated by a direct activation of GCGR-expressing vagal afferent nerve terminals[585] since glucagon has limited access at the blood brain barrier [586,587] and vagotomy blunts the anorectic effect of glucagon[588]. Subcutaneous glucagon injection increases c-Fos immunoreactivity in the area postrema, the cadual NTS, the central nucleus of the amygdala and the parabrachial nucleus, the same regions within the brainstem and the hypothalamus that are activated by peripheral GLP-1 administration[388], which has well-characterized satiating effects[69,575,589,590]. Co-administration of glucagon and GLP-1 at doses that were insufficient to cause significant neuronal activation in these areas significantly reduced food intake in mice and increased c-Fos expression in brain regions that are implicated in appetite control[591], such as the area postrema and central nucleus of the amygdala[388]. Thus, it is possible that the two proglucagon-derived peptides share the same CNS targets to induce satiety and reduce food intake postprandially. Moreover, the anorectic effect of glucagon could be mediated by the central melanocortin system, as GCGR co-localises on hypothalamic orexigenic AgRP neurons[592] and injection of glucagon into the arcuate nucleus decreases *AgRP* mRNA levels[593]. Glucagon may also induce satiety by promoting intestinal gluconeogenesis[470], which substantially increases portal glucose concentrations and subsequently activates glucose sensors along the portal vein and relays the signal to hypothalamic nuclei involved in appetite regulation to reduce food intake[594-596]. The fact that a protein rich meal is a potent stimulant of glucagon secretion *in vivo*[479,597,598] and dietary protein is a potent activator of intestinal gluconeogenesis[595,599] strongly supports the notion that

glucagon-induced intestinal gluconeogenesis is implicated in the satiating effects of dietary proteins[595].

1.3.5.5.2.2 Glucagon increases energy expenditure

Parenteral administration of glucagon increases energy expenditure in humans[600-604] likely by stimulating the secretion of fibroblast growth factor-21[605-607], which directly stimulates lipolysis in adipose tissue[608], and promotes thermogenesis, white adipose tissue browning and weight loss[609]. On the other hand, glucagon is a prominent regulator of hepatic lipid metabolism. GCGR signalling is essential for the suppression of hepatic lipogenesis[610] and triglyceride synthesis and secretion[611], while glucagon also promotes β-oxidation of hepatic free fatty acids[611,612]. Glucagon action is implicated in exercise-induced reversal of fatty liver in mice[613]. Although GCGR knockout mice display a lean phenotype[557], several animal models with disrupted GCGR signalling have increased hepatic lipid accumulation[553,560] and increased levels of LDL[557,611]. Moreover, a recent phase II clinical trial demonstrated that the chronic use of an orally available GCGR antagonist was associated with significant increase in hepatic fat accumulation[579]. Taken altogether, these reports suggest glucagon plays an important role in preventing lipid deposition in the liver and the development of non-alcoholic fatty liver disease.

1.3.5.5.2.3 Therapeutic potential of GCGR activation

GCGR signalling is a highly attractive anti-obesity target as glucagon has anorectic effects and can increase energy expenditure. However, its potent hyperglycaemic effect proves to be a major obstacle for the use of GCGR agonists alone as a weight loss strategy. Nevertheless, a series of proof-of concept experiments demonstrated that the metabolic benefits of glucagon could be safely harnessed when GLP-1R was concomitantly activated to circumvent its hyperglycaemic effects[614]. GLP1-R agonists (GLP-1RAs) have recently been approved as an anti-obesity treatment[389], although the clinically relevant weight loss achieved (5-10 %) is generally less than what is desirable in the severely obese. Pocai and colleagues were the first to reveal that GCGR activation by a GLP-1R/GCGR co-agonist, administered on alternate days, caused significant and additional reductions in food intake and body weight in DIO mice over 13 days compared to GLP-1RA treatment alone. These effects were predominantly driven by the loss of fat mass and mice also displayed marked improvements in glucose tolerance[615]. A single injection of a GLP-1R/GCGR co-agonist dose-dependently reduced bodyweight and fasting blood glucose levels of DIO mice over a week without acute dysglycaemia[616]. Follow-up experiments showed these effects were sustained over a month upon weekly injections at a lower dose, with mice showing marked improvements in glucose tolerance and insulin sensitivity, accompanied by increased energy expenditure and reduced fat mass[616]. Furthermore, these effects were also observed in rats, indicating they were not species-

specific. The anorectic effects of GCGR agonism are likely to be mediated by satiety signalling of peripheral sensory nerves, as centrally administered glucagon to the arcuate nucleus was ineffective in reducing food intake in DIO rats[593]. Importantly, the co-agonist caused significant weight loss and reduced fat mass in G*lp1r* deficient mice, indicating that GCGR agonism was the driver of weight loss in these DIO animals. Interestingly, *Glp1r* deficient mice treated with the co-agonist showed a tendency toward hyperglycaemia, suggesting that GLP-1R agonism may confer protection against hyperglycaemia induced by GCGR activation[615,616].

Further studies revealed the GLP-1R/GCGR co-agonist was effective in reversing leptin resistance in DIO mice, which is likely to contribute to weight loss in addition to that driven by GCGR/GLP1R co-agonism since leptin itself is a potent anorectic hormone[617,618]. Indeed, exogenous leptin significantly reduced food intake in co-agonist treated DIO animals, compared to animals treated by the co-agonist alone[447]. The metabolic benefits of exogenous leptin were not sustained upon discontinuation of the co-agonist treatment in these animals, suggesting GCGR agonism preserved leptin sensitivity in the DIO model. To further improve the profile of the co-agonist, a GCGR/GLP1R/GIPR tri-agonists were subsequently developed to take advantage of the additional benefits of GIPR stimulation[448] in improving glucose tolerance but with minimal effect on body weight[238]. The tri-agonist demonstrated superior efficacy in both glucose tolerance and weight loss in DIO mice, and the authors convincingly showed using various loss-of-function models that GCGR activation was the main driver for the observed improvement in a range of metabolic parameters and it did not exacerbate HFD-induced hyperglycaemia due to the protection conferred by GLP-1R/GIPR co-agonism[448].

Stage I clinical experiments confirmed the metabolic benefits conferred by GCGR agonism observed in rodent models could be translated to human, as co-infusion of GLP-1 and glucagon in healthy human volunteers significantly attenuated the hyperglycaemic effect of glucagon while preserving desirable effects on energy expenditure and appetite suppression[601]. Consistent with rodent studies, co-infused glucagon and GLP-1 exert synergistic effects on satiety and reduce food intake in healthy volunteers[601,602]. The pivotal role of GLP-1R agonism in balancing the hyperglycaemic effects of GCGR agonism may, however, limit the use of such a therapy in insulin-deficient or severely resistant patients, since much of the desirable glucose-lowering effect of GLP-1 is attributed to its insulinotropic and glucagonostatic effects. However, phase 1-2 clinical trials of alternative GCGR agonists with agonist activity at various other receptors are currently underway[449].

1.3.6 Peptide YY (PYY)

While not structurally related to PGDPs, Peptide YY (PYY) is co-localized with GLP-1 in enteroendocrine L cells[107,619-621] and is co-released with GLP-1 postprandially in proportion to caloric intake[620,622]. Human PYY circulates in two active forms: PYY_{1-36} and PYY_{3-36}, the latter being a cleavage product of the former by DPP4[623]. In contrast to GLP-1, which is present in sufficient amounts in the duodenum to account for the immediate postprandial surge, PYY abundance is very low in the upper gut and increases distally from the ileum to the colon[624,625]. Thus, postprandial PYY release under normal physiological conditions is likely to be mediated through paracrine and neural mechanisms[626]. The exaggerated postprandial PYY response observed in post-gastric bypass or Endobarrier patients is likely attributed to direct nutrient stimulation of PYY cells as there is an increased flow of nutrients into the PYY-rich distal gut[627,628]. The physiological effects of PYY are mediated through a family of NPY receptors (termed Y1, Y2, Y3, Y4, Y5 receptors), which are differentially expressed in a wide range of tissues such as enterocytes, myenteric and submucosal neurons and extrinsic primary afferent nerve fibres[626]. PYY_{1-36} and PYY_{3-36} are important regulators of GI functions; such as the ileal and colonic brake[629]. PYY also inhibits gastric and pancreatic secretion while increasing fluid and electrolyte absorption throughout the GI tract[630].

1.3.6.1 PYY and energy balance

PYY_{3-36} is a potent anorectic hormone with administration of exogenous PYY significantly reducing food intake in both obese and lean humans[631,632]. While the "ileal brake" contributes to this satiating effect[633], PYY_{3-36} induces satiety primarily by targeting the hypothalamus. PYY_{3-36} is highly selective for Y2Rs found on NPY/AgRP neurons in the arcuate nucleus. Activating these receptors suppresses the release of the orexigenic neuropeptides NPY and AgRP, subsequently disinhibiting the release of the anorectic α-MSH from neighbouring POMC neurons to reduce food intake[408,632]. *Pyy*-deficient mice are hyperphagic and obese[634] while overexpression of *Pyy* protects mice against DIO or leptin deficiency[635]. PYY may also increase energy expenditure[635-637]. As such, the exaggerated postprandial PYY response observed in post-bariatric patients has been proposed as one of the underlying mechanisms for the dramatic weight loss achieved by the procedure[390,638].

1.3.6.2 PYY and glucose homeostasis

Studies aimed at investigating the glycaemic effects of PYY have also yielded conflicting results. Acute PYY administration improved intraperitoneal glucose tolerance in mice[639], although a lack of effect has also been reported[640]. Acute PYY treatment also augments insulin-mediated glucose disposal in high-fat fed mice[641]. However, PYY infusion in humans had no significant effect on plasma glucose, insulin or glucagon levels[631,642], nor did it affect glucose excursion and insulin level upon intravenous glucose challenge[643]. Despite the potent inhibitory effects of PYY on gastric emptying[644],

which in principle would reduce postprandial glucose excursions[645], acute PYY administration did not appear to have any significant impact on oral glucose tolerance in rodents[646] or humans[636]. PYY may restore normal islet function after RYGB as the peptide was identified as the humoral factor from RYGB rats that could correct impaired glucose-induced hormone secretion in islets isolated from diabetic rats[647]. Moreover, treating human islets with high concentrations of PYY significantly improved GSIS, although the *in vivo* physiological relevance remains to be determined as the PYY concentrations used in these experiments were several orders of magnitude higher than normal postprandial plasma PYY concentrations. While there have been studies demonstrating potential trophic effects of PYY on pancreatic β cells[648], such effects are believed to be mediated by islet-derived, rather than gut-derived PYY[649].

1.4 Why study L cells?

Enteroendocrine L cells are of special interest because their secretory products, namely GLP-1 and PYY, are implicated in a range of favourable metabolic functions and have been attributed to the metabolic gains from gastric bypass surgeries[77,90-92]. Thus, one of the approaches to create a "bariatric mimetic" would be to develop a specific L cell secretagogue, which could reproduce the exaggerated secretory responses observed in post-gastric bypass patients. However, the underlying mechanisms that regulate the secretory function of L cells remains largely unknown, due to the lack of a suitable and easily translatable model. To date, there is no means to study primary enteroendocrine L cells in viable pure culture. In the following section, the current models that are used to study L cell physiology are discussed.

1.4.1 GLUTag cells (Murine)

GLUTag is a cell line derived from a colonic tumour induced in the GLUTag transgenic mice. This lineage of transgenic mice was generated using the GLUTag2.3 transgene, which was constructed with approximately 2000 bp of the rat proglucagon 5'-flanking region aligned to the SV40 large T antigen (TAg) gene[650]. The SV40 TAg is an oncoprotein commonly used for transformation and immortalization of primary cells[651]. The inclusion of the 2000 bp segment proved to be pivotal for intestinal-specific expression of the large T antigen as an earlier experiment using only 800-1000 bp of the rat proglucagon 5'-flanking region (designated GLUTag1.3) only resulted in expression by pancreatic cells and specific neurons in the brain of the transgenic mice[652]. The transgenic mice expressing the large T antigen in the intestinal epithelia developed tumours in the large bowel and the tumour fractions were used to inoculate nude mice. The inoculated mice subsequently developed tumours in the colon and immunohistochemistry of these tumours revealed that tumour cells stained for both glucagon and GLP-1 (but only a limited portion of tumour cells stained for

PYY)[650]. The authors later described the processing of proglucagon-derived peptide in GLUTag cells as "somewhat aberrant and intermediate between pancreatic α cells and intestinal L cells", as it is a cell line that produces both glucagon and GLP-1[653]. Despite the significant differences in post-translational processing of the proglucagon peptide between native L cells and GLUTag cells, the cell line has been widely used as a model for enteroendocrine L cells[654-671] and commonly referred to as a "murine L cell line"[672-685]. GLUTag cells contain multiple other hormones including CCK and 5-HT[686], possibly because these tumour cells were not well-differentiated from their pluripotent stem cell precursors. Alternatively, it could be due to the plasticity of endocrine tumour cells.

Numerous studies have been conducted using GLUTag cells to elucidate mechanisms that could potentially trigger GLP-1 release in L cells. Consistent with previous observations using the enriched canine L cell culture and Foetal Rat Intestinal Culture (FRIC) models (discussed in sections 1.4.3 and 1.4.4, respectively), GLP-1 release by GLUTag cells was stimulated upon increased intracellular cAMP levels[655] and the subsequent activation of PKA[682,683]. GLUTag cells also express PC1/3, the enzyme responsible for post-translational processing of the proglucagon peptide in primary L cells[69]. It is interesting to note that the expression of PC1/3 in GLUTag cells also appeared to be induced by PKA activation[682], which is consistent with an earlier observation in the FRIC model[687]. Later studies demonstrated PC1/3 expression in GLUTag cells was also modulated by glucose concentration[678]. GLUTag cells are highly sensitive to glucose and this sensitivity appeared to be mediated by K_{ATP} channels, SGLT1 & 3, GLUT1 and GLUT5[657]. GLUTag cells are also responsive to both insulin[672] and GIP[683]. However, the physiological implications of these observations might be questionable as the concentrations of insulin and GIP used in these studies were supraphysiological[688,689]. Notably, PYY is not detected in GLUTag cells[690], which is in contrary to native L cells[620,691,692]. This finding strongly refutes the notion of GLUTag as a valid "L cell line". Nevertheless, GLUTag cells remain one of the most widely used models to study L cell secretion.

1.4.2 NCI-H716 cells (Human)

The NCI-H716 cell line was first established by Park *et al.* in 1987 from a human colorectal tumour[693]. These cells were described as being poorly differentiated and displayed enteroendocrine characteristics. Further characterizations suggested the cell line comprised a mixture of exocrine and endocrine cells, as evident by mucin- and chromogranin A-staining, respectively. Interestingly, some of NCI-H716 cells were classified as amphicrine, as they contained both mucin vacuoles and dense-core secretory granules. It was also found that the NCI-H716 cells express gastrin, 5-HT and somatostatin receptors[694]. The endocrine characteristics could be further enhanced by co-culturing with fibroblasts or culturing in native extracellular matrices (ECM) (amnion membrane, colonic ECM and Basement Membrane Matrigel)[695]. This cell-line was not very intensively studied since its

establishment until Reimer *et al*. suggested that the NCI-H716 cell-line might serve was a human cellular model for studying the secretion of GLP-1[696]. Given the intense clinical interest in GLP-1, the NCI-H716 cell line has since been extensively used as a model for studying GLP-1 secretion in the intestine. However, whilst an array of published work conveniently referred the NCI-H716 cell-line as a human enteroendocrine L cell line[249,673,678,697-700], such a reference was never made by either Park *et al.* or Reimer *et al.*. NCI-H716 cells are aneuploid, containing from 55 to 64 chromosomes[694] and it is not known how these extra chromosomes contribute to cellular processes in the NCI-H716 cells. Moreover, there is major disparity between the secretory repertoires of native L cells and NCI-H716 cells. The fact that PYY secretion cannot be detected in NCI-H716 cells strongly argues against its use as a reliable model of human L cells[690].

1.4.3 STC-1 cells (Murine)

STC-1 is a GLP-1 secreting cell line derived from a murine invasive small intestine neuroendocrine tumour[701]. This cell line secretes a wide range of gut hormones, especially those characteristic of the stomach, such as secretin and gastrin[702] and has been deemed to bear the least resemblance to native L cells, when compared with GLUTag and NCI0H716[690]. As such, STC-1 cells are less often used for studying L cell physiology.

1.4.4 Enriched Canine L cell culture

This is one of the earliest protocols for L cell enrichment in primary culture. Briefly, canine intestinal mucosa was isolated, minced and enzymatically digested. Enrichment was achieved by subjecting the mucosal cell suspension to elutriation[703]. Further optimization of the protocol resulted in a purity of 20-25 % of L cells culture[704]. However, this model was not too widely adopted due to the relative high cost of producing only partly purified L cell cultures.

1.4.5 Foetal Rat Intestinal Culture (FRIC)

This is a model originally developed by Brubaker et al. where the whole intestine of a foetal rat was dissected, minced, enzymatically digested and filtered to achieve separation of intestinal cells[705]. This resulted in a mixture of single cells, cell clusters and small fragments of the dissected intestine. Due to the heterogeneity of cells in culture, it is therefore difficult to differentiate direct and indirect effects of test reagents on L cells. Although no longer widely used, this *in vitro* model did provide valuable information of L cell physiology. It enabled early studies of L cell secretion and showed that GLP-1 secretion could be stimulated by activation of the adenylate cyclase pathway[687], which was later supported by studying primary murine L cells[692].

1.4.6 Fluorescent protein-tagged L cells in transgenic mouse

This is one of the better models for human L cells currently available as it is a primary culture of murine L cells. This model was developed from the generation of a lineage of transgenic mice, in which a modified yellow fluorescent protein, Venus (YFP-Venus), is expressed under the control of the proglucagon promoter, resulting in targeted expression in L cells in the intestine, pancreatic α cells and a subpopulation of NTS neurons in the brainstem[692]. This strategy was later modified to express different fluorescent proteins[660,691] and in a different enteroendocrine cell population, such as CCK-secreting I cells[706] and GIP-secreting K cells[707]. Using Fluorescence-Assisted Cell Sorting (FACS), the researchers were able to sort the intestinal epithelial cells based on the expression of the fluorescent protein. FACS-sorted cells could subsequently be used for gene expression and patch-clamp studies. It was through this approach that the glucose-sensing machinery of L cells was elucidated. Although L cells isolated and purified by FACS did not survive long in culture, they could be maintained for several days in a mixed-cell culture (either small intestinal or colonic culture) for GLP-1 secretion studies. Based on this method, single primary L cells were studied in detail for the first time and comparisons with GLUTag cells could be made. While the authors reported that many characteristics observed in the GLUTag cell line closely resembled those observed in primary murine L cells, such as glucose-sensing ability[657,692] and glutamine-stimulated GLP-1 release[656,708], there are major differences in the gene expression profiles between these cells. *Kir6.2* and *Sur1* (encode for different K_{ATP} channel subunits) and *Sglt1* (encodes for the sodium-glucose co-transporter, SGLT1), were found to be much more highly expressed in these isolated primary L cells than in GLUTag cells[692]. Moreover, *Glut2* (encodes for the facilitative glucose transporter, GLUT2) mRNA was detected in primary L cells, but not in GLUTag cells[657]. The two populations were also found to expressed different members of the adenylyl cyclase family[669] and have different Na^+ channel subunit composition[709]. Another noticeable difference between the two populations is their sensitivity to short-chain fatty acids (SCFAs), commonly produced by gut flora. Significant SCFA-stimulated GLP-1 secretion was observed in primary L cells but not in GLUTag cells[710]. Such differences further highlight the significant divergence of cell lines from primary cells. The development of this protocol also revealed the fragility of primary murine L cells, in that although they are electrically excitable, this excitability was only observed when the L cells were cultured with epithelial cells.

1.4.7 *Ex vivo* models

Although *in vitro* models have provided invaluable insight into L cell physiology, there are significant drawbacks with using cell cultures. Cells are removed from their natural environment by digestion (typically a combination of EDTA, trypsin and collagnase) and cell-cell contacts are lost in the

process. Indeed, detachment from the basement membrane is often a trigger for apoptosis in intestinal cells[711,712]. For the portion of cells that survive the isolation process, the loss of cell-cell contacts could still have dramatic functional ramifications. The significance of this is evident in islet biology, although researchers could purify individual cell populations and study them in pure culture, they are more often study in intact islets. Secretory responses of isolated α and β cells significantly differ to that of intact islets[713,714] and it is often very difficult to determine if this is an experimental artefact. Several *ex vivo* methods have been employed to overcome these issues.

1.4.7.1 Intestinal organoid culture

Although the intestinal epithelium is capable of self-renewing, primary cultures derived from the intestine often have limited lifespan. To overcome this, Sato and colleagues developed a sophisticated method that allows crypts isolated from murine intestinal tissue to be grown in culture[715]. Intestinal crypts were dissociated through EDTA-chelation of Ca^{2+} and Mg^{2+}; the isolated crypts are then resuspended in Matrigel or other basement membrane matrices and plated. The "stemness" of the isolated crypt is preserved by ensuring the composition of the culture medium resembles that of the stem cell niche, such that optimal Lgr4/5, EGF, Notch and Wnt signalling is achieved enabled while BMP (bone morphogenetic protein) signalling is attenuated. Further optimization efforts allowed crypts isolated from human colonic epithelial tissue to be grown into organoids in a similar fashion[716]. These organoids can be passaged at 1:5 ratio weekly and intriguingly, it appears that the cultures can be maintain indefinitely as the phenotype and karyotype of the organoids remained unchanged for at least 18 months[717], which makes it far superior over immortalized cell lines, which are highly susceptible to genetic mutations. In addition, organoid stem cell differentiation closely mimics that of *in vivo* stem cells, as evident by the presence of most intestinal epithelial cell types including enteroendocrine cells[716] in developed organoids. Organoids also preserve the features of the intestinal segments that the crypt originates from[718], e.g. only ileal organoids express the Apical Sodium-dependent Bile acid Transporter (ASBT), which is only expressed in the terminal ileum *in vivo*. The major advantage of using organoids over primary cultures of isolated intestinal cell is that the polarity is somewhat maintained. As the organoid develops, it undergoes "budding" that results in the formation of crypt-like structures within the organoid: microvilli are present on the "luminal side" of the organoid with terminally-differentiated, apoptotic cells shedding into this central lumen[715]. It was demonstrated that intestinal organoids are excellent models for studying the physiology of enteroendocrine cells; 5-HT secretion from EC cells[719] and L cell differentiation[720-722] have since been studied in detailed using this technique.

1.4.7.2 Isolated perfused animal intestine

Isolated perfused intestine models of various species have been widely used to study the secretion of gut hormones since the 1970s[723,724]. Optimization efforts by multiple groups[723,725-733] have made it one of the most physiologically relevant models to study gut hormone release[734]. After sacrificing of the experimental animal, the intestine preparation is arterially perfused with oxygenated buffer through the mesenteric artery, the gut lumen is cannulated and perfused with isotonic saline and an automated fraction collector collects the vascular effluent from the portal vein at pre-set time intervals for downstream analysis. This allows for the interrogation of the mechanisms underlying gut hormone secretion in response to changes in the intestinal lumen or vasculature in a manner that resembles native physiological environment as the integrity of the organ and local neural circuits are preserved. Since the intestine is perfused with oxygen and nutrients, it is not uncommon for the preparation to be viable for hours after isolation, with the integrity of the epithelial brush border fully maintained after up to 5 hours of perfusion[723]. This model also allows specific sections of the gut to be studied in isolation. Test reagents can be added to either the vascular or luminal compartments to discriminate apical from basolateral stimulation. Another major advantage of the isolated perfused intestine model is its high versatility; technically, any gut hormone could be measured in the collected effluent fractions from one single experiment, provided reliable assays are available for the analytes of interest. This model has since been used to study nutrient-induced neurotensin secretion from the rat small intestine[728,735], pro-GIP processing in mouse intestine[736], nutrient-induced GLP-1 secretion in rat small intestine[186,733,737], GIP-induced GLP-1 release from the rat colon[727] and duodenal hormone-induced somatostatin and GLP-1 release in porcine ileum[732], just to name a few. Intriguingly, the perfused rat small intestine model correctly predicted the existence of another incretin other than GIP, several years before GLP-1 was discovered[726], highlighting the physiological relevance of this model.

1.4.7.3 Ussing Chamber

Although isolated perfused animal intestines are physiologically relevant, findings in animal models may not be fully translated in humans. GLP-1 secretion from the human gut has also been studied using a modified Ussing chamber setup with intestinal biopsies collected endoscopically from subjects and mounted onto the apparatus[738,739]. This setup differs from the conventional Ussing chamber set up in that it does not measure electrical current moving across the epithelium but simply provides a means to maintain polarity of the tissue and the volumes of the two reservoirs are significantly smaller than that of conventional setups (0.5-1.5 mL versus 5-10 mL), which enables the researchers to quantify the amount of hormone released. Interestingly, results from this approach showed that luminal exposure to pea and wheat proteins significantly triggered GLP-1 and CCK

release from human duodenal mucosal preparations but not in rats[738], emphasising the importance of studying human tissue whenever possible for better translational potential.

1.5 Lost in translation?

Besides the obvious differences in appearance, there are well documented differences between the physiology of rodents and humans[740-743]. Although "75 % of mouse genes have 1:1 orthologs in the human genome", "mice are not miniature humans"[744]. These differences are especially widely-reported in the field of immunology. For example, it was found that Killer immunoglobulin-like receptor molecules on murine NK cells are sugars whereas their equivalent in humans are proteins[740]. The translation of efficacy in treatment protocols from animal models to clinical settings has more often resulted in disappointment than success and many of the failures can be attributed to the ignorance that pathways elucidated in animal models do not neccessarily exist in human[741]. Thus, potential blockbuster treatment protocols developed on the basis of these pathways often produced disappointing results in clinicial testing, and in some cases were, potentially dangerous. One such example is the failed clinical trial where altered peptide ligand (APL) peptide was tested as a potential treatment for multiple sclerosis. The APL peptide was hypothesized topotentially suppress T cell responses via antagonism (or partial agonism) of T cell receptors based on extensive studies in animal models. However, the peptide turned out to act as a full agonist in a subgroup of patients and thereby exacerbating their disease[745]. The authors later pointed out that in retrospect, such an event could have been predicted had a human polyclonal cell population been used prior to clinical testing[746].

The loss in translation is not only observed in immunology. In the area of endocrinology and specificly L cell physiology, there are also cases where successes in the laboratory based on *in vitro* and *in vivo* rodent models failed to translate into beneficial clinical outcomes. One prominent example is the potential of GPR119 agonists to be used as secretagogues to stimulate GLP-1 secretion by intestinal L cells in humans[747]. *GPR119* mRNA was detected in human intestinal pancreas and intestine[667] and its receptor agonists were shown to increase GLP-1 secretion *in vitro* using both human and murine cell lines[667,674,680,748] and *in vivo* mouse models[749-751]. However, the observed efficacy did not translate into favourable clinical outcomes, as evident by the underwhelming performance of the oral GPR119 agnoist, JNJ-38431055. Although well-abosrbed and well-tolerated, the drug failed to significantly increase active GLP-1 level in plasma following meal-tolerance and oral glucose tolerance tests. Nor did it improve glycaemic control, when compared with placebo, and it was inferior to the DPP-IV inhibitor, sitagliptin[752]. It was later reported that a different GPR119 agonist, AR231453, did not trigger GLP-1 release in human primary

colonic culture, despite promising results from murine cultures employing similar protocols[620]. While the underlying reasons of such disappointing results are yet to be elucidated, there are several possible explanations to the problem. Although the the human and mouse GPR119 share an 82% amino acid sequnce identity[753], they are not identical. It is speculated the lack of efficacy can be due to different receptor-ligand binding interactions between human and rodents[754] but this cannot explain the efficacy of GPR119 agonists observed in transfected GLUTag cells that expressed human GPR119[674]. Another possible explanation is that there may be fundamental differences in L cell signalling between human and rodents. Indeed, it is worthwhile to point out that there is no published evidence that supports the expression of GPR119 in human L cells. The closest proof of such speculation was the detection of *GPR119* mRNA in human intestinal tissue[667] but the result was not backed by immunostaining for the GPR119 protein in human intestinal tissues, not to mention co-staining with proglucagon. GPR119 could well be an excellent target in rodents but in human L cells. Failure to bridge such major gaps of knowledge between models in the laboratory and human physiology in the clinic will result in more such failures and hinder the development of new treatment strategies in combating diseases, not just diabetes. It is therefore crucial to develop systems that closely resemble the human *in vivo* environment.

1.6 Aims and significance

The primary aim of this project is to develop a reliable platform that allows for detailed interrogation of the mechanims underlying the secretion of GLP-1 and PYY from L cells in the human gut. The use of human intestinal tissue as a starting point completely mitigates the issue of species difference and thus, such a platform can serve as a valubale intermediary step between rodent studies and clinical studies, significantly reducing the risk of expensive failure of clinical developments of any therapetuics. The second aim of the project is to determine whether various reported pathways that govern GLP-1 secretion in rodents exist in humans. Specifically, pathways governing GLP-1 secretion in response to glucose, to the anti-diabetic drug metformin, to endogenous and synthetic agonists of the melanocortin 4 receptor, and to interleukin-6 will be investigated. The role of the classical endocytotic protein, dynamin, in mediating L cell secretion will also be investigated. The third aim of this project is to address the recent report of the presence of extrapancreatic glucagon in human[463] and I hypothesize that the human gut epithelium is a source of fully-processed glucagon.

2 Optimization of methods to measure L cell secretion from human gut tissue

2.1 Isolation and enrichment of human L cells

2.1.1 Introduction

The secretory responses of L cells to a range of stimuli have been studied predominantly using GLUTag cells[654-658,668] and primary mixed intestinal epithelial cell cultures[95,660,708-710,755-757], owing to the fact that FACS-isolated primary murine L cells were not viable for functional studies[692]. The first aim of this project is to develop a method to isolate L cells from human colonic epithelial specimens as a highly purified culture such that their secretory responses to various nutrients can be studied in isolation. Pioneering work from our laboratory has enabled the isolation of the most abundant enteroendocrine cells, the serotonin-secreting enterochromaffin cells from mice, guinea pigs, and humans using a Percoll density gradient[109]. Importantly, these highly-enriched primary enterochromaffin cell cultures can be kept viable for a considerable duration (typically up to 48 hours), which is sufficient time for a range of different functional experiments such as single-cell carbon fibre amperometry[109,116], flow cytometry[115] and secretion studies[115,127] to be conducted. Based on the reliability of this method, I hypothesized that a cell culture highly-enriched in L cells can be obtained using a Percoll density gradient, similar to enterochromaffin cells isolation.

2.1.2 Methods

2.1.2.1 Primary culture human intestinal epithelial cells

Fresh anonymised surgical specimens of human colon were obtained from the Gastroenterology surgical department of Flinders Medical Centre and Flinders Private Hospital, Bedford Park, South Australia, stored in Krebs buffer and processed within 15-30 minutes of surgery. Tissue samples were cleaned of luminal contents and dissected of muscles and connective tissues. Blood cells present on the mucosa were gently scraped off. The mucosal layer was carefully torn off the basal laminae and chopped finely in Krebs's buffer into 1-2 mm pieces. The minced mucosa was then centrifuged at 500 x *g* for 5 minutes to remove the Krebs's buffer. The mucosa was digested with 8 mL of 0.5% Trypsin-EDTA (Sigma Aldrich, Australia) and 3.6 mL of 1 mg/mL Collagenase A (Roche, Australia) in a shaking water bath at 37°C for 40 minutes. The digestion was stopped by adding iced-cold culture medium (DMEM supplemented with 10% (v/v) fetal calf serum) and passed through a stainless steel mesh filter followed by a 40 µm cell strainer (EASYstrainer™, Greiner Bio One International). The introduction of a stainless steel mesh filter greatly reduced the time required for the digest to pass through the much finer cell strainer by up to 30 minutes. It was observed that a

thick mucus layer gradually developed as the digestion progressed and greatly hindered the digestion efficacy as the mucous layer prevented the digestive enzymes from accessing the mucosal tissue pieces. This mucous layer could not be removed with centrifugation and greatly hindered the passage of the digest through the subsequent filtration steps. Thus, 1 mM 1,4-dithiothreitol (DTT) was added to the digest as DTT is an effective mucolytic agent[758] and is generally suitable for use in cell culture[759]. The filtrate was centrifuged at 800 x *g* at room temperature for 4 minutes to pellet cells. The supernatant was removed and the cell pellet was resuspended in DMEM, plated in a 10 cm cell culture dish and let to recover in the incubator (37°C, 95% O_2/5% CO_2) for at least 30 minutes. The whole digestion and filtration process was repeated up to five times to maximize the number of cells obtained per specimen.

2.1.2.2 Fractionation of isolated mixed epithelial cells using a Percoll density gradient

After recovery, the cell suspension was put through a thirteen-step Percoll density gradient. Isotonic Percoll stock solution (referred to as Percoll SIP) was prepared by adding 9 parts (v/v) of undiluted Percoll (P1644, Sigma Aldrich) to 1 part (v/v) 1.5 M NaCl. Each 1 mL fraction of the Percoll gradient was then made up of varying ratios of Percoll SIP and 0.15 M NaCl as per manufacturer's instruction (Table 2.1). Our laboratory has previously reported the success of using an eight-step Percoll density gradient to obtain highly-enriched cultures of serotonin-secreting enterochromaffin cells from guinea pig and human colonic mucosae[109,116]. As alluded before, this method was subsequently modified and optimized for the successful isolation of murine enterochromaffin cells[113,115]. Thus, this same protocol formed the basis for my attempt to isolate L cells. Enteroendocrine cells make up approximately 1% of the epithelial cells of the gut epithelium[760] and enterochromaffin cells constitute approximately 50% of the enteroendocrine cell population[123]. As such, the original eight-step Percoll density gradient was further fractionated to a thirteen-step gradient in the hope of capturing the less-abundant L cells in these new density fractions. Fraction density was calculated using a formula provided by the manufacturer (Cell Separation Media Handbook 18-1115-69, GE Healthcare):

$$\rho = \frac{V_i\rho_i + V_y\rho_y}{V_y + V_i}$$

Where V_y = volume of diluting medium (mL), V_i = volume of Percoll SIP (mL), ρ_i = density of Percoll SIP (1.123 g/mL), ρ_y = density of 0.15 M NaCl (1.0046 g/mL).

Fraction Number	Volume of Percoll SIP (µL)	Volume of 0.15 M NaCl (µL)	Density of fraction (g/mL)
F1	1000	0	1.123
F2	600	400	1.07564
F3	550	450	1.06972
F4	500	500	1.0638
F5	450	550	1.05788
F6	400	600	1.05196
F7	350	650	1.04604
F8	300	700	1.04012
F9	250	750	1.0342
F10	200	800	1.02828
F11	150	850	1.02236
F12	100	900	1.01644
F13	50	950	1.01052

Table 2.1.1 Composition and density of each fraction of the Percoll density gradient.

The Percoll density gradient was constructed in a 15 mL centrifuge tube: 1 mL of F1 was added to the bottom of the tube with standard 1 mL pipette and subsequent fractions were carefully layered using 1 mL syringe through a 23G needle (the needle was positioned just under the meniscus of the top fraction). 1 mL of cell suspension was then carefully layered onto the Percoll density gradient and centrifuged at 1100 x *g* for 8 minutes with slow braking at 20°C. The bottom of the 15 mL tube was then pierced with a 25 G needle and fractions were collected into new 15 mL tubes at 1 mL intervals. 10 mL 1X phosphate-buffered saline (PBS) (Gibco, Life Technologies) was added to all 13 collected fractions and centrifuged at 800 x *g* for 4 minutes to wash off Percoll and pellet cells. The cell pellets from each tube were then resuspended in 1 mL fresh DMEM and plated in cell culture dishes for recovery in the incubator for at least 3 hours. Cell viability was assessed using the Trypan Blue exclusion method. Using the isolation method described above, cell viability of greater than 90% was typically achieved.

2.1.2.3 Immunocytochemical analysis of L cell enrichment

Since immunocytochemical analysis was used for assessing the purity of the enriched enterochromaffin culture in previous work from our laboratory[109,113,115], I sought to use a similar approach to determine the enrichment efficacy of the aforementioned method. Cells from each of the 13 fractions were grown overnight on glass coverslips that were pre-coated with laminin and poly-D-lysine. Media was aspirated before Zamboni's fixative was added and the cells fixed overnight

(18-20 hours) at 4°C. The fixative was then removed and cells were permeabilized by a series of 5-minute washes as follows: 4 X 80% EtOH, 2 X 100% EtOH, 3 X DMSO, 4 X PBS. Fixed cells were then blocked with 10% normal donkey serum in antibody diluent (290 mM NaCl, 7.5 mM Na_2HPO_4, 2.6 mM $NaH_2PO_4 \cdot 2H_2O$, 0.1% NaN_3, pH 7.1) in a humidity chamber for 30 minutes followed by a 24 hour incubation with a goat polyclonal antibody against GLP-1 (sc-7782, Santa Cruz, 1:400). The primary antibody was washed three times with 1X PBS (5 minutes each) before a secondary antibody (donkey anti-goat IgG tagged with Cy3, Jackson Immunoresearch, 1:200) and DAPI (Sigma Aldrich, 1:500) was added to the fixed cells and incubated in the dark. After a 2 hour incubation, cells were washed three times with 1X PBS (5 minutes each). The coverslips were then mounted onto glass slides in buffered glycerol and visualized using an upright fluorescence microscope (Olympus BX50). As no staining with the anti-GLP-1 antibody was observed with 12 cell preparations (Figure 2.1.1), it was determined that this protocol was not suitable for this primary antibody.

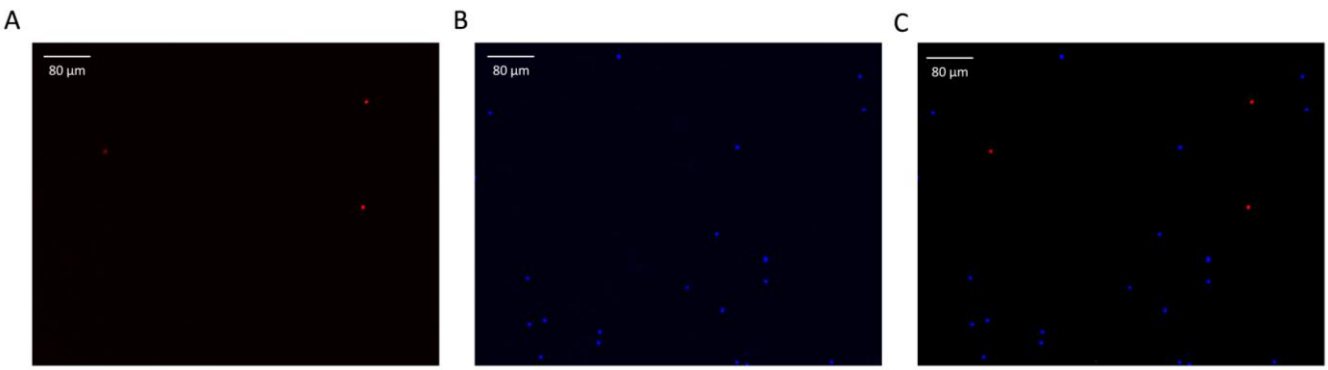


Figure 2.1.1 A representative image of the immuocytochemical staining of GLP-1 (A), DAPI (B) and overlay (C). Note there is a lack of co-localization of GLP-1 and DAPI.

An alternative immunocytochemical method was subsequently tested. Instead of Zamboni's fixative, cells were fixed for 30 minutes in 4% paraformaldehyde at room temperature. The fixative was then aspirated and the cells washed three times with 1X PBS (5 minutes each). The cells were then blocked and permeabilized with a blocking buffer (5% normal donkey serum, 0.2% TritonX 100 in 1X PBS) for 90 minutes at room temperature. Anti-GLP-1 antibody (as above) was then diluted in antibody dilution buffer (2.5% normal donkey serum, 0.1% TritonX 100 in 1X PBS) and added to fixed cells. The cells were left in humidity chamber overnight at room temperature before they were washed with 1X PBS three times. Secondary antibody (as above) and DAPI was then diluted with the same antibody dilution buffer and added to cells. The cells were left to incubate with the secondary antibody in the dark for 2 hours at room temperature before excess antibody was washed three times with 1X PBS. The coverslips were then mounted on glass slides and visualized as described above. As depicted in Figure 2.1.2, L cells were detected.

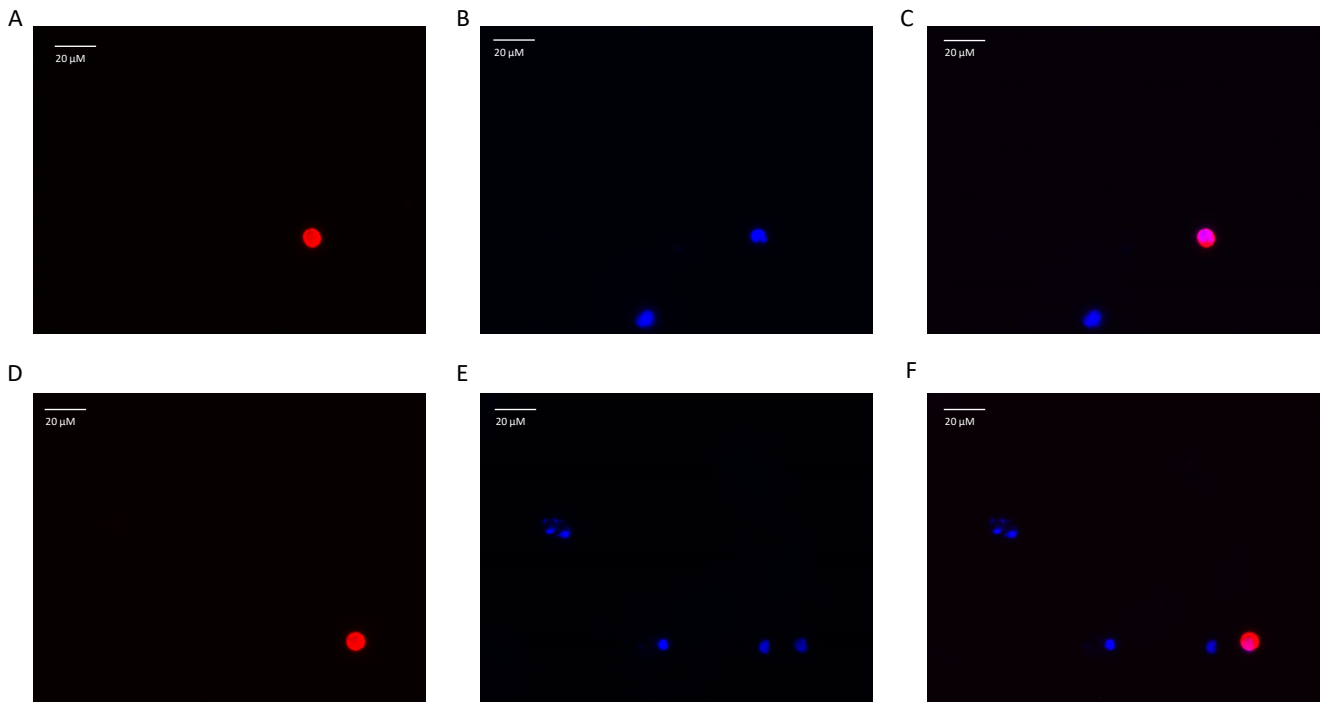


Figure 2.1.2 Representative images of immunocytochemistry using an alternative method from two different field of views at 40X magnification. (A, D) GLP-1; (B, E) DAPI; (C, F) overlay.

2.1.2.4 ELISA analysis of L cell enrichment

While the immunocytochemical protocol was successful in detecting individual L cells, immunocytochemical analysis was unsuitable for determining the enrichment efficacy of the cell culture protocol as L cell abundance was equally low across all fractions. Therefore, cells from each fraction from three separate preparations were lysed with CelLytic M buffer (C2978, Sigma Aldrich), supplemented with a DPP4 inhibitor (DPP4-010, Merck Millipore, 1:50 as per manufacturer's instruction) and the active GLP-1 content of each fraction was assayed using a commercially available ELISA kit (EGLP35k, Merck Millipore). Lysis experiments performed on cells obtained from each fraction of the Percoll gradient indicated active GLP-1 enrichment was observed at 1.0756 g/mL, 1.06972 g/mL and 1.0638 g/mL (Figure 2.1.3). Cells in fractions F2, F3 and F4 were pooled for subsequent experiments.

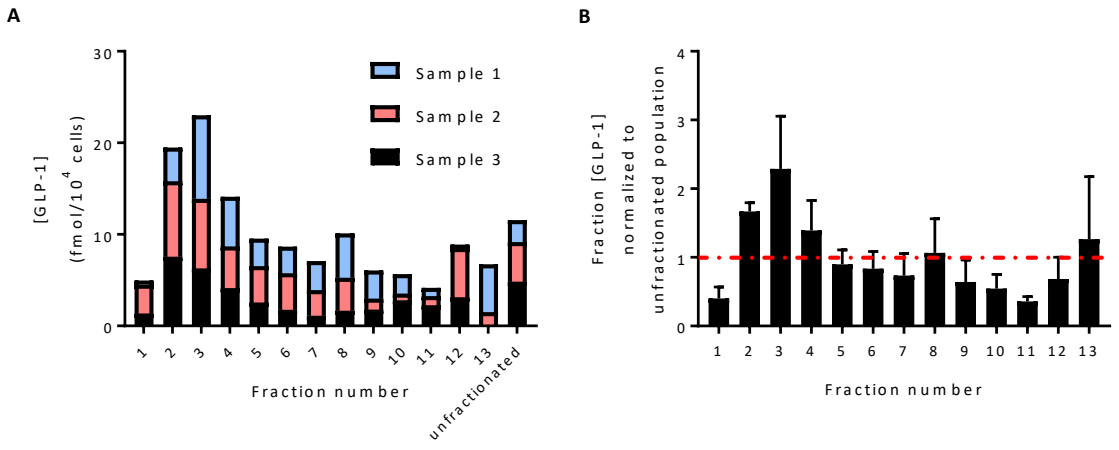


Figure 2.1.3 (A) GLP-1 content of each fraction from the Percoll density gradient obtained from three separate cell preparations. (B) GLP-1 content of the three samples pooled for each fraction and normalized to the GLP-1 content of unfractionated fraction (above red line: ratio > 1 ie enrichment in the fraction).

2.1.2.5 In vitro secretion experiments using enriched L cells

Secretion studies were performed on 24-hour old cultures. 200 µL of Geltrex® (A1569601, Life Technologies) was added to each well of a 24-well cell culture plate using pre-cooled pipettes, left at 37°C for one hour and at room temperature for one hour to coat the wells. Excess liquid from each well was then carefully aspirated immediately before cells were seeded. Up to $1x10^5$ cells were plated in each Geltrex-coated well and let to recover for 10-18 hours in 300 µL DMEM. Immediately before secretion experiment, culture media was removed and 250 µL of Krebs buffer containing various glucose concentrations, supplemented with 1:50 DPPIV-inhibitor (DPP4-010, Merck Millipore) and 0.1% fatty acid-free BSA, was added to each well. Cultures were incubated for 2 hours at 37°C, 5% CO2. At the end of the incubation, the media was removed and assayed for GLP-1 content with ELISA. Results from the 5 mM glucose treated well were used as control for basal secretion in the 2 hour incubation period. At the end of each incubation, cells were lyzed using 250 µL of DPPIV-inhibitor-supplemented Cellytic-M buffer (Sigma-Aldrich) and assayed for total active GLP-1 content.

2.1.3 Results and Discussion

2.1.3.1 Lack of glucose response of colonic mixed cell preparations

Increasing concentrations of glucose did not evoke significant dose-related GLP-1 secretion from colonic L cells after incubation of two hours, in contrast to that reported in primary murine colonic mixed cell culture[692]. GLP-1 secretion with 100 mM glucose stimulation was significantly lower than that of 10 mM glucose stimulation ($p < 0.05$, One-way ANOVA with Turkey's post-hoc comparison, Figure 2.1.4).

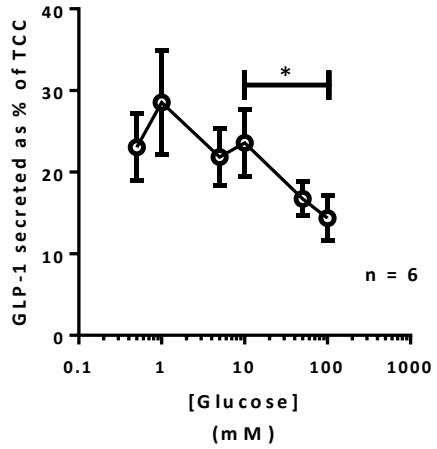


Figure 2.1.4 GLP-1 release in response to stimulation by increasing glucose concentrations. Data presented as total secreted amount as a percentage of total cell content (TCC).

2.1.3.2 Effect of short chain fatty acids on human colonic cell preparations

Short chain fatty acids (SCFAs), produced by bacterial fermentation of undigestible fibre, are potent murine L cell secretagogues *in vitro*[710,761]. Therefore, the SCFAs acetate, butyrate and propionate were tested for their effect on GLP-1 secretion in human colonic mixed epithelial cell culture. Unexpectedly, except for 1 mM acetate, all other concentrations of acetate, butyrate and propionate tested significantly inhibited GLP-1 secretion after 2-hour incubation, compared with control.

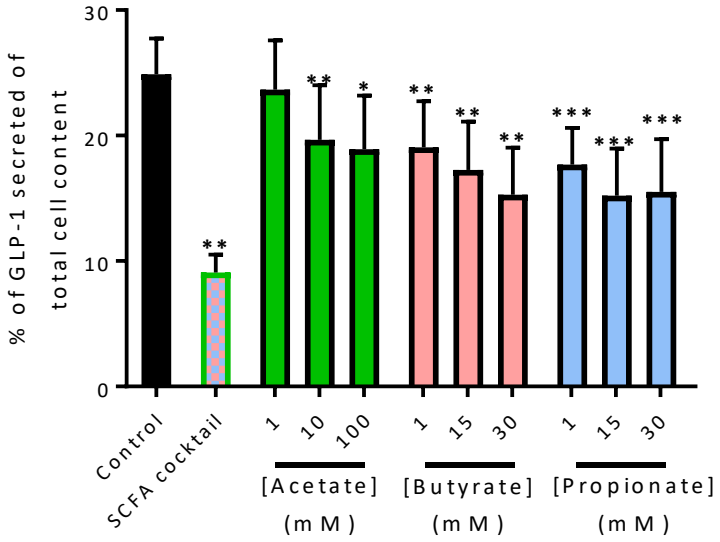


*Figure 2.1.5 GLP-1 release from mixed colonic epithelial cell culture was significantly inhibited upon exposure to SCFAs stimulation. *p < 0.05, **p < 0.01, ***p < 0.001, n = 6 -12.*

2.2 Whole tissue *ex vivo* secretion platform

2.2.1 Introduction

The lack of GLP-1 response to glucose and SCFA stimulation cast considerable doubt over the validity of the abovementioned primary human colonic epithelial cell culture as a suitable model for studying L cell secretion. Moreover, the entire isolation protocol was a low throughput process, with each preparation taking 8-12 hours to complete. Thus, an alternative platform that is of better physiological relevance was needed to study GLP-1 secretion from human L cells. A biorelease assay that reliably measured gut hormone release from murine and human colonic mucosal biopsies was described by Symonds *et al*.[117] and I sought to adopt a similar approach to study GLP-1 secretion from the human colon and ileum.

2.2.2 Methods

Human colonic and ileal specimens were obtained after consent from patients undergoing cancer resection or stoma reversal at the Flinders Medical Centre and Flinders Private Hospital colorectal operating theatres as described previously. Mucosal tissue was obtained as intact sheets by careful dissection using a stainless spatula and blunt-end forceps. The mucosal layer was then cut into 5 mm pieces, placed in microfuge tubes filled with a customized culture medium (10X stock solution obtained from Ms Nicole Isaacs, University of Adelaide. Medium composition as follow (in mM): NaCl 122, KCl 5.9, $NaHCO_3$ 48.9, $CaCl_2$ 2.0, NaH_2PO_4 1, $MgSO_4$ 0.9, HEPES 27.8, D-glucose 6.1, L-glutamine 4.4, pyruvic acid 1.1, $Fe(NO_3)_3$ 275 nM, 1% BSA, pH 7.4) supplemented with a DPP4 inhibitor, PK44 phosphatase (50 mM, 4145, Tocris) and weighed individually. The mucosal pieces were then transferred to a 96-well plate for static incubation at 37°C, 95% O_2/5% CO_2 for 2 hours. After incubation, supernatants were collected from each well, stored at -20°C and subsequently assayed for GLP-1 content.

As the melanocortin 4 receptor (MC4R) was shown to be expressed by enteroendocrine L cells and its endogenous ligand, α-melanocyte-stimulating hormone (α-MSH) was shown to be an L cell secretagogue *ex vivo* in both murine and human colonic epithelia[762], the peptide was used to stimulate GLP-1 secretion in this *ex vivo* biorelease platform. However, α-MSH did not significantly trigger GLP-1 secretion from baseline using this protocol (Figure 2.2.1). Thus, further method optimization was undertaken.

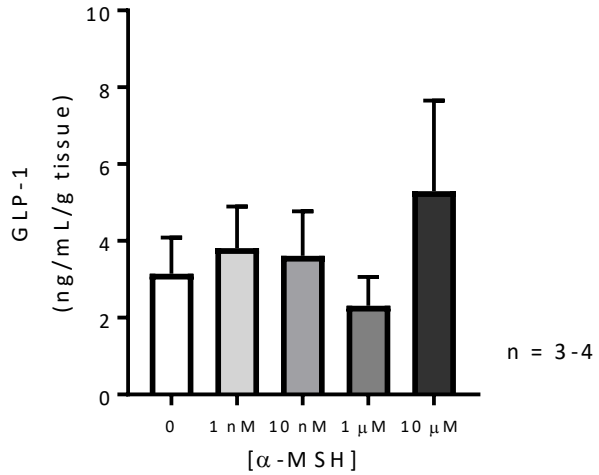


Figure 2.2.1 α-MSH did not significantly trigger GLP-1 secretion from human colonic mucosae beyond basal levels after a 2-hour static incubation.

2.2.2.1 Method optimization: DPP4 inhibitor

Since the ELISA detects only active GLP-1, the efficacy of the DPP4 inhibitor is likely to be an important factor that would potentially influence the outcome of the secretion experiment. Therefore, I compared the efficacy between two DPP4 inhibitors, PK44 and sitagliptin (gift from Pfizer Inc.). Colonic mucosal tissue was obtained and prepared from one colonic specimen as described above. 12 pieces of mucosal tissue were placed in biorelease buffer supplemented with 50 nM PK44 and 12 were placed in buffer supplemented with 1 µM sitagliptin (IC_{50} = 17 nM[763]). Active GLP-1 levels were significantly higher from samples supplemented with sitagliptin (Figure 2.2.2).

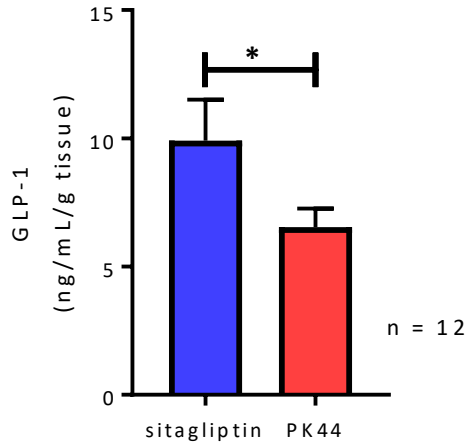


Figure 2.2.2 Active GLP-1 levels were significantly higher in samples supplemented with 1 µM sitagliptin than with 50 nM PK44 phosphatase.

Thus, sitagliptin was used instead of PK44 in subsequent experiments.

2.2.2.2 *Method optimization: secretion assay buffer*

I next aimed to optimize the secretion assay buffer such that the colonic mucosae would release GLP-1 upon stimulation by known physiological stimulants. Since the tissue was not responding to known physiological stimulants using the biorelease assay buffer used by Symonds *et al.*[117], I investigated if a modified Krebs buffer that is similar to that used for an *ex vivo* rat small intestine perfusion method[733] would be more appropriate. A modified Krebs buffer with the following composition (in mM): NaCl 138, KCl 4.5, $NaHCO_3$ 4.2 $CaCl_2$ 2.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 10, D-glucose 5.0, 0.1% BSA, pH 7.4, was thus tested against the original biorelease assay buffer in a colon and an ileum specimen obtained from the same patient that had undergone right hemicolectomy, in which the terminal ileum was resected as part of the procedure. As glucose is a known stimulant for GLP-1 release from the small intestine[733] and the combination IBMX and forskolin is a widely used positive control for GLP-1 secretion from primary colonic epithelial cell cultures[620,692], they were used to stimulate the tissue. As illustrated in Figure 2.2.3, 300 mM glucose potently triggered GLP-1 secretion from the ileal mucosae that was incubated in the modified Krebs buffer but not the biorelease assay buffer. Similarly, IBMX/FSK only triggered GLP-1 secretion from the colonic mucosae that was incubated in the modified Krebs buffer.

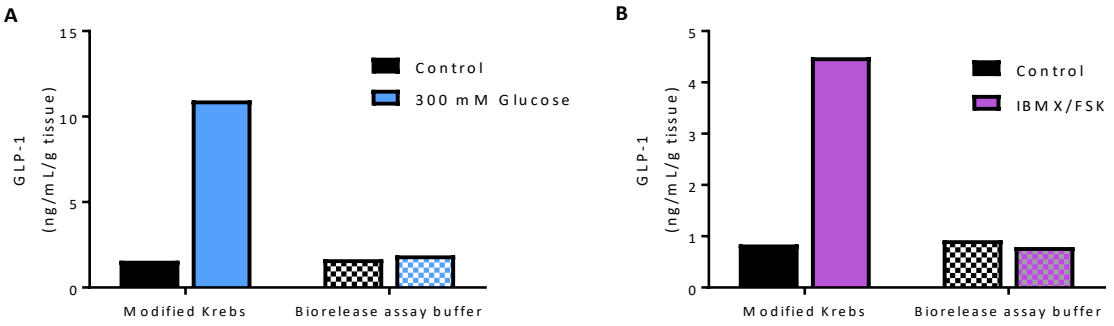


Figure 2.2.3 Comparison of the GLP-1 responses between tissue that were incubated a modified Krebs buffer and the original biorelease assay buffer. (A) 300 mM triggered GLP-1 secretion from ileal mucosae, (B) IBMX/FSK triggered GLP-1 secretion from colonic mucosae in the modified Krebs buffer but not in the biorelease assay buffer.

Therefore, the modified Krebs buffer was used for subsequent experiments.

2.2.2.3 *Method optimization: Incubation time*

Results from *ex vivo* perfused rat small intestine studies showed glucose-[733] and α-MSH[762] stimulated GLP-1 secretion occurs only transiently and that stimulatory effects were not sustained throughout the duration of the time in which the intestine was exposed to the stimulants. Therefore, mucosal pieces obtained from one colonic specimen were stimulated by a range of stimuli for different durations (30 minutes, 60 minutes and 120 minutes). In the hope of better capturing the stimulatory effect of MC4R activation, a more potent analogue of α-MSH, NDP-α-MSH was also used to

stimulate GLP-1 release. In addition, IBMX and forskolin were used in combination to serve as a positive control at concentrations previously described[620,692]. As depicted in Figure 2.2.4, GLP-1 release was above basal levels after 30 minute incubation with most stimuli tested and this stimulatory effect was not observed with longer incubation times. Thus, it was determined that shorter incubation time was crucial for capturing stimulatory effects on GLP-1 release.

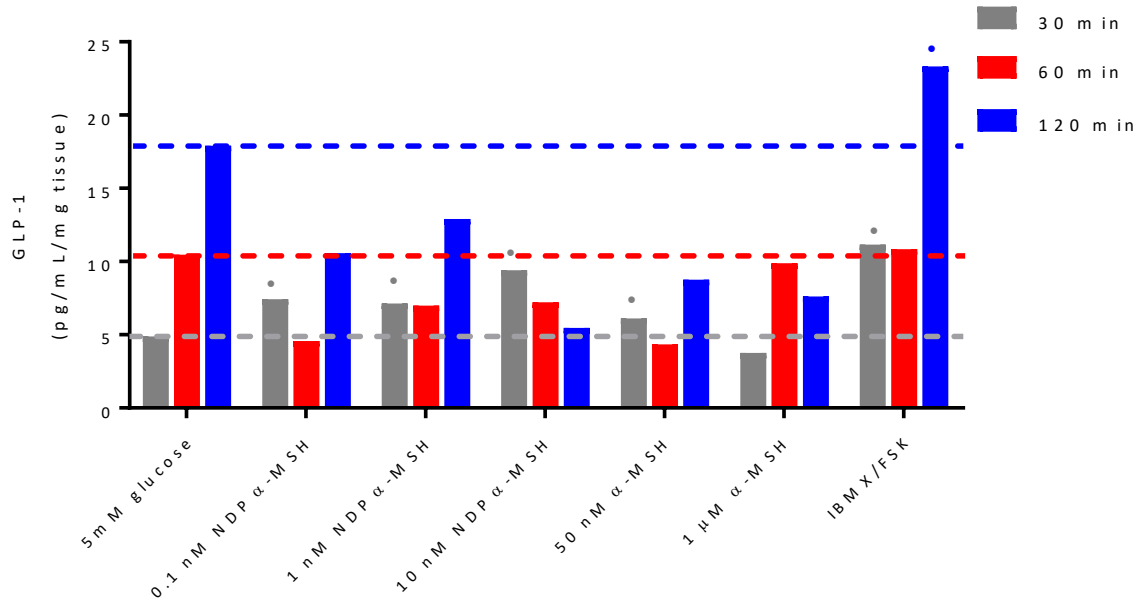


Figure 2.2.4 30 minute incubation was best to capture stimulated GLP-1 release. (Dots above bars indicate levels of GLP-1 were above basal levels; dashed lines indicate basal GLP-1 release during their corresponding incubation time)

I next sought to determine if the stimulatory effect of IBMX/FSK after 30-minute incubation could be reliably repeated and whether the stimulatory effect could be detected at even earlier time point. Indeed, IBMX/FSK significantly triggered GLP-1 secretion from colonic mucosae after 15-minute and 30-minute incubation. However, no stimulatory effects were detected after 5-minute incubation (Figure 2.2.5).

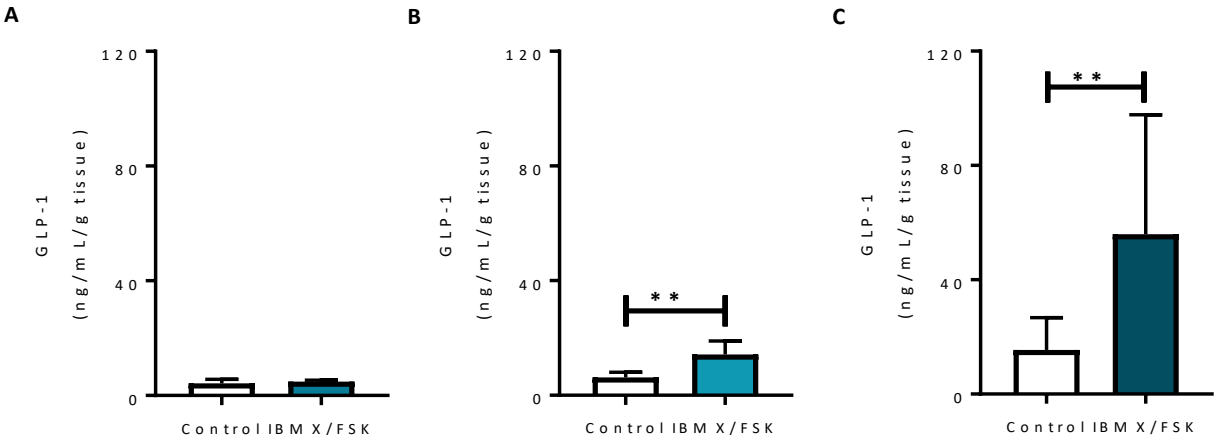


*Figure 2.2.5 The stimulatory effect of IBMX/FSK on GLP-1 secretion from human colonic epithelia after (A) 5 minutes (n = 3), (B) 15 minutes (n = 4), (C) 30 minutes (n = 4). **p < 0.01, paired-ratio t-test.*

Therefore, 15-minute incubation time was chosen for subsequent secretion experiments.

2.2.3 Results and Conclusion

Finally, to validate the optimized whole tissue *ex vivo* secretion platform, known L cell secretagogues were used to stimulate GLP-1 release from human colonic mucosae. The bile acid, taurodeoxycholic acid (TDCA, 100 µM) is a potent stimulant of GLP-1 release *in vitro* from primary mixed colonic epithelial cell culture and *ex vivo* from perfused rat small intestine[186]. Thus, TDCA, alongside the combination of 10 µM of IBMX and forskolin, and a SCFA cocktail (acetate 5 mM, butyrate 1 mM, propionate 1 mM) were used to stimulate GLP-1 secretion from human colonic mucosae, using a modified Krebs buffer supplemented with 1 µM sitagliptin and incubated for 15 minutes. All three stimuli significantly triggered GLP-1 secretion from the tested tissue samples (Figure 2.2.6).

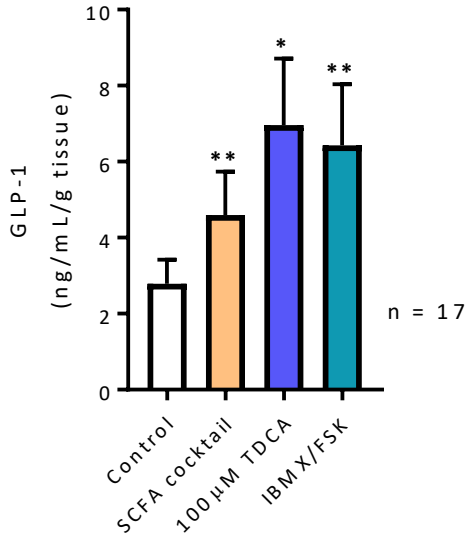


*Figure 2.2.6 Human colon GLP-1 secretory response to known L cell secretagogues. *p < 0.05, **p < 0.01, one-way ANOVA with Fisher's LSD test for comparisons between treatments.*

The above results demonstrated that with these optimized conditions established in this chapter, the whole tissue *ex vivo* secretion assay is a suitable platform to study secretory responses of L cells upon exposure to different stimulants.

3 GLP-1 and PYY responses in human *ex vivo* gut tissue to known L cell secretagogues

3.1 Mechanisms controlling glucose-induced GLP-1 secretion in human small intestine.

3.1.1 Introduction

As discussed in detail in previous chapters, GLP-1 is secreted by enteroendocrine L cells postprandially to potently enhance glucose-induced insulin release from pancreatic β-cells. Together with the other incretin hormone, GIP, it is estimated the incretins are responsible to 50 – 70 % of insulin secretion following oral glucose administration in healthy individuals[68]. In addition to its insulinotropic effect, GLP-1 is trophic to β cells[764] and confers glucose-sensitivity to glucose-resistant β cells[765]. Moreover, GLP-1 exerts potent inhibitory effects on glucagon secretion to suppress hepatic glucose output[220,322,766]. The extensive use of GLP-1-based anti-diabetic agents, namely DPP4 inhibitors and GLP-1 receptor agonists (GLP-1RAs) highlights the important role of GLP-1 in maintaining glucose homeostasis. In addition, the GLP-1RA liraglutide is efficacious in weight management[389], likely through central actions, and has recently been approved as an anti-obesity agent. Furthermore, there is increasing acceptance the metabolic benefits of gastric bypass surgeries are partly attributed to significantly elevated GLP-1 levels in patients after surgeries[314].

L cells are predominantly located in the epithelia of the distal small intestine and colon; although there are also a substantial population of L cells dispersed along the duodenum[251]. These proximal populations are likely to be responsible for the early phase of GLP-1 secretion upon meal ingestion. Luminal nutrient exposure to L cells in the intestinal epithelium is a potent stimulant for GLP-1 release and the underlying mechanisms of nutrient-sensing have been investigated in an array of experimental models[767]. The availability of fluorescent protein-tagged L cells generated from transgenic mice[692] has been instrumental in broadening our understanding of basic L cell physiology. In addition, the development of the rat small intestine perfusion model[733] has enabled the study of L cell secretion in a setting that closely resembles its native physiological environment. Based on studies by others using primary murine intestinal mixed cell cultures[660,692,768], transgenic mouse model *in vivo*[769] and an *ex vivo* rat model[733], glucose-induced GLP-1 release by L cells in the small intestine is thought to be primarily mediated by the sodium glucose co-transporter SGLT1 and to a lesser extent, glucose transporter GLUT2. Intracellular glucose metabolism upon glucose internalization and the subsequent closure of K_{ATP} channels are also shown to be pivotal. In addition,

sweet taste receptor signalling has also been shown to be involved in glucose-induced GLP-1 release[697,770].

However, fundamental species differences may hinder the translation of experimental results from these *in vitro* and *in vivo* rodent models to human *in vivo;* similar mechanistic examinations in human L cells are lacking. In this study, the mechanisms controlling glucose-induced GLP-1 release in human gut tissue were examined. Through the use of an *ex vivo* static secretion model in human gut intestinal mucosa, I demonstrated that glucose potently triggered GLP-1 release in duodenal, ileal, but not colonic, mucosae at concentrations equivalent to postprandial luminal, but not plasma, glucose levels.

3.1.2 Methods

3.1.2.1 Human tissue collection

For *ex vivo* secretion experiments, patients gave consent for tissue donation from resected terminal ileum and colon at Flinders Medical Centre and Flinders Private Hospital approved by the Southern Adelaide Clinical Human Research Ethics Committee. Ileal and colonic tissue specimens were collected from patients undergoing bowel resection for cancer or stoma reversal. In the case of resection specimens, samples were obtained from sites at least 10 centimetres away from the tumour location. Specimens from patients that were indicative of any form of inflammatory bowel disease were excluded from this study. Characteristics of the patient cohort are listed in Table 3.1. The specimens were immediately placed in iced-cold Krebs buffer (in mM, NaCl 138, KCl 4.5, $CaCl_2$ 2.6, $NaHCO_3$ 4.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 10, Glucose 5) and transported to the laboratory for dissection within 15 minutes. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently dissected off from the submucosae as intact sheets using a stainless steel spatula, cut into 5 mm pieces and weighed individually. The mucosal pieces were then transferred to a 96-well plate for secretion assays.

	Ileum specimen	Colon specimen
N	36	24
Age	71 ± 2	65 ± 2
Sex	15M : 11F	13M : 11F
BMI (kg/m^2)	29 ± 1	29 ± 1
History of T2D (yes/no)	6/ 30	7/ 17

Table 3.1 Characteristics of specimen donors for ex vivo experiments. BMI – body mass index, T2D – type 2 diabetes, M – male, F – female. Data are mean ± SEM.

3.1.2.2 Secretion experiments

Mucosal pieces were incubated with 250µL of buffer (control) or buffer containing test agents in a 96-well plate for 15 minutes. The buffer was a modified Krebs buffer as previously described in chapter 2, with the addition of sitagliptin 1 µM, 0.1% BSA (A1595, Sigma Aldrich) at pH 7.4. Following incubation at 37°C in 95%O_2/5%CO_2, supernatants were collected and stored in aliquots at -20^{o}C. Active GLP-1 levels were quantitated using a commercially available ELISA kit, according to manufacturer's instructions (EGLP-35K, Merck Millipore).

3.1.2.3 Materials

The following compounds were purchased from Sigma Aldrich: Diazoxide (D9035), Tolbutamide (T0891), 2,4-dinitrophenol (2,4-DNP) (D198501), Nifedipine (N7634), Lignocaine (L7737), Phlorizin (P3449), Phloretin (P7912), methyl α-D-glucopyranoside (α-MG) (M9376), sucralose (69293), D-Mannitol (M4125). To aid solubility, diazoxide, tolbutamide, 2,4-DNP, nifedipine, lignocaine, phlorizin and phloretin were first dissolved in DMSO and then diluted further in Krebs buffer, with DMSO concentrations never exceeding final concentration of 0.1%.

3.1.2.4 Statistical analysis

All statistical analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual. A paired-ratio Student's t-test was used for single comparisons and a paired one-way ANOVA with Fishers Least Significant Difference post-hoc test used for multiple comparisons. Statistical significance was $p < 0.05$. All data are shown as mean ± SEM.

3.1.3 Results

3.1.3.1 Glucose triggers GLP-1 release in the ileum but not in the colon

I established a dose-response relationship of glucose-induced GLP-1 secretion from ileal epithelial tissue; release was only triggered above 200 mM. 300 mM gave the highest GLP-1 secretion of the glucose concentrations tested (Figure 3.1.1.A). In contrast to the robust glucose responsiveness in the ileum, GLP-1 secretion from human colonic mucosal tissue did not increase in response to high glucose stimulation (Figure 3.1.1.B).

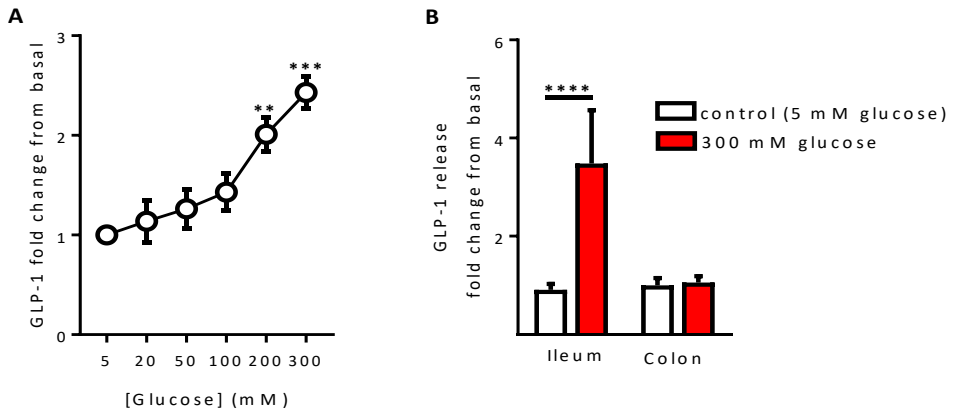


*Figure 3.1.1 (A) Concentration-response curve for GLP-1 secretion in response to increasing glucose in human ileum tissue, glucose potently triggered GLP-1 release from the human ileum but at 200 - 300 mM but not a lower concentrations, n = 5; **p < 0.05, ***p < 0.01. (B) 300 mM glucose significantly increased GLP-1 secretion from the human ileum (n = 21) but not colon (n = 24), ****p < 0.0001.*

3.1.3.2 High glucose-induced GLP-1 secretion from the ileum was not due to osmotic stress

300 mM D-mannitol did not increase GLP-1 release (Figure 3.1.2), which indicates osmotic stress did not drive the observed response to glucose.

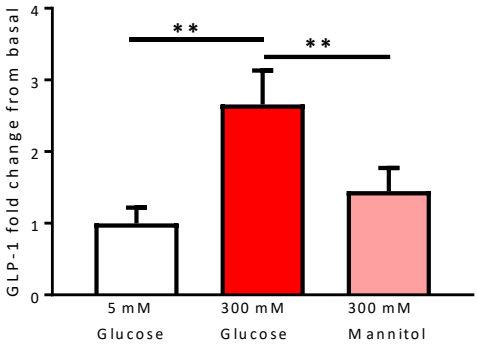


*Figure 3.1.2 300 mM glucose significantly increased GLP-1 secretion from human ileum but 300 mM D-mannitol did not cause significant release (n = 8, **p < 0.01).*

3.1.3.3 Glucose-induced GLP-1 secretion from the ileum did not change with diabetic status

I observed no changes in basal (5 mM glucose), or stimulated (300 mM glucose) GLP-1 release, or magnitude of stimulation, between specimens from non-diabetic (ND) and type 2 diabetic (T2D) donors (Figure 3.1.3). Similarly, the above measures were not correlated to the BMI of specimen donors.

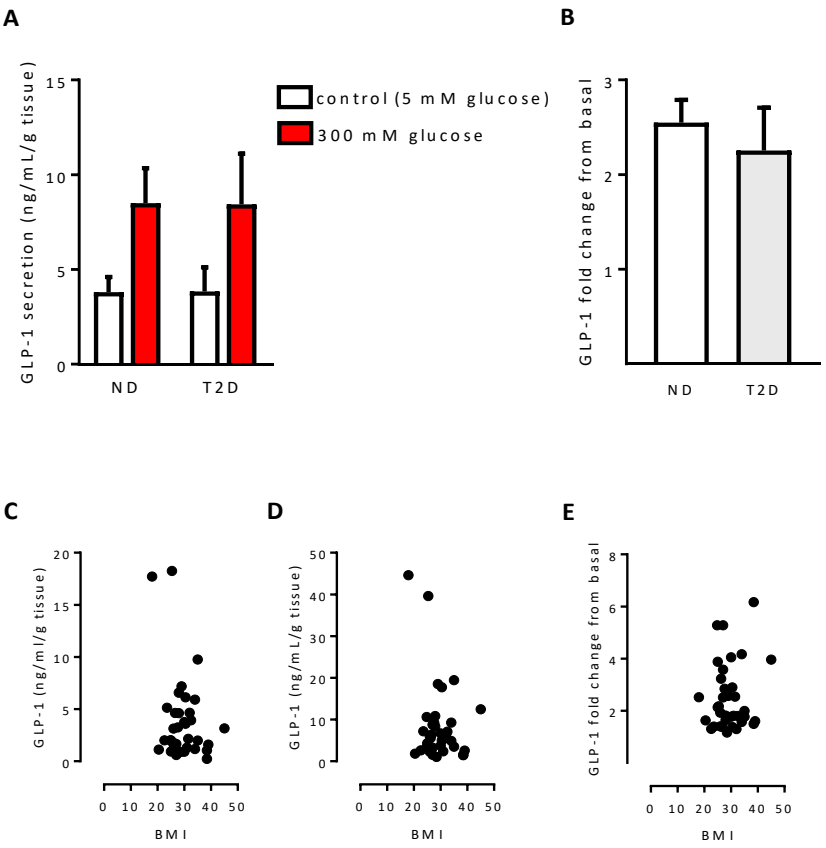


Figure 3.1.3 (A, B) Type 2 diabetes did not affect basal and stimulated GLP-1 release from human ileal preparations (ND: n = 30, T2D: n = 6). (C-E) BMI of specimen donors did not affect basal (C) and high glucose-stimulated (D) GLP-1 release (n = 36).

3.1.3.4 Mechanism regulating glucose-induced GLP-1 secretion

High glucose-induced GLP1 secretion was inhibited by the presence of 1 mM phlorizin, an inhibitor of the sodium glucose co-transporter SGLT1. The non-metabolizable SGLT1 substrate, α-MG, also induced GLP-1 secretion from basal levels, albeit to a lesser extent than equimolar glucose, and the stimulatory effects of α-MG was abolished by 1 mM phlorizin. Blockade of the passive glucose transporter, GLUT2, with 1 mM phloretin, attenuated high glucose-stimulated GLP-1 secretion (Figure 3.1.4.A). Glucose-stimulated GLP-1 secretion was completely abolished when extracellular sodium was substituted with equimolar NMDG (Figure 3.1.4.B), which indicates Na^+ is pivotal for glucose-induced GLP-1 secretion. The K_{ATP} channel opener, diazoxide and the proton ionophore, 2,4-DNP both abolished glucose-induced GLP-1 secretion. The K_{ATP} channel antagonist tolbutamide did not stimulate GLP-1 release (Figure 3.1.4.C). The voltage-gated Na^+ channel blocker, lignocaine and L-type Ca^{2+} channel blocker, nifedipine, both attenuated glucose-induced GLP-1 secretion (Figure 3.1.4.D). The non-caloric sweetener, sucralose, stimulated GLP-1 release, indicating a role for STRs in GLP-1 secretion. The STR antagonist, lactisole, did not affect glucose-induced GLP-1 secretion, indicating glucose-induced GLP-1 secretion is independent of this pathway (Figure 3.1.4.E).

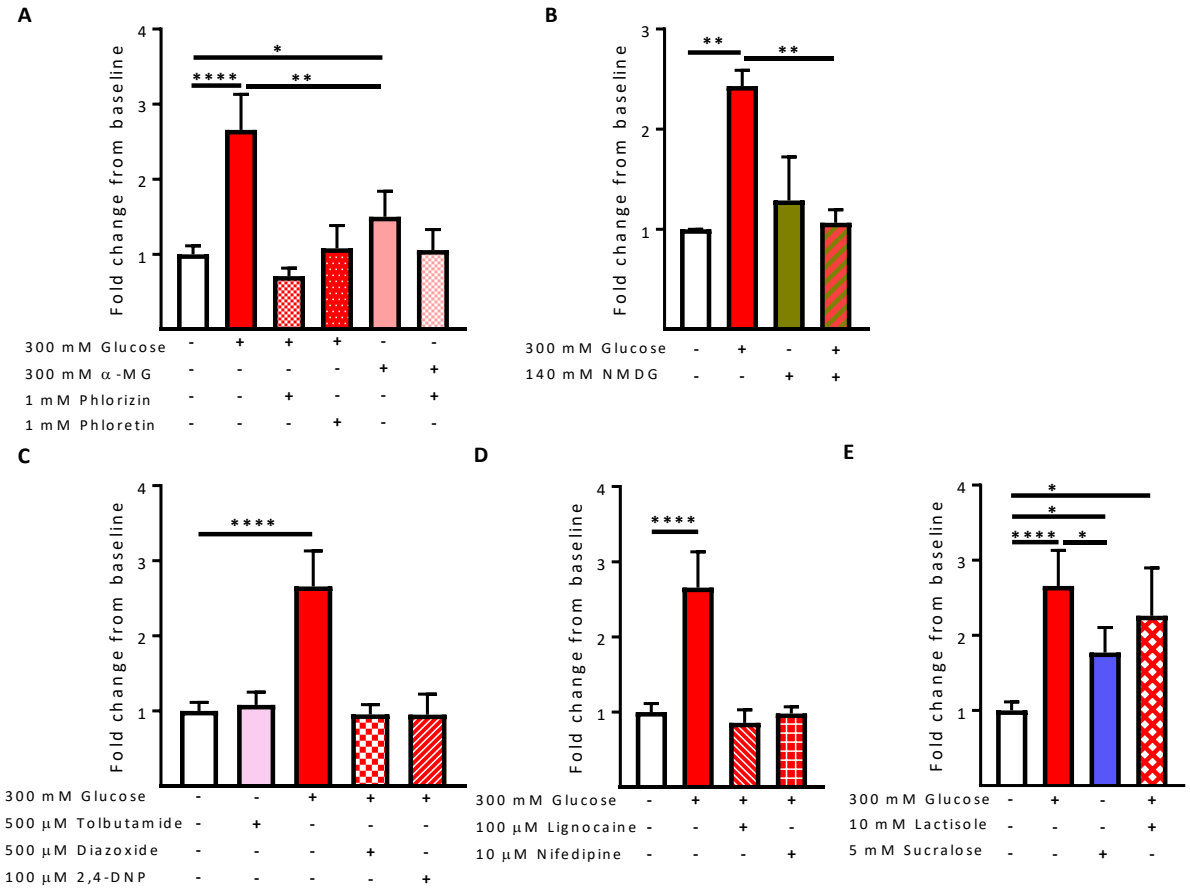


*Figure 3.1.4 (A) Phlorizin potently suppressed high glucose-induced GLP-1 secretion (n = 8). 300 mM of the SGLT1 substrate, α-MG significantly increased GLP-1 secretion from basal but to a lesser extent than 300 mM glucose and its stimulatory effect is sensitive to the blockade of SGLT1 by phlorizin (n = 8; *p < 0.05, **p < 0.01). The GLUT2 inhibitor, phloretin, abolished high glucose-induced GLP-1 secretion from human ileum; (B) Replacing extracellular Na+ with equimolar NMDG completely abolished high glucose-induced GLP-1 secretion (n = 5); (C) Glucose-induced GLP-1 secretion was attenuated by 500 µM Diazoxide and 100 µM 2,4-DNP (n = 8) while closure of KATP channels with 500 µM tolbutamide did not trigger significant GLP-1 release from basal levels (n = 8); (D) 100 µM lignocaine and 10 µM lignocaine both attenuated glucose-induced GLP-1 secretion (n = 8). (E) 5 mM sucralose alone significantly induced GLP-1 secretion but to a significantly lesser extent to 300 mM glucose (n = 8) while 10 mM of the sweet taste receptor blocker, lactisole, did not significantly attenuate high glucose-induced GLP-1 secretion from the human ileum (n = 8). *p < 0.05, **p < 0.01, ****p < 0.0001.*

3.1.4 Discussion and Conclusion

In this study, I established that glucose concentrations equivalent to postprandial intraluminal, but not plasma levels, were capable of triggering GLP-1 secretion from human ileum *ex vivo*, but not colon. Moreover, I identified the mechanisms underlying this glucose response in human ileal L cells and showed SGLT1 is central to this pathway.

The current *ex vivo* model demonstrates that exposure of human ileal mucosa to glucose triggers GLP-1 secretion, independent of neural inputs, gut contractions and osmotic stress. While I was

unable to acquire total GLP-1 content from the current samples to observe potential differences in type 2 diabetes patients, secretion was similar in both groups, enabling me to pool the *ex vivo* data from all patients. I showed that, similar to the glucose response in perfused rat small intestines; luminal, but not vascular, infusions of high glucose triggered substantial GLP-1 secretion[733]. This is in contrast to the results from recent reports showing colonic enterochromaffin cells are glucose-sensitive[115,127]. While the result in colon contrasts that from murine colonic mixed cell culture[660,692], it does support clinical findings that glucose-induced GLP-1 secretion is not affected by colon resection, and that rectally administered glucose did not trigger GLP-1 release[771].

This study defined pivotal roles of electrogenic and facilitative glucose transport via SGLT1 and GLUT2, respectively, in GLP-1 release from the human ileum. The significant GLP-1 release triggered by equimolar amounts of the non-metabolizable SGLT1 substrate, α-MG, reversal of α-MG-induced GLP-1 release by phlorizin and blockade of glucose response by substituting external Na^+ with NMDG, all support a central role of SGLT1 in driving this glucose response[733,772]. Thus, the present results confirm the critical role of electrogenic sodium-dependent glucose uptake by SGLT1 in causing membrane depolarization and subsequent GLP-1 release in human L cells, similar to that shown in rodent models[660,733,768,769,772]. GLUT2 blockade by phloretin also attenuated glucose-stimulated GLP-1 secretion. Although this is also observed in perfused rat small intestines[733,773], it may be a species-specific pathway; GLUT2 activity was not implicated in glucose-induced GLP-1 secretion in mouse models[660,769]. GLUT2 may be important in human L cells by facilitating glycolytic and/or mitochondrial metabolism for metabolism-dependent, K_{ATP} channel-independent, glucose-induced GLP-1 release can occur, as it is implicated in mediating K_{ATP} channel-independent GLP-1 secretion by other secretagogues including lipids and bile acids[773].

It has been proposed that glucose induces GLP-1 release through glucose internalization, ATP production via oxidative phosphorylation to close K_{ATP} channels, and subsequent membrane depolarization[767]. In this study, I demonstrate that the K_{ATP} channel opener, diazoxide, potently reduced glucose-induced GLP-1 secretion in humans, consistent with *in vitro*[692,709] and *ex vivo*[733] rodent data. Inhibiting intracellular ATP synthesis with 2,4-DNP abolished glucose-induced GLP-1 secretion in human ileum, consistent with results in rat small intestine[733]. Tolbutamide did not increase GLP-1 secretion in this present model, in contrast to that same study and other *in vitro* experiments[692], but is consistent with *in vivo* findings that sulfonylureas do not trigger GLP-1 secretion in humans[774,775]. Diazoxide increases K^+ permeability and subsequently clamps membrane potential below the K^+ equilibrium potential. This hyperpolarization must override any membrane depolarization induced by the inward Na^+ current associated with SGLT1 activity.

Na^+-dependent action potentials and voltage gated L-type Ca^{2+} currents are implicated in mediating basal and stimulated GLP-1 release in murine L cells *in vitro*[709]. My study supports the role of both channels in glucose-induced GLP-1 release from the human ileum. Blockade of voltage-gated Na^+ channels after intravenous lignocaine administration failed to attenuate glucose-induced GLP-1 secretion from rat small intestine[733]. Such differing results may highlight a shortcoming of the current approach as cell polarisation is lost in this model, rendering us unable to differentiate between apical and basolateral pathways. Future experiments using human vascularly perfused tissue, or using Ussing chambers, could mitigate these shortcomings.

Intestinal sweet taste receptors have recently emerged as an important regulator of gut hormone secretion. Reimann *et al*. showed that GLP-1 secretion from primary murine small intestine mixed cell cultures increased upon exposure to the artificial sweetener, sucralose[692]. Immunohistochemistry showed that GLP-1 co-localizes with the sweet taste receptor T1R3 and its coupling protein α-gustducin in human small intestine and the sweet taste receptor antagonist, lactisole, markedly reduced glucose-stimulated GLP-1 release in humans *in vivo*[770]. Thus, I investigated the effect of the artificial sweetener sucralose on GLP-1 secretion. In contrast to negative results in a clinical study[776] and that from rat a *ex vivo* perfusion model[733], I observed a modest but significant increase of GLP-1 secretion from human ileal mucosae upon exposure of the sweetener alone. However, further interrogation of this pathway was unsuccessful as inhibition of sweet taste receptor signalling did not significantly reduced glucose-stimulated GLP-1 secretion. The considerable variance in the lactisole-treatment group may have prevented us from detecting any inhibitory action of the drug on glucose-induced GLP-1 secretion. It is also possible that sucralose is a more potent activator of the STRs at this dose, or that a small portion, if any, of the glucose-stimulated GLP-1 secretion is mediated by this pathway[692,733,770,776].

Not all pathways shown to govern GLP-1 secretion in rodents were implicated in this study, highlighting the importance of species differences in studying L cell physiology. One of the notable differences was the inability for high glucose to invoke GLP-1 secretion in human colon mucosae in this study, contrasting findings in mice *in vitro*[692] and rat colon *ex vivo*[777]. One of the major limitations of the current study was the inability to differentiate between apical and basolateral effect of the reagents tested. Nonetheless, this study clearly demonstrated that glucose-induced GLP-1 secretion in human ileum is mediated by the electrogenic activity of SGLT1. It additionally involves a component reliant on intracellular glucose metabolism and is dependent on voltage-gated Na^+ and Ca^{2+} channels.

3.2 Metformin directly triggers GLP-1 and PYY secretion in human colon and ileum

3.2.1 Introduction

For more than half a century, the biguanide, Metformin, has been the first-line treatment of type 2 diabetes due to its low cost, proven efficacy and positive side effect profile. Despite wide use, its exact mechanisms of action are still a subject of ongoing investigation[778]. Metformin decreases hepatic gluconeogenesis and improves peripheral insulin sensitivity[779] through activation of 5' AMP-activated protein kinase (AMPK) in metabolically active organs including the liver[780], skeletal muscles[781] and adipose tissue[782]. The weight-neutral or in many cases, modest weight-reducing effect associated with metformin use was recognized soon after it was first used as an efficacious anti-diabetic agent[783] but the underlying mechanism was unclear. There is an increasing uptake of metformin in various subgroups of non-diabetic patients; metformin was recently shown to decrease maternal weight gain in obese pregnant women without type 2 diabetes[784] and is increasingly used in women with Polycystic Ovarian Syndrome (PCOS)[785], in which modest weight loss is commonly achieved[786]. Furthermore, there are ongoing investigations to explore metformin's place as an adjunct therapy to reduce weight gain secondary to atypical antipsychotic treatments[787-789].

A more recently appreciated mechanism of action of metformin is focused on the GI tract[778,790]. This view is supported by reports that showed the effect of metformin on hepatic glucose production is observed with oral, but not parental administration of the drug[778,790-792]. This is further supported by trials that both extended- and delayed-release formulations of metformin have more potent effects in type 2 diabetes patients on lowering fasting blood glucose levels than immediate release formulations of equivalent doses, without increasing the incidence of adverse effects[793,794]. Such data associates the prolonged exposure of the GI tract to metformin with improved efficacy of the drug. Interestingly, delayed-release formulation of metformin was shown to be superior in lowering fasting blood glucose level (BGL) over the extended-release formulation in a randomized controlled trial, further strengthening this association[795].

In rodents[662,796] , acute metformin treatment activates L cell secretion and increases plasma GLP-1 without affecting DPP4 activity. Additionally, clinical studies report the positive effect of chronic metformin treatment in type 2 diabetes patients on fasting and post-prandial GLP-1 and PYY levels[794,797]. An early clinical study showed that three days of metformin treatment in healthy normal weight females was sufficient to significantly increase fasting PYY level and that six months of metformin treatment in overweight women with PCOS elevated fasting PYY levels by 50 % in more

than half the participants[798]. Later studies confirmed chronic metformin treatment is associated with elevated fasting and postprandial levels of PYY[799,800]. However, evidence supporting a direct effect of metformin on human L cells is lacking. To ascertain whether a direct effect on L cell secretion exists, *ex vivo* mucosal tissue sections could be used so as to remove any potential confounding effects from factors such as bile acids, gut contraction, extrinsic and intrinsic nervous input or the microbiome. Sufficiently large amounts of human mucosal to undertake such secretion experiments are most readily acquired through surgical tissue specimens.

The aim of this study was to determine if metformin directly triggers the release of GLP-1 and PYY from human L cells in an *ex vivo* tissue model. We demonstrate that metformin causes acute release of GLP-1 and PYY in human gut mucosa via transport through PMAT and SERT and activation of AMPK. This is the first evidence supporting a direct action of metformin to trigger GLP-1 and PYY release from human L cells. The co-release of these two gut peptides may, in part, explain the anti-diabetic and weight loss effects of metformin.

3.2.2 Methods

3.2.2.1 Human tissue collection:

Patients gave consent for tissue donation from resected small and large intestine at Flinders Medical Centre and Flinders Private Hospital approved by the Southern Adelaide Clinical Human Research Ethics Committee. Ileum and colon tissue specimens were collected from patients undergoing bowel resection for cancer or stoma reversal. In the case of resection specimens, samples were obtained from sites at least 10 centimetres away from the tumour location. Specimens from patients that were indicated for any form of inflammatory bowel disease were excluded from this study. Characteristics of the patient cohort are listed in Table 3.2. The specimens were immediately placed in iced-cold Krebs buffer (in mM, NaCl 138, KCl 4.5, $CaCl_2$ 2.6, $NaHCO_3$ 4.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 10, Glucose 5) and transported to the laboratory for dissection within 15 minutes. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently dissected off from the submucosae as intact sheets using a stainless steel spatula, cut into 5 mm pieces and weighed individually. The mucosal pieces were then transferred to a 96-well plate for secretion assays.

	Colon specimen	Ileum specimen
N	46	10
Age (years)	67 ± 13 (38 - 87)	70 ± 10 (52 - 83)
Sex (male/female)	25/ 22	3/ 7
BMI (kg/m^2)	29.3 ± 6.7 (19 - 55)	31.6 ± 6.3 (25 - 45)
History of type 2 diabetes (yes/no)	11/ 35	3/ 7
Metformin-treated (yes/no)	6/ 40	2/ 8

Table 3.2 Characteristics of the specimen donors (data is average ± SEM and range in parentheses)

3.2.2.2 Secretion experiments:

Mucosal pieces were incubated with 250µL of buffer (control) or buffer containing test agents in a 96-well plate for 15 minutes. The buffer was a modified Krebs buffer described previously with the addition of sitagliptin 1 µM, 0.1% BSA (A1595, Sigma Aldrich) at pH 7.4. Following incubation at 37°C in 95%O_2/5%CO_2, supernatants were collected and stored in aliquots at -20 ^oC. Active GLP-1 and total PYY levels were quantitated using commercially available ELISA kits, according to manufacturer's instructions (EGLP-35K & EZHPYYT66K, Merck Millipore).

3.2.2.3 Test agents:

3-isobutyl-1-methylxanthine (IBMX) and forskolin (I5879 & F6886, Sigma Aldrich) (10 µM each) and 70 mM KCl were used as positive controls. For the 70 mM KCl solution, an equimolar amount of NaCl was removed to maintain osmolarity. The following compounds were purchased from Sigma Aldrich: Metformin (PHR1084), Lopinavir (SML1222), Quinine hydrochloride dehydrate (Q1125), Fluoxetine (F132). The AMPK inhibitor, dorsomorphin was from Merck Millipore (171260). Only samples that show positive response to at least one positive control (70 mM KCl or 10 µM IBMX/FSK) were included in analysis.

3.2.2.4 Statistical analysis:

All statistical analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual. A paired Student's t-test was used for single comparisons and a paired one-way ANOVA with Fishers Least Significant Difference post-hoc test used for multiple comparisons. Statistical significance was $p < 0.05$. All data are shown as mean ± SEM.

3.2.3 Results

3.2.3.1 Metformin triggers GLP-1 and PYY secretion

Exposure to high external K^+ or to a combination of known activators of L cell secretion forskolin and IBMX increased intact GLP-1 release from colonic epithelial tissue by 1.79 fold and 3.01 fold,

respectively (n= 22, $p < 0.001$, Figure 3.2.1.A). Acute exposure to metformin (10 µM) also triggered an increase in intact GLP-1 levels in supernatant by 3.95 fold (n = 46, $p < 0.01$, Figure 3.2.1.B). A similar increase in GLP-1 release in response to metformin in ileal tissue by 4.1 fold was observed (n = 10, $p < 0.05$, Figure 3.2.1.C). PYY was also reliably released in the presence of known L cell secretogogues; high external K^+ and FSK/IBMX increased PYY release by 2.13 and 1.82 fold respectively (n = 22, $p < 0.001$, Figure 3.2.1.D). Acute metformin exposure triggered PYY release in colonic (n = 46, $p < 0.001$, Figure 3.2.1.E) and ileal (n = 10, $p < 0.05$, Figure 3.2.1.F) tissue by 1.67 and 2.01 fold respectively. Thus, metformin increases GLP-1 and PYY release within 15 minutes from human colonic and ileal L cells.

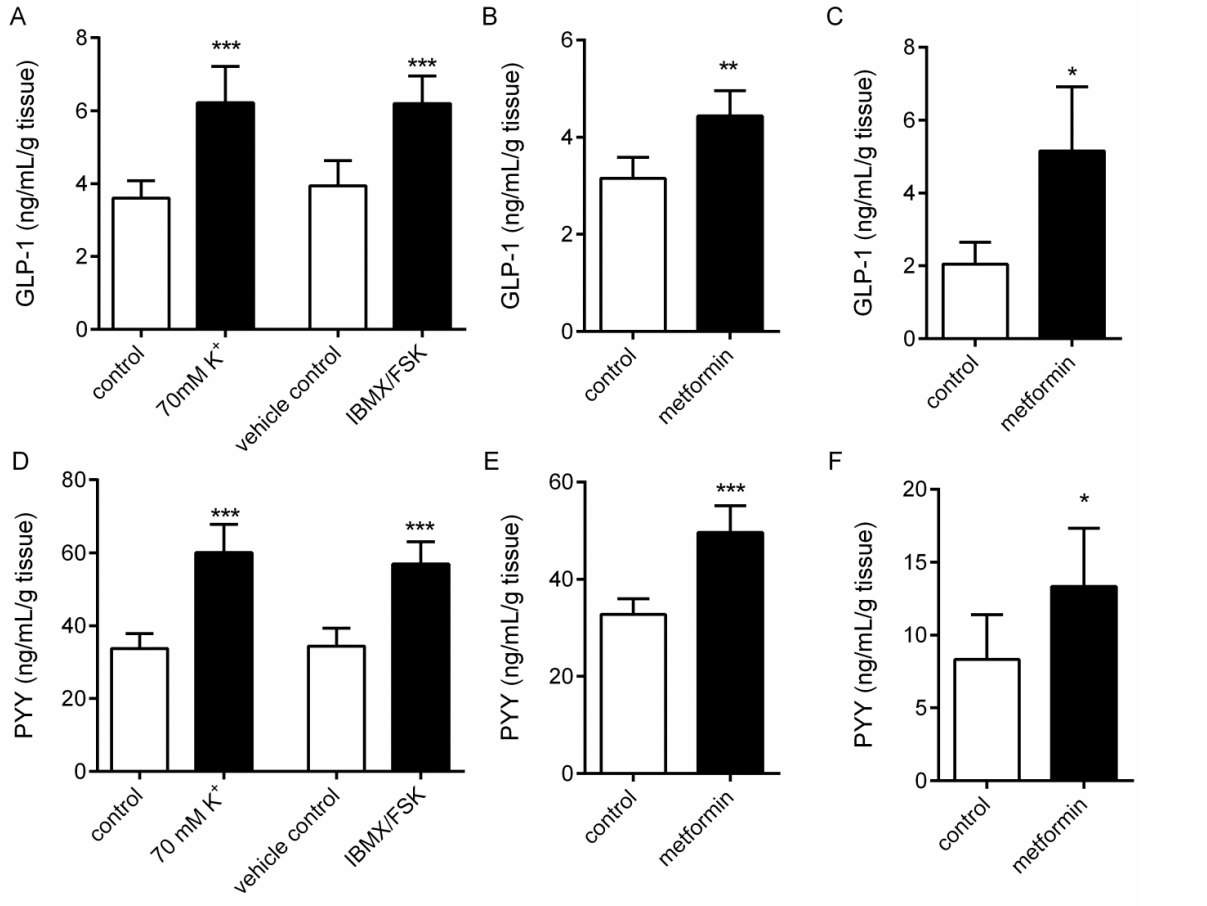


*Figure 3.2.1 Metformin-induced secretion in human L cells. (A) Colonic epithelial preparations readily secrete GLP-1 in response to high (70 mM) external K+ or to a combination of IBMX and forskolin (n = 22). Metformin (10 µM) increases GLP-1 release after 15 minutes in epithelial tissue from human (B) colon (n = 46) and (C) ileum (n = 10). D-F are the same as A-C but represent PYY release. Bar graph data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 compared to respective control groups.*

3.2.3.2 Metformin-induced L cell secretion is unrelated to BMI or diabetes status

To identify whether the effect of metformin on L cell secretion was altered in human obesity or type 2 diabetes, I examined responses to metformin in our colonic preparation across BMI and in samples obtained from patients with type 2 diabetes. Neither the basal release of GLP-1 (Figure 3.2.2.A), nor

the degree of metformin-stimulated GLP-1 released (Figure 3.2.2.B), correlated with BMI (n = 46). No difference was seen in either basal (Figure 3.2.2.C) or stimulated GLP-1 release (Figure 3.2.2.D) between tissue obtained from non-diabetes (n = 35) or type 2 diabetes individuals (n = 11). The same result was observed when similarly stratifying this data for PYY release (Figure 3.2.2.E-H). Thus, basal L cell secretion, and the response of L cells to metformin, do not change across BMI and are unrelated to diabetic status.

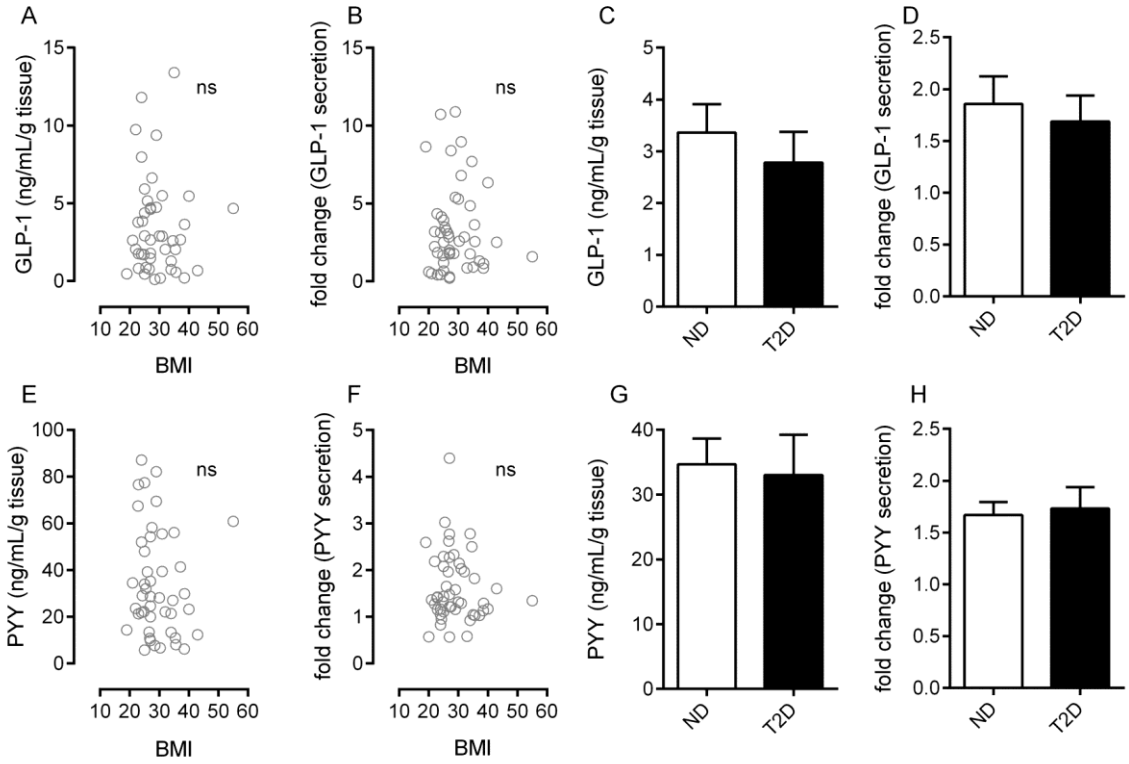


Figure 3.2.2 Metformin-induced colonic L cell secretion does not change in obesity or diabetes. (A) No correlation exists between BMI and (A) basal and (B) stimulated GLP-1 release (n = 46). (C) Basal and (D) stimulated GLP-1 release are similar in samples from non-diabetes (ND, n = 35) and type 2 diabetes (T2D, n = 11) patient samples. E-H are the same as A-D but represent PYY release. Bar graph data are mean ± SEM, ns indicates no significant correlation.

3.2.3.3 Metformin is transported into L cells and activates AMPK

I investigated the mechanism by which metformin triggers L cell secretion. AMP kinase has been associated with metformin action[780], and inhibiting AMPK activity using 10 µM dorsomorphin blocked metformin-induced PYY and GLP-1 secretion (Figure 3.2.3.A and C). I then used a series of membrane transporter antagonists to identify the mechanism of metformin internalisation in human colonic L cells. Quinine (Organic Cation Transporter 1 (OCT1) inhibitor) had no effect on metformin-induced PYY release, while lopinavir (Plasma membrane monoamine transporter (PMAT) inhibitor) and fluoxetine (serotonin transporter (SERT) inhibitor) both blocked metformin-induced PYY release (Figure 3.2.3.B). Metformin-induced GLP-1 release is blocked by fluoxetine but not quinine or lopinavir (Figure 3.2.3.D).

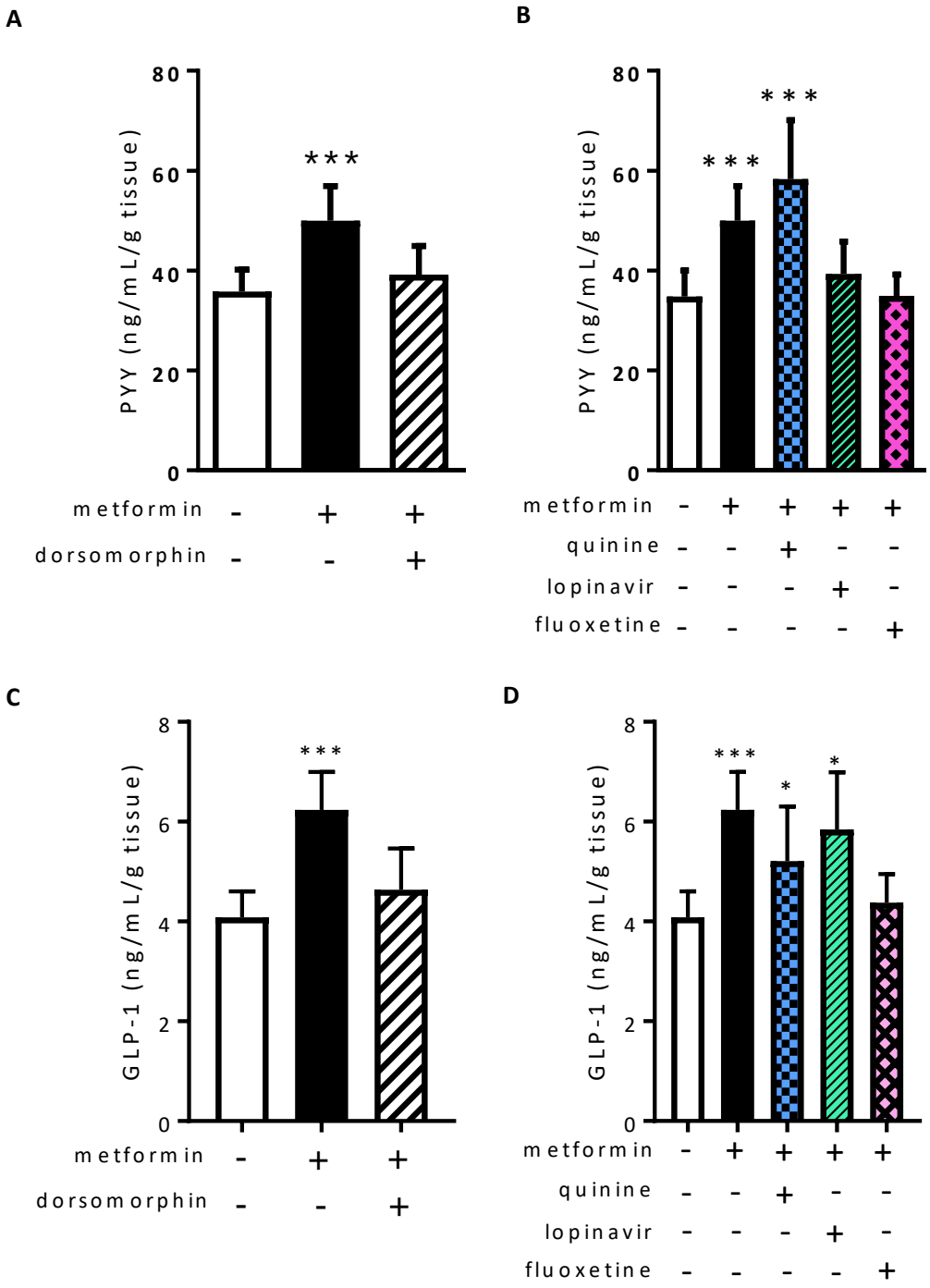


*Figure 3.2.3 Mechanisms controlling metformin-induced secretion in human colonic L cells. The AMPK inhibitor dorsomorphin blocks metformin-induced PYY (A, n = 20) and GLP-1 (C, n = 18) release. (B) Metformin-induced PYY release is blocked by the PMAT inhibitor lopinavir and the SERT inhibitor fluoxetine (n = 18). (D) Metformin-induced GLP-1 release is blocked by the serotonin transporter (SERT) inhibitor fluoxetine (n = 18). Bar graph data are mean ± SEM, **p < 0.01, ***p < 0.001 compared to respective control conditions, ns indicates no significant effect compared to controls.*

3.2.4 Discussion and Conclusion

This is the first report of metformin directly triggering GLP-1 and PYY release from L cells within human intestinal epithelium, and the mechanisms by which it occurs. Others have previously shown that chronic oral metformin treatment increases fasting and post-prandial plasma GLP-1 and PYY

levels in humans[794,797-799,801] and that acute oral administration of the drug in mice increases plasma GLP-1 in the absence of glucose administration[802]. However, these previous studies did not exclude the potential effects of metformin administration on the autonomic and enteric nervous systems or blood borne factors that influence GLP-1 and PYY actions, such as DPP4. Nonetheless, one of the major limitations to my experimental setup is the inability to exclude the possibility that metformin could act on absorptive enterocytes or other enteroendocrine cells to trigger L cell secretion in a paracrine fashion.

The oral bioavailability of metformin is approximately 50 % with an estimated 30 % of an ingested dose recoverable in faeces[803]. This incomplete absorption means the lower intestine, the body's richest source of GLP-1 and PYY, is exposed to a considerable portion of an oral metformin dose. Considering the importance of GLP-1 and PYY in maintaining energy homeostasis[69,804], results from this study could serve to explain why delayed release formulations of metformin have a more potent glucose-lowering effect than immediate and extended release formulations of equivalent doses, albeit achieving lower plasma concentrations of metformin when compared with these formulations[794,795]. Since higher plasma metformin levels are associated with increased incidence of lactic acidosis, a rare but severe adverse drug reaction associated with metformin use[805], the use of delayed release formulations can therefore minimize systemic exposure to metformin but maintain a comparable, if not superior, anti-diabetic effect. In addition, there were no differences in metformin-induced GLP-1 or PYY release between control and type 2 diabetes patients and no relationship was evident between release and BMI, indicating the potency of metformin to trigger GLP-1 and PYY release is preserved in obese patients and in patients with type 2 diabetes. Given the incretin effect elicited by GLP-1 is preserved in type 2 diabetes patients[806], results from this study provide additional mechanistic explanation for the efficacy of metformin in lowering blood glucose levels in type 2 diabetes patients.

It is widely accepted that metformin improves peripheral insulin sensitivity and activates AMPK in the liver[780], skeletal muscles[781] and adipose tissue[782], all of which contribute to its hypoglycaemic effect. Therefore, I hypothesized that a gut-based anti-diabetic mechanism would also require AMPK activation. This hypothesis was supported in this current study, given that inhibition of AMPK by dorsomorphin significantly attenuated the metformin response. This is consistent with acute AMPK activation being sufficient to cause GLP-1 release *in vivo* in rats[796]. Based on the current knowledge of the membrane transporters involved in intestinal uptake of metformin[807] and that genetic variations in OCT1[808,809] and SERT[810] are associated with variations in therapeutic response and GI adverse reactions to the drug, I also hypothesized that metformin's stimulatory effect depends on active transport of the drug into the cell. In line with this hypothesis, I observed the attenuation of

the stimulatory effect of metformin on colonic GLP-1 and PYY secretion by pre-treatment with PMAT and SERT inhibitors lopinavir and fluoxetine, respectively. However, the possibility of an indirect stimulatory effect of metformin on L cells via paracrine actions should not be discounted, especially considering the lack of direct stimulatory effect of metformin on several *in vitro* L cell models including NCI-H716 cells[796]. It is possible that metformin causes serotonin release from local enterochromaffin cells[109,119] and transportation of 5-HT into L cells activating release should not be discounted, although this seems less likely however given the major signaling pathways of serotonin involve binding to and activation of plasma membrane serotonin receptors.[796] Despite the link between OCT1 polymorphisms and metformin response[808,809], there were no changes to metformin response in the presence of the OCT1 inhibitor, quinine, suggesting OCT1 polymorphisms may affect metformin response through alternate mechanisms.

Despite the increasing appreciation for the role of the gut plays in mediating the anti-diabetic effects of metformin, it is important to acknowledge that the biguanide exerts an array of effects on different metabolically important organs. Such effects help to explain its long-held place as first-line therapy in treating type 2 diabetes. It is accepted that metformin potently suppresses hepatic glucose output[792], possibly through a hepatic AMPK-dependent pathway[780], by activating duodenal AMPK[811], or by suppressing hepatic glucagon signalling[812]. Interestingly, the current findings may also serve to explain metformin's suppressing effect on hepatic glucose output since GLP-1 is a known potent inhibitor of hepatic gluconeogenesis[69]. Although metformin's actions on other metabolically active organs could contribute to its weight loss effects, it is likely that weight-reducing effect of metformin is at least partly mediated by increased release of PYY, which then acts centrally on the hypothalamic feeding circuit to induce satiety and reduce food intake. Although this hypothesis has not yet been directly tested, Kim *et al.* showed that oral metformin administration in mice caused significant increase in c-Fos immunoreactivity within the brainstem NTS neurons of obese[813], which is an important target for peripherally administered PYY_{3-36} to reduce food intake in mice[814]. It would be informative to investigate if metformin-induced weight loss and reduced food intake are attenuated in *Pyy* knockout mice. It is worth noting that metformin has been shown to attenuate the release of the orexigenic gut hormone, ghrelin from primary rat gastric cell culture in an AMPK-dependent fashion[815]. Thus, metformin may well have opposite effect on the secretion on the anorectic PYY and orexigenic ghrelin to synergistically reduce food intake. Increased food intake is believed to be one of the major reasons why many patients gain significant amount of bodyweight as a result of atypical antipsychotic treatments[816,817], metformin's direct stimulatory effect of PYY secretion can therefore explain why it is an efficacious option in limiting atypical antipsychotic-induced weight gain[787-789].

In summary, I have provided direct evidence that metformin elicits GLP-1 and PYY release in human colon and ileum mucosae, independent of the autonomic and enteric nervous systems and blood-borne factors such as DPP4. Although metformin has been shown to positively affect the gut microbiota composition[818] and bile acid metabolism[799], both of which could contribute to increased GLP-1 and PYY release[186], my results suggest the drug's acute action on gut hormone secretion can occur independently of these factors. The increased release of these two gut peptides is of significant metabolic benefit as both hormones are implicated in the success of type 2 diabetes remission and marked weight loss achieved by bariatric surgeries[314,390,804,819]. This is in-line with the growing acceptance of the view that metformin's primary site of action is within the GI tract.

3.3 The Melanocortin-4 Receptor regulates the secretion of PYY and GLP-1 from the human gut epithelia

3.3.1 Introduction

The central melanocortin system is one of the most important regulators of energy homeostasis in mammals. The melanocortin-4 receptor (MC4R) is extensively expressed throughout the brain[408] and plays a pivotal role in regulating feeding drive and energy expenditure[409]. Central MC4R function largely depends on the interplay between the two neuronal populations in the arcuate nucleus (ARC) of the hypothalamus, the anorectic proopiomelanocortin (POMC) and orexigenic Neuropeptide Y/ Agouti-related peptide (NPY/AgRP) neurons, both of which project widely throughout the brain. Upon stimulation by satiety signals such as insulin and leptin, POMC neurons release alpha-melanocyte-stimulating hormone (α-MSH), the endogenous ligand of MC4R, to activate MC4R-positive neurons in different brain regions. NPY/AgRP neurons, on the other hand, are stimulated by orexigenic signals such as ghrelin and release the endogenous MC4R antagonist, AgRP to antagonize the anorectic actions of POMC neurons on MC4R-positive neurons. NPY/AgRP neurons also secrete NPY, which acts primarily on the Y1 receptor (Y1R) in various brain regions to drive feeding[626]. In addition, there is a unidirectional control at the level of the ARC; NPY/AgRP neurons directly exert inhibitory effects on neighbouring POMC neurons via GABA and NPY release but POMC neurons do not directly affect the firing of NPY/AgRP neurons. Considering the importance of appetite control in energy homeostasis, it is not surprising that deficiencies in POMC or MC4R result in overt obesity that is characterized by severe hyperphagia and hyperinsulinaemia. Indeed, mutation in the *MC4R* gene is the most common, known monogenetic cause of human obesity[820,821].

The obesogenic effect of *MC4R*-deficiency appears to be primarily driven by increased feeding; male *Mc4r*$^{-/-}$ mice pair-fed to wild type controls showed close to normal body weight[822]. Of all the MC4R-containing brain regions, the paraventricular nucleus (PVN) of the hypothalamus appears to be the most critical in mediating the effect of MC4R on feeding behaviours. Selective reactivation of *Mc4r* in PVN neurons in otherwise *Mc4r*-deficient mice completely rescued the hyperphagic phenotype and reduced the bodyweight of these animals by 60 %[823,824]. On the other hand, the central melanocortin system also regulates energy expenditure, partly through acting on the sympathetic nervous system[825]. MC4R expressed by sympathetic preganglionic neurons are partly responsible for diet- and cold-induced thermogenesis in brown adipose tissue (BAT) and beiging of white adipose tissue (WAT)[826]. However, impaired MC4R signalling in both the PVN and sympathetic nervous system does not fully account for the obese phenotype in whole body *Mc4r*$^{-/-}$ mice, which indicates MC4Rs expressed elsewhere could also contribute to maintaining energy homeostasis. Moreover,

peripherally-administered α-MSH and other MC4R peptide agonists are effective in reducing bodyweight[823,827-831], despite the fact that these peptides do not readily cross the blood brain barrier[832,833], further supporting this notion.

The role of the gut in regulating energy homeostasis is exemplified by the remarkable success rate of bariatric surgeries in achieving clinically significant weight loss in obese patients; anatomical manipulations of the GI tract confer metabolic benefits that are not simply due to nutrient malabsorption. Despite the fall in circulating levels of the anorectic hormone, leptin, secondary to reduced fat mass, post-gastric bypass patients often report the restoration of a sense of satiety after surgery[834]. It is well-established that postprandial levels of various satiety-inducing gut hormones increase dramatically in post-bariatric patients. In particular, postprandial levels of GLP-1 and PYY increase dramatically to almost pharmacological levels in post-gastric bypass patients and are both implicated in inducing weight loss by potently suppressing appetite. Interestingly, *in vivo* MC4R activation significantly induced GLP-1 and PYY secretion in mice[762] and humans[835]. In addition, a recent rodent study demonstrated the anorectic effect of chronic dual activation of GLP-1R/MC4R was similar to GLP-1R activation alone[830], indicative of potential overlap of the two pathways. Murine gene expression analysis showed that *Mc4r* is expressed in L cells[762], vagal efferents innervating the myenteric plexus[836] and enteric glial cells[837]. It remains unclear if MC4R activation directly triggers L cell secretion from the intestinal epithelium, independent of any neural inputs. Moreover, although the human gut has been demonstrated to express MC4R[838], it is unknown if the receptor is also enriched in L cells, and if so, how does it contribute to regulating the secretory function of human L cells.

The aim of this study is to determine if MC4R activation directly triggers GLP-1 and PYY secretion from the human gut epithelium using the *ex vivo* static incubation model prepared from endoscopic biopsies and surgically resected human gut tissue, as described in previous sections. Based on *in vivo* results from mice and humans, I hypothesized that MC4R activation would significantly trigger GLP-1 and PYY secretion from the human intestinal epithelium.

3.3.2 Methods

3.3.2.1 Human tissue collection:

Morphologically normal ileal and colonic specimens were collected from consented patients (Table 3.3) undergoing bowel resections for cancer or stoma reversal at Flinders Medical Centre and Flinders Private Hospital. In the case of resection specimens, samples were obtained from sites at least 10 centimetres proximal to the tumour location. Specimens from patients with clinical or macroscopic evidence of inflammatory bowel disease were excluded from this study. The specimens

were immediately placed in iced-cold Krebs buffer and transported to the laboratory for dissection within 15 minutes as previously described. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently dissected off from the submucosae as intact sheets using a stainless steel spatula, cut into 5 mm pieces and weighed individually.

	Ileum Specimens	**Colon Specimens**
N	14	24
Gender (male/female)	4/10	13/11
Age (years)	71.3 ± 2.7 (52 – 83)	68.7 ± 2.8 (38 – 87)
BMI (kg/m^2)	30.5 ± 1.6 (23 – 45)	29.4 ± 1.6 (19 – 55)
Type 2 diabetes (Yes/No)	2/12	7/17

Table 3.3 Specimen donor characteristics. Data presented as mean ± SEM.

3.3.2.2 ex vivo secretion experiments:

The biopsies and tissue pieces were incubated with 250 µL of pre-warmed Krebs buffer (control) or with test reagents in a 96-well plate for 15 minutes at 37°C in 95 O_2/CO_2. Following incubation, the supernatants were collected and stored in aliquots at -20 °C. Active GLP-1 and total PYY levels were quantitated using separate commercially available ELISA kits (EGLP-35K and EZHPYYT66K, respectively, both from Merck Millipore). For ileal secretion experiments, 300 mM glucose in Krebs solution was used as positive control. For colonic secretion experiments, a combination of IBMX and forskolin (I5879 & F6886, Sigma Aldrich, 10 µM each) were used as positive control. These conditions were chosen as they were shown to reliably trigger GLP-1 secretion from human *ex vivo* mucosal preparations[252].

3.3.2.3 Materials:

α-MSH (M4135, Sigma Aldrich), PF06732395, setmelanotide and LY2112688 (all from Pfizer Inc.) were dissolved in DMSO at 20 mM and stored at -20 °C. [Nle4, D-Phe7]-α-MSH (NDP-α-MSH)(043-06, Phoenix Pharmaceuticals Inc.) and AgRP (83-132) (003-53, Phoenix Pharmaceuticals Inc.) were dissolved directly in Krebs buffer on the days of experiments.

3.3.2.4 Statistical analysis:

All statistical analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual to relevant control conditions. A paired ratio Student's t-test was used for single comparisons. As secretion experiments did not always include a full sequence of all conditions used in this study, a paired one-way ANOVA with Fishers Least Significant Difference post-hoc test

was used for multiple comparisons. Statistical significance was $p < 0.05$. All data are shown as mean ± SEM.

3.3.3 Results

3.3.3.1 MC4R agonsim in human ex vivo gut epithelia significantly increased GLP-1 and PYY secretion:

The endogenous MC4R ligand, α-MSH caused significant increase in GLP-1 secretion from ileal and colonic preparations at concentrations tested (Figure 3.3.1).

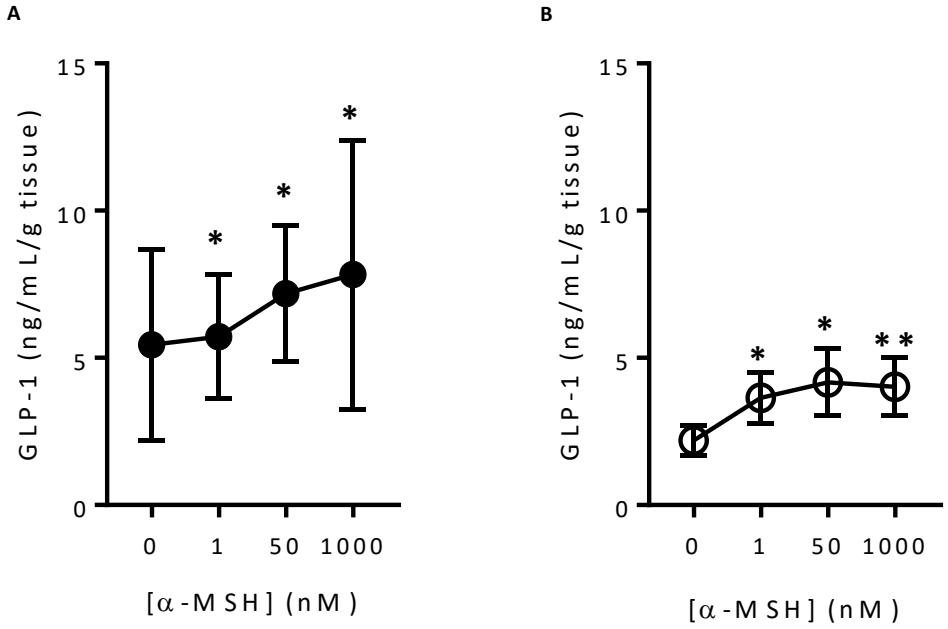


*Figure 3.3.1 α-MSH significantly triggered GLP-1 release from human ileal (A, n = 13 – 14, *p < 0.05, **p < 0.01) and colonic (B, n = 14, *p < 0.05, **p < 0.01) mucosae.*

α-MSH also significantly increased PYY secretion from colonic preparations by more than 2-fold at all concentrations tested (Figure 3.3.2.B) but none of the concentrations tested caused significant increase in PYY secretion from ileal preparations (Figure 3.3.2.A).

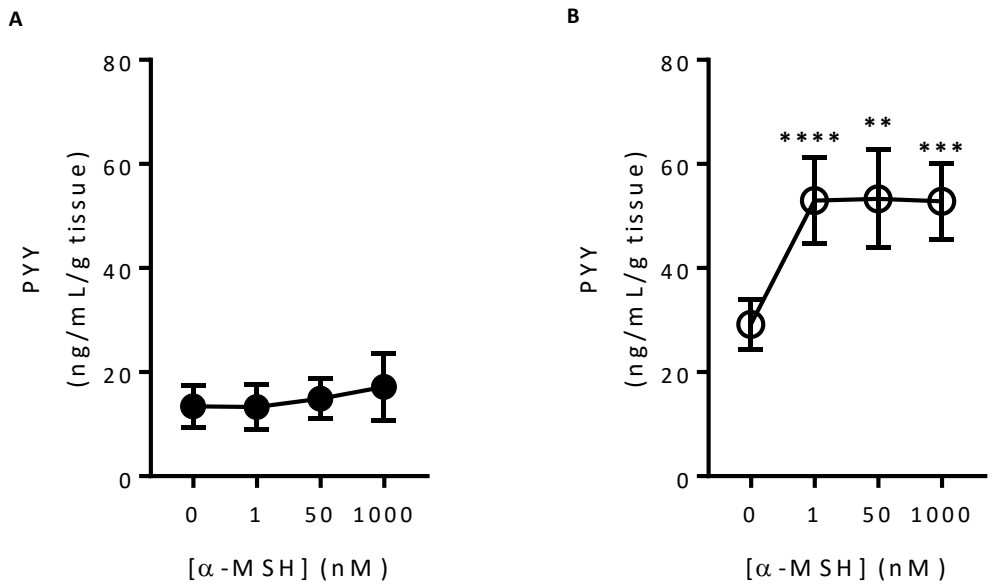


*Figure 3.3.2 α-MSH did not significantly triggered PYY release from human ileum (A, n = 12 - 14) but significantly increased PYY secretion from colon (B, n = 20, **p < 0.05, ***p < 0.01, ****p < 0.001).*

A more potent and stable analogue of α-MSH, NDP-α-MSH[839], also stimulated GLP-1 secretion from ileal and colonic preparations (Figure 3.3.3).

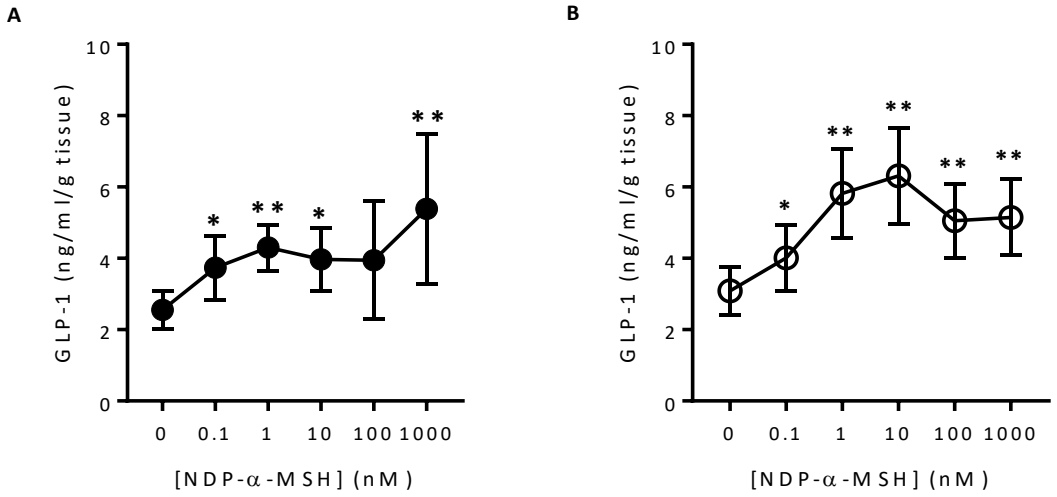


*Figure 3.3.3 NDP-α-MSH stimulated GLP-1 release from human ileum (A, n = 10 - 14, *p < 0.05, **p < 0.01) and colon (B, n = 20, *p < 0.05, **p < 0.01).*

NDP-α-MSH caused significant increase in PYY secretions from ileal preparations at 1 µM but not at lower concentrations (Figure 3.3.4.A). NDP-α-MSH caused significant increase in PYY secretion from colonic preparations at all concentrations tested (Figure 3.3.4.B).

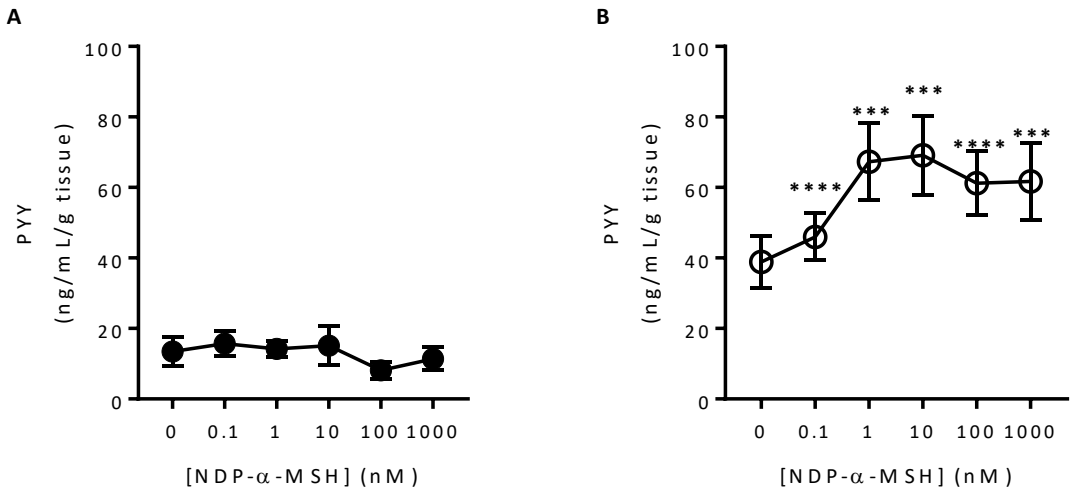


*Figure 3.3.4 NDP-α-MSH significantly triggered PYY release only at 1 µM in ileum (A, n = 9 – 14, *p < 0.05). In the colon, NDP-α-MSH significantly triggered PYY release at all concentrations tested (B, n = 21 - 14, ***p < 0.001, ****p< 0.0001).*

There were no significant differences in the magnitude of GLP-1 and PYY secretion in response to 10 nM NDP-α-MSH between type 2 diabetic patients and non-diabetic patients. The magnitude of stimulation of either hormone was not correlated to the BMI of specimen donors (Figure 3.3.5).

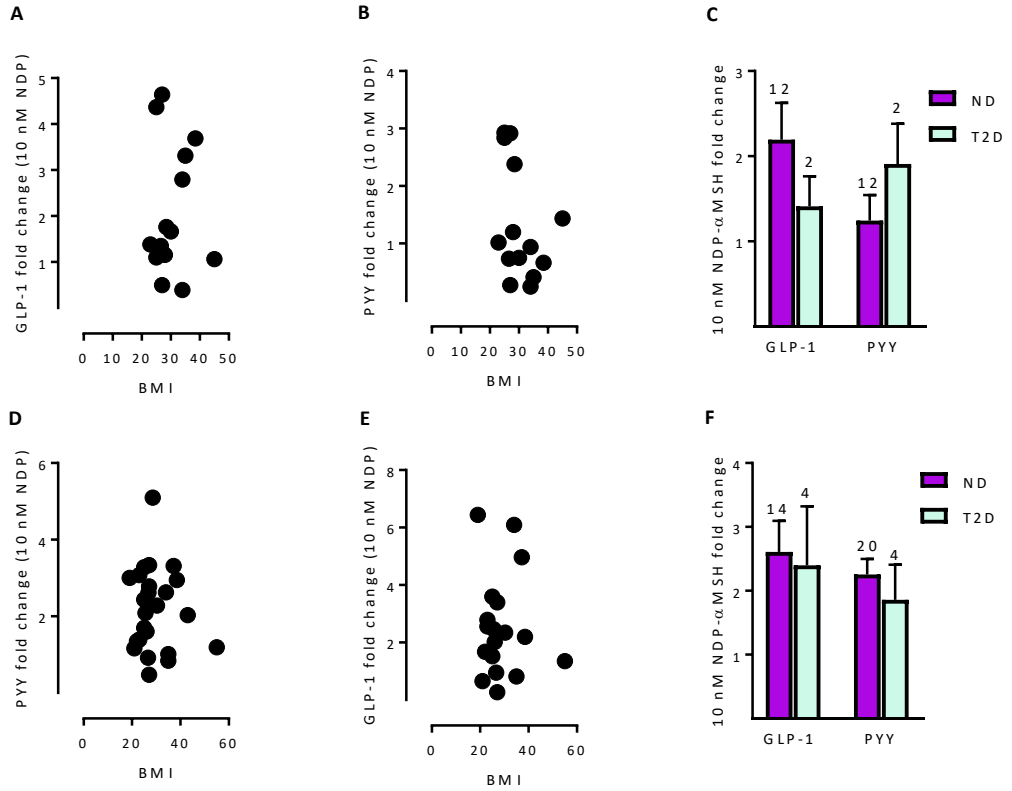


Figure 3.3.5 BMI and T2D status of specimen donor did not affect the magnitude of GLP-1 and PYY secretion in response to 10 nM NDP-α-MSH (A - C: ileum; D - F: colon. Number above bars indicate n for each group).

Small molecule and peptide agonists of MC4R also caused significant GLP-1 (Figure 3.3.6) and PYY secretions from ileal and colonic preparations (Figure 3.3.7).

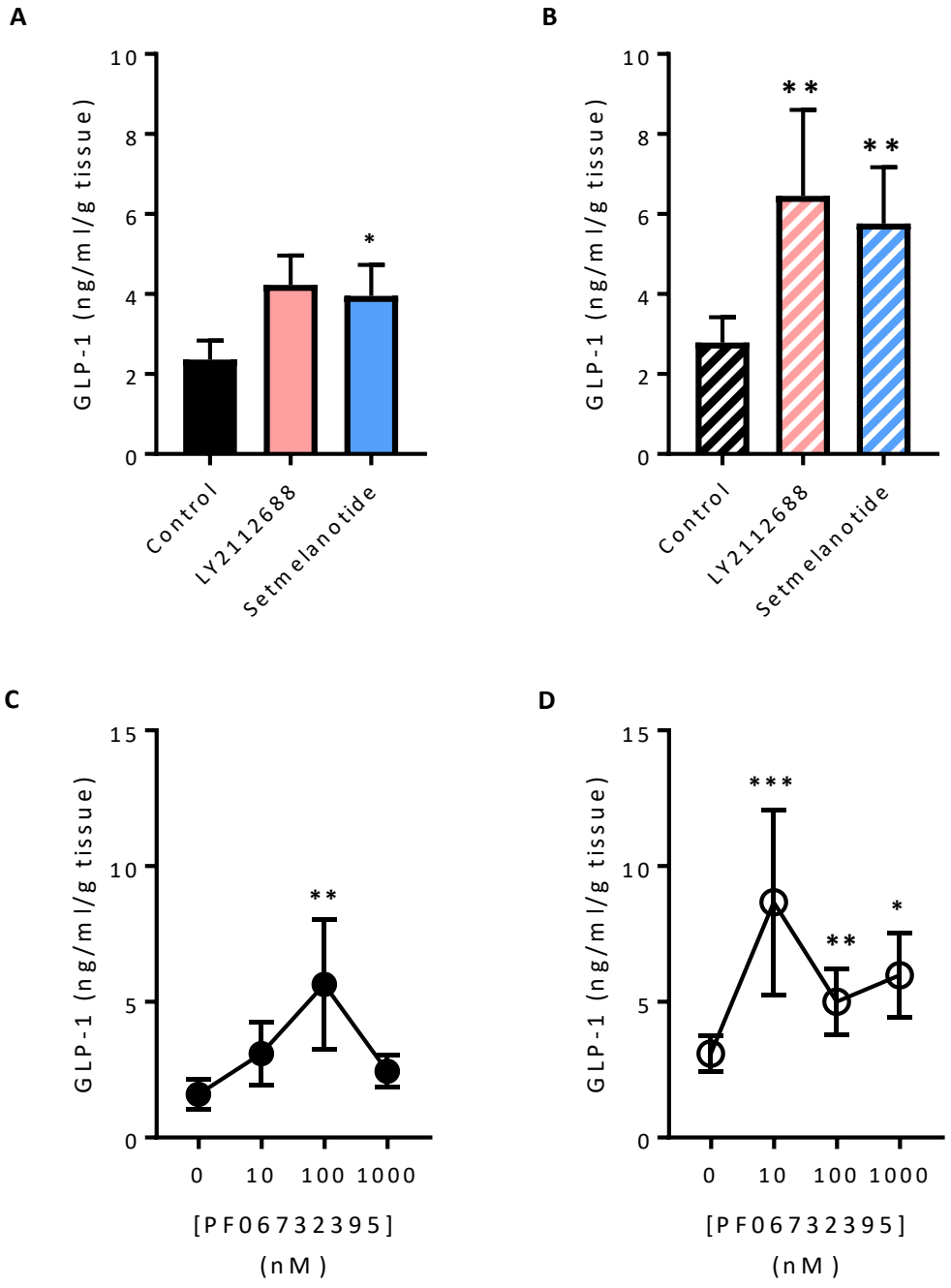


*Figure 3.3.6 (A) The peptide MC4R agonist setmelanotide (1 nM) significantly triggered GLP-1 release from human ileum but not LY2112688 (1 nM) (n = 15). (B) Both LY2112688 and setmelanotide significantly triggered GLP-1 secretion from human colon at 1 nM (n = 17). (C) The small molecule MC4R agonist PF06732395 triggered GLP-1 release from human ileum at 100 nM and 1 µM (n = 8 – 14) and (D) from human colon at all concentrations tested (n = 15-18). *p < 0.05, **p < 0.01, ***p < 0.001*

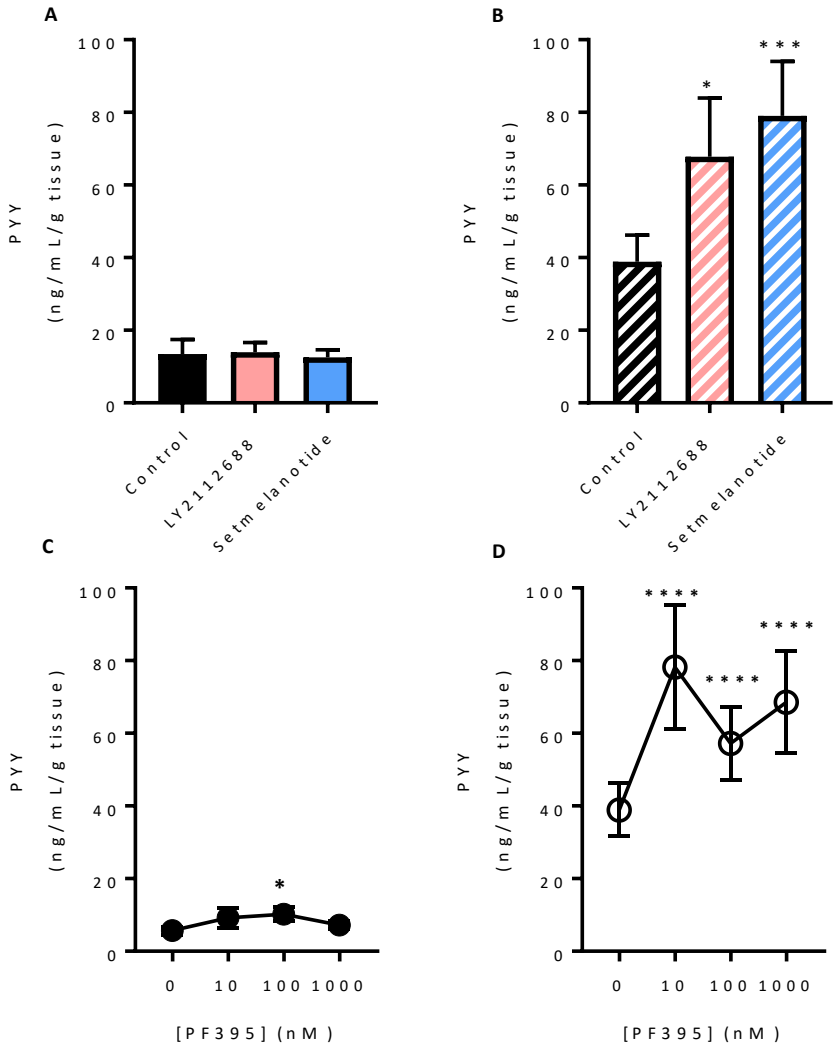


*Figure 3.3.7 (A) The small peptide MC4R agonists LY2112688 and setmelanotide did not significantly triggered PYY release from human ileum (n = 13-14). (B) Both LY2112688 and setmelanotide significantly triggered PYY secretion from human colon (n = 24). (C) The small molecule MC4R agonist PF395 triggered PYY release from human ileum at 100 nM (n = 8 – 14) and (D) from human colon at all concentrations tested (n = 21-24). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*

3.3.3.2 MC4R antagonism by AgRP attenuated NDP-α-MSH induced PYY secretion from human colonic epithelium:

Since POMC neurons in the ARC exert a tonic inhibitory effect on PVN neurons to suppress feeding drive[409], we sought to investigate if L cell secretion was also under tonic control from MC4R. Surprisingly, addition of the endogenous MC4R antagonist, AgRP, significantly stimulated PYY secretion at 1 and 10 nM (Figure 3.3.8 A). 10 nM AgRP significantly attenuated NDP-α-MSH, but not α-MSH-stimulated PYY secretion from colonic preparations (Figure 3.3.8 B).

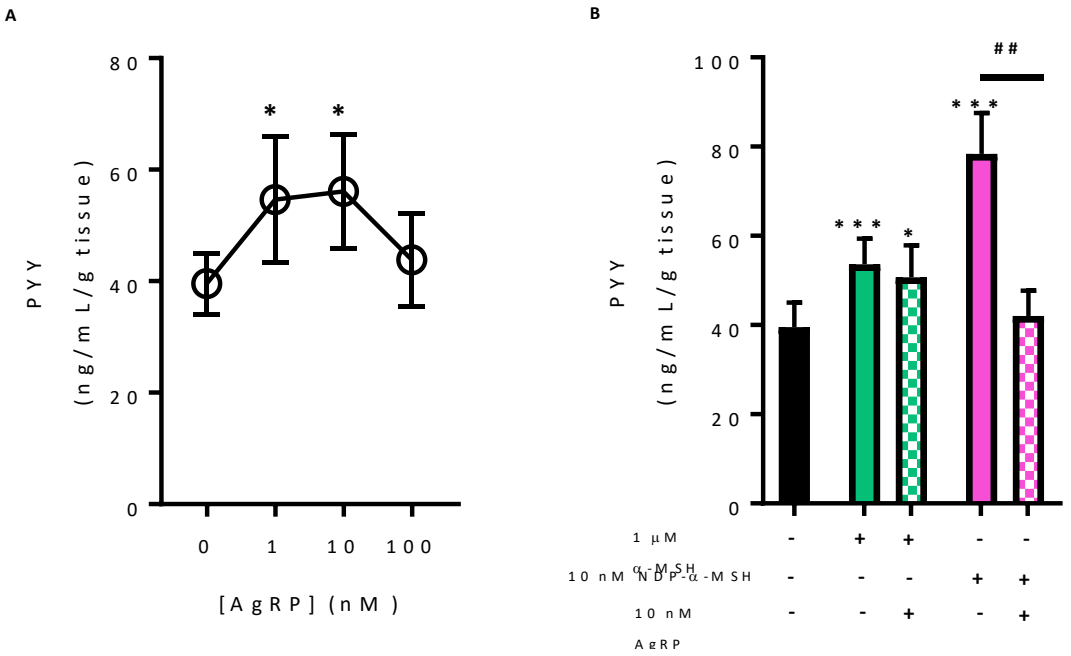


*Figure 3.3.8 The endogenous MC4R antagonist AgRP significantly stimulated PYY release from human colonic epithelial preparations at 1 and 10 nM but not at 100 nM (A, n = 10 – 13, *p < 0.05). α-MSH significantly triggered PYY secretion from basal (B, ***p < 0.001) and 10 nM AgRP did not attenuate this stimulation (*p < 0.05 compared to basal). 10 nM NDP-α-MSH also significantly triggered PYY release from basal (B, n = 13, **p< 0.01) but the stimulatory effect was blocked by equimolar AgRP (##p < 0.01 compared to 10 nM NDP-α-MSH alone).*

3.3.4 Discussion and Conclusion

The aim of this experiment was to investigate if MC4R regulates GLP-1 and PYY secretion from the human intestinal epithelia in a similar fashion to that reported in the mouse[762]. Although Panaro and co-workers demonstrated that MC4R agonism significantly reduced chloride secretion across human intestinal mucosae[762], which serves as a surrogate for PYY secretion due to its anti-secretory effect[840], PYY and GLP-1 secretion from these mucosal preparations were not reported. Results from this current study are the first to provide direct evidence supporting a role of MC4R in regulating acute GLP-1 and PYY secretion from the human intestinal epithelium. The reasons for MC4R activation to cause less robust PYY secretion from ileal preparations when compared to colonic preparation is likely due to the fact that the predominant secretory product from ileal L cell is GLP-1, rather than PYY. PYY was secreted in the ileum at levels that were near the lower limit of quantitation of the ELISA assay used in this study and thus, the high signal-to-noise ratio may have hindered the detection of possible effects of MC4R activation.

This study clearly demonstrated that α-MSH and other MC4R agonists have the capacity to directly stimulate GLP-1 and PYY release from the gut epithelia. The functions of MC4R-regulated GLP-1 and PYY release can be readily examined the effects of α-MSH in mouse models with attenuated GLP-1R

and Y2R signalling, with either genetic knockout animals or receptor antagonists such as exendin 9-39 for GLP-1R and BIIE0246 for Y2R, the preferential receptor of PYY_{3-36}[626]. Another approach would be to use gut-specific *Mc4r* knockout animal models. Such experiments will provide valuable insights how MC4R-regulated GLP-1 and PYY secretion could potentially be implicated in the satiating actions[827,828,831,841-844] and glucose-lowering effect[831,845-847] of peripherally administered α-MSH and other MC4R agonists.

While the results from the current study support my hypothesis that α-MSH directly triggered GLP-1 and PYY secretion from the human gut epithelium, it raises the immediate question of what could be the source of endogenous MC4R ligands that regulate L cell secretion. Given the intense focus on the metabolic implications on ARC-derived α-MSH, it is tempting to suggest that centrally-derived α-MSH may somehow activate MC4Rs on L cells, causing the release of GLP-1 and PYY to reinforce the satiating effects of α-MSH. However, α-MSH is a small peptide that does not readily cross the blood brain barrier to reach the peripheral circulation[832,833]. Although the ARC is believed to have greater exposure to peripherally-derived blood-borne factors than other hypothalamic nuclei due to its close proximity to the median eminence, nuclei targeted by POMC fibres reside in anatomical locations that are protected by an intact blood brain barrier[848]. POMC-neurons in the ARC are not known to have projections other than the forebrain, midbrain and periaqueductal grey matter[408]. Whilst a smaller population of POMC-neurons resides within the NTS, their projections appear to be confined to within the brainstem, specific nuclei in in the hypothalamus and the amygdala[849]. Altogether, it is unlikely for centrally derived α-MSH to act on MC4R expressed by L cells under physiological conditions.

POMC is highly expressed by endocrine cells in the anterior pituitary gland and was once thought to be produced exclusively by melanotropes in the intermediate lobe[850,851]. However, emerging evidence suggests that corticotrophs in the anterior lobe of the anterior pituitary also produce α-MSH, albeit in significantly smaller amount[846]. Importantly, pituitary-derived α-MSH accounts for approximately 70 % of circulating α-MSH in humans and this circulating α-MSH follows a prandial pattern that resembles those of insulin and gut hormones including GLP-1 and PYY[846]. While immunohistochemical data from an early study suggested anterior pituitary corticotrophs only express very low levels of glucokinase, if any[852], Enriori and colleagues recently demonstrated that the glucose-induced α-MSH surge was abolished in mice with POMC-specific K_{ATP} channel signalling disruption[846], which supports the notion that glucose is a regulator of peripheral α-MSH secretion. Intriguingly, acute intravenous infusion of α-MSH significantly reduced glucose excursion in mice during intraperitoneal glucose tolerance test and markedly increased glucose infusion rate during hyperinsulinaemic-euglycaemic clamp studies, an effect that was not blocked by

intracerebroventricular administration of AgRP. This indicates α-MSH regulates glucose homeostasis through peripheral melanocortin receptors[846]. Although α-MSH was demonstrated to increase glucose uptake into skeletal muscles[846,853], it is possible that this is not the only pathway through which α-MSH acutely improves glucose tolerance. The postprandial α-MSH surge could also trigger GLP-1 and PYY release from the distal small intestine and colon in an endocrine fashion. Postprandial plasma levels of GLP-1 and PYY increase rapidly, long before ingested nutrients arrive in the L cell-rich distal ileum and colon to directly trigger GLP-1 and PYY secretion[854]. Although the duodenum has considerable populations of L cells[251] that are activated upon glucose exposure[252], it does not exclude the possibility that humoural factors such as α-MSH also regulate postprandial gut hormone release. However, circulating α-MSH in humans occurs in the picomolar range[846,855-858] while the concentrations of α-MSH used in this current study, as well as in other published work that adopted *ex vivo* approaches[762,846,853] were in the high nanomolar to micromolar range, nearly a thousand-fold higher than normal physiological levels of the endogenous hormone. The notion of pituitary-derived α-MSH stimulating L cell secretion is further challenged by the fact that circulating α-MSH is highly susceptible to enzymatic degradation[859], which makes it even less unlikely for substantial amounts of pituitary-derived α-MSH to reach the basolateral membranes of L cells to activate MC4R and subsequently trigger GLP-1 and PYY release.

Most binding studies consistently showed hMC4R to be activated by α-MSH with an EC_{50} in the nanomolar range[762,860-864]. This suggests endogenous ligands for MC4R expressed on L cells most likely originate from sources of relative close proximity to the gut epithelium. Interestingly, the intestinal mucosa do not only express *Pomc*[865], but also produce α-MSH[866]. Therefore, it is plausible that intestinal POMC cells secrete substantial α-MSH to activate neighbouring L cells in a paracrine fashion, in addition its inhibitory effect on the release of pro-inflammatory cytokines in the gut[855,867]. Although the factors governing α-MSH secretion from POMC-containing gut epithelial cells remain unclear, the fact that the magnitude of the postprandial α-MSH surge is proportional to the caloric load[846] supports the notion that these cells have nutrient sensing capacity, much like L cells and other enteroendocrine cells, and are well-situated to account for the postprandial α-MSH surge.

One unexpected finding in the current study is that while AgRP significantly attenuated NDP-α-MSH-induced GLP-1 and PYY secretion from the human colonic intestinal epithelial preparations, it has stimulatory effect on the preparations in the absence of α-MSH and NDP-α-MSH. This finding was later confirmed by follow-up experiments performed by others in our laboratory. Enigmatically, this appears to be an AgRP-specific effect as another MC4R antagonist, SHU9119, did not stimulate L cell secretion at similar or higher concentrations (Keating *et al.*, unpublished observations). Results from the AgRP dose-response experiment in this present study suggests AgRP could be a partial agonist at

MC4R on human L cells, as it stimulated GLP-1 and PYY by 1.5 fold, compared with NDP-α-MSH, which stimulated secretion by more than two fold. However, such interpretation is complicated by the observation that 10 nM AgRP completely abolished stimulatory effects of NDP-α-MSH; if it is indeed a partial agonist, PYY and GLP-1 secretion should still be significantly elevated to the levels seen with AgRP alone, though to a lesser extent than that induced by equimolar NDP-α-MSH. The nature of AgRP-MC4R interaction has been a subject of ongoing investigation as it has been suggested that rather than a pure competitive antagonist, AgRP acts as an inverse agonist on MC4R *in vitro*[868] and *in vivo*[869]. α-MSH activates MC4R activation in a $G_{\alpha s}$-dependent manner, which results in adenylyl cyclase activation and elevated intracellular cAMP levels[862,864,870]. If AgRP is a pure inverse agonist, its binding to MC4R would lead to decoupling of $G_{\alpha s}$ from the receptor and thereby attenuate any constitutive activities. However, emerging evidence support AgRP as a biased agonist at MC4R in that it favours $G_{\alpha i/o}$ activation, which leads to inhibition of adenylyl cyclase[871]. Whilst the concepts of AgRP being a pure competitive antagonist, an inverse agonist or a biased agonist can all sufficiently explain AgRP-attenuated NDP-α-MSH-stimulated GLP-1 and PYY release, none provides an adequate explanation of why would AgRP paradoxically stimulate GLP-1 and PYY secretions. Interestingly, AgRP increases forskolin-induced cAMP elevations in hypothalamic GT1-7 cells, a cell-line that endogenously express MC4R, in contrast to other *in vitro* experiments based on overexpression of the MC4R[862]. The authors attributed this observation to the differential G-protein regulation of different families of adenylyl cyclase and that AgRP sensitizes group 2 adenylyl cyclases by releasing βγ-subunits from $G_{i/o}$ proteins[862]. It is possible that AgRP induces L cell secretion via this pathway. Another possibility is that AgRP acts on other enteroendocrine cells through an unidentified receptor to regulate the secretion of other hormones, which subsequently acts on neighbouring L cells in a paracrine fashion to stimulate the release of GLP-1 and PYY.

The magnitude of GLP-1 and PYY response to the stimulation by 10 nM NDP-α-MSH did not show any significant correlation to the BMI of the specimen donors, which suggest MC4R activated- L cell secretion is preserved in obese individuals. Obese individuals have higher circulating plasma levels of α-MSH and AgRP[857,858,872,873] but the underlying mechanism and the physiological implications of such increases remain unclear. The fact that the anorectic effects of subcutaneously infused MC4R agonists are preserved in obese humans[835] and non-human primates[829] suggests that unlike the leptin pathway, the MC4R signalling pathway is intact in obese individuals. Interestingly, the elevated AgRP level could be a protective mechanism against potential MC4R desensitization from prolonged activation secondary to elevated α-MSH levels[874,875]. The binding of AgRP to MC4R has been shown to promote receptor endocytosis[861], which is essential for maintaining MC4R responsiveness to the stimulatory effects of α-MSH[876]. Thus, AgRP could be an integral component

to normal MC4R function. Nonetheless, the physiological implications of such observations are yet to be determined.

In summary, results from this experiments confirmed the findings in mice from Panaro *et al*., which demonstrated GLP-1 and PYY secretion from the gut is under the control of MC4R[762]. Endogenous and synthetic MC4R agonists all triggered significant GLP-1 and PYY release from human intestinal epithelial preparations. However, it is to my surprise that AgRP, an antagonist, or perhaps an inverse agonist of MC4R, also triggered significant GLP-1 and PYY release from these preparations. It is crucial to identify the source of the endogenous ligand that activates L cell MC4R as this will provide invaluable insight to potentially new functions of the receptor that have not been previously described.

3.4 gp130 cytokines stimulate GLP-1 secretion in human gut epithelia

3.4.1 Introduction

Chronic low-grade inflammation is one of the hallmarks of type 2 diabetes[232] and is believed to be a driver of obesity-induced peripheral insulin resistance[231,877]. Hyperglycaemia[878], increased levels of circulating saturated fatty acids[879] and ceramide[880] are all potent triggers of the inflammatory response, likely through the action the Toll-like receptors (TLRs). Activation of TLRs results in increased production of pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) and interleukin (IL)-1[881]. These cytokines can further recruit other immune cells, thereby amplifying the initial inflammatory response. Macrophage infiltration in adipose tissue[234,882], skeletal muscle[883] and pancreatic islets[884,885] all have deleterious effects on metabolism. Circulating levels of various pro-inflammatory cytokines are significantly elevated in obese and T2D individuals, compared to healthy controls[886-891]. Attenuating the actions of TNF-α and IL-1 resulted in significantly improvements in insulin sensitivity and glucose tolerance in diabetic and diet-induced obesity animal models [892-894], and humans with type 2 diabetes[895].

The cytokine, IL-6, is produced by all immune-competent cells and is crucial in mediating immune responses against infections[896,897]. However, exaggerated IL-6 action also underlies the pathogenesis of many inflammatory conditions such as rheumatoid arthritis[898,899]. Notably, elevated levels of IL-6 in T2D patients have been consistently reported[233,890,900,901], which supports the view that IL-6 could have detrimental effects on metabolism and be a driver of T2D pathogenesis[902]. This notion is further supported by the fact that serum levels of IL-6 are positively correlated with increasing adiposity[903-908], as adipose tissue is also a prominent source of IL-6[906]. However, this view is challenged by a study that showed attenuated IL-6 action causes obesity and impaired glucose tolerance in various mouse models[909-911]. Importantly, such disturbed metabolic phenotypes could be rescued by exogenous supplementation of IL-6 in *Il6* knockout mice[909], which suggests IL-6 plays an important role in the maintenance of energy balance and glucose homeostasis. Furthermore, circulating IL-6 levels are profoundly increased during endurance running[912,913] , an activity with known benefits in weight loss and glycaemic control. Seminal studies later demonstrated that exercise-induced surges in IL-6 levels are produced by actively contracting skeletal muscles[914-916], rather than by adipocytes or circulating immune cells[917]. Skeletal muscle-derived IL-6 increases insulin-stimulated glucose uptake and fatty acid oxidation in skeletal muscles[918-921]. Indeed, it has been proposed that the metabolic benefits of exercise, such as improved insulin resistance, are conferred by skeletal muscle-derived IL-6[922,923] as animals with attenuated IL-6 function did not

benefit from metabolic gains secondary to exercise[924-926]. Intriguingly, increased GLP-1 secretion was shown to contribute to the metabolic benefits of exercise-induced IL-6 in mice[95]; acute IL-6 treatment was also shown to increase GLP-1 secretion in mice[95,927]. Although it remains unclear if IL-6 directly causes GLP-1 secretion in humans, increased GLP-1 levels in critically ill patients are strongly associated with elevated IL-6 levels[927,928]. However, despite its many desirable metabolic benefits, IL-6 proves to be a less-than-ideal therapeutic target as it is a potent pro-inflammatory cytokine and enhancing its function would have undesirable consequences such as increasing tumourigenesis[929-932].

IL-6 belongs to the gp130 cytokine family, named after the membrane-bound signal-transducing protein glycoprotein 130 receptor β (gp130Rβ) that is central to signal transduction of all of the different cytokines in the gp130 cytokine family. The binding of IL-6 to its receptor, IL-6Rα, triggers the homodimerization of two gp130Rβ subunits and subsequently signals through the JAK/STAT pathway[897]. In addition to signalling through membrane-bound IL-6R (referred to as "classical signalling"), IL-6 can also signal through a soluble form of IL-6R (sIL-6R), which lacks the cytoplasmic and transmembrane domains (referred to as "*trans* signalling")[933]. The circulating IL-6/sIL-6R can signal gp130Rβ-expressing cells that do not express IL-6R. As gp130Rβ is ubiquitously expressed in almost all cell types and IL-6R expression is more specific[934], many of the undesirable pro-inflammatory effects of IL-6 have been attributed to *trans*-signalling[930,935]. Therefore, an IL-6R agonist that does not trigger *trans*-signalling could circumvent the negative effects of IL-6R signalling.

Interestingly, another member of the gp130 cytokine family, ciliary neurotrophic factor (CNTF), is also a ligand of IL-6R, albeit of a lower affinity than IL-6; the CNTF/IL-6R complex causes dimerization of gp130Rβ and the leukaemia inhibitory factor receptor (LIFR), instead of gp130Rβ homodimerization as with IL-6/IL-6R[936]. CNTF is a neural cytokine expressed predominantly by astrocytes in the CNS[937] and Schwann cells in the PNS[938]. In addition to IL-6R, CNTF also signals through the CNTF receptor (CNTFR) and triggers the dimerization of LIFR and gp130Rβ as it would through IL-6R binding. Whilst CNTF can signal through IL-6R in humans, IL-6 does not signal through CNTFR[934]. Contrary to many cytokines, the CNTF protein is sequestered within the cytoplasm and is only released upon cell lysis as the protein itself lacks a secretory signal sequence peptide[939]. Interestingly, there is evidence to suggest that GLP-1R could be a downstream effector of CNTF, at least in the CNS, as CNTF treatment increased proglucagon expression in primary mouse hypothalamic neurons and the neurotrophic effects of CNTF were abolished in *Glp1r*$^{-/-}$ mice[940]. Originally trialled as a treatment for amyotrophic lateral sclerosis due to its pro-survival effects on injured neurons, recombinant human CNTF did not improve disease outcome in the clinical trial but

significant weight loss was observed in treated patients[941], prompting an interest in using CNTF as an anti-obesity treatment. Indeed, CNTF was highly efficacious in promoting weight loss in pre-clinical animal models, especially in leptin-resistant animal models, through increased satiety and increased energy expenditure[942-949]. Moreover, CNTF improved glucose homeostasis in non-obese mouse model of T2D[950]. As these favourable effects of CNTF treatment are similar to that of GLP-1 receptor agonists, it is possible that GLP-1 mediates some of the metabolic benefits of CNTF. Nonetheless, despite its significant weight-loss efficacy in humans, the development of CNTF as an anti-obesity agent was not pursued as many treated patients developed high levels of auto-antibodies against the CNTF analogue used[951], likely due to the non-secretory nature of endogenous CNTF[934]. In addition, due to the low level of CNTFR expression, coupled with its low affinity for the more widely expressed IL-6R, high concentrations of CNTF would be required to achieve beneficial metabolic outcomes *in vivo*.

An IL-6/CNTF chimeric protein, IC7, was developed in order to overcome the pro-inflammatory effects of IL-6R signalling and the immunogenicity and low potency of CNTF[952]. This protein has higher affinity for IL-6R than endogenous CNTF but retains a specific LIFR binding epitope such that it only signals through the gp130Rβ/LIFR heterodimer, instead of the gp130Rβ homodimer[936], thereby preventing the undesirable IL-6R *trans* signalling. It was believed that such a "designer cytokine" would retain the desirable features of the two parent proteins[953], such as the GLP-1 secretagogue effect of IL-6. Indeed, in high-fat fed mice, IC7 treatment significantly reduced fat mass and improved glucose tolerance. Importantly, such effects are partially attenuated in *Glp1r* knockout mice, indicating GLP-1 mediates some of the metabolic benefits conferred by IC7 treatment (personal communication, Professor Mark Febbraio). However, it remains to be determined if targeting IL-6R signalling would stimulate GLP-1 secretion in humans. As such, the first aim of this study was to determine if IL-6 directly triggers L cell secretion from human gut epithelial tissue. I hypothesize that IL-6 would trigger GLP-1 secretion from the human gut epithelia, based on results from seminal rodent studies[95,920]. The second part of this study was done in collaboration with the developer of IC7, Professor Mark Febbraio. A huge body of data concerning the pharmacodynamics and pharmacokinetics of IC7 had been gathered using various preclinical models but it remained unclear whether these preclinical findings could be translated in to the clinic. As GLP-1 was shown to be implicated in some of the metabolic benefits of IC7, I aimed to determine if IC7 triggers GLP-1 secretion in the human colonic epithelia.

3.4.2 Methods

3.4.2.1 Human tissue collection for ex vivo studies in human colonic mucosae

Patients gave written informed consent for colon tissue donation from resected large intestine at Flinders Medical Centre and Flinders Private Hospital approved by the Southern Adelaide Clinical Human Research Ethics Committee. Human ileal and colonic tissue were obtained from patients undergoing bowel resection for cancer and stoma reversal. In cases of cancer resection, tissue samples were obtained from sites at least 10 centimetres away from the tumour location. Specimens from patients that were indicative for any form of inflammatory bowel disease were excluded from this study. Characteristics of the patient cohort are listed in Table 3.4. None of the patients had diabetes. The specimens were immediately placed in iced-cold Krebs buffer and transported to the laboratory for dissection within 15 min as described previously. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal content and dissected clear of adipose, muscular and connective tissue. The mucosae were gently dissected off from the submucosae as intact sheets using a stainless steel spatula, cut into 5 mm pieces and weighed individually. The mucosal pieces were then transferred to a 96-well plate for secretion assays.

3.4.2.2 Secretion experiments

Mucosal pieces were incubated with 200 µL of buffer (control) or buffer containing IL-6, or various concentrations of IC7 (from 1 ng/mL up to 1 µg/mL) or positive controls in a 96-well plate for 15 min. The buffer was a modified Krebs buffer described above with the addition of sitagliptin 1 µM, 0.1% BSA (A1595, Sigma Aldrich) at pH 7.4. Following incubation at 37°C in 95% O_2/5% CO_2, supernatants were collected and stored in aliquots at -20°C. Active GLP-1 levels were quantitated using commercially available ELISA kits, according to manufacturer's instructions (EGLP-35K, Merck Millipore, Billerica, MA, USA).

3.4.2.3 Test agents

Recombinant human IL-6 (I1395, Sigma Aldrich) was dissolved in 0.1 % BSA in PBS as a 100 µg/mL stock and stored at -20°C as single-use aliquots. 100 ng/mL dose was chosen for secretion experiments as it was shown to be effective in triggering GLP-1 secretion from GLUTag cells[95]. IC-7 was a gift from Professor Mark Febbraio from the Garvan Institute of Medical Research under a Material Transfer Agreement. IC7 was dissolved in 0.1% BSA in PBS as a 10 mg/mL stock and stored at -20°C as single-use aliquots. IC7 concentrations were selected based on concentrations that were sufficient to cause significant increased glucose uptake in *ex vivo* mouse soleus muscle[953].

300 mM glucose was used as positive control for ileal preparations, as it was demonstrated in previously in section 3.1 that ileal GLP-1 secretion is sensitive to high glucose. IBMX and forskolin

(FSK) (I5879 & F6886, Sigma Aldrich) (10 µM each) and 70 mM KCl were used as positive controls for colonic preparations. For the 70 mM KCl solution, an equimolar amount of NaCl was removed to maintain osmolarity. Only colonic samples that show positive response to at least one positive control (70 mM KCl or 10 µM IBMX/FSK) were included in analysis.

3.4.2.4 Statistical analyses

All statistical analyses were conducted as paired analyses, comparing responses in tissues obtained from the same individual to relevant control conditions. A paired-ratio *t*-test was used for single comparisons. A paired one-way ANOVA with Fishers Least Significant Difference post-hoc test used for multiple comparisons. Statistical significance was $p < 0.05$. All data are shown as mean ± SEM.

	Ileum specimens	Colon specimens
N	8	29
Gender (male/female)	(4/4)	(19/10)
Age (years)	73.4 ± 3.2 (57 – 83)	64.6 ± 2.5 (38 – 93)
BMI (kg/m^2)	29.58 ± 1.6 (25 – 38.5)	29.6 ± 1.5 (19 – 55)

Table 3.4 Characteristics of the specimen donors

3.4.3 Results

Acute treatment of 100 ng/mL IL-6 significantly triggered GLP-1 secretion by two fold from human ileal and colonic epithelial preparations (Figure 3.4.1).

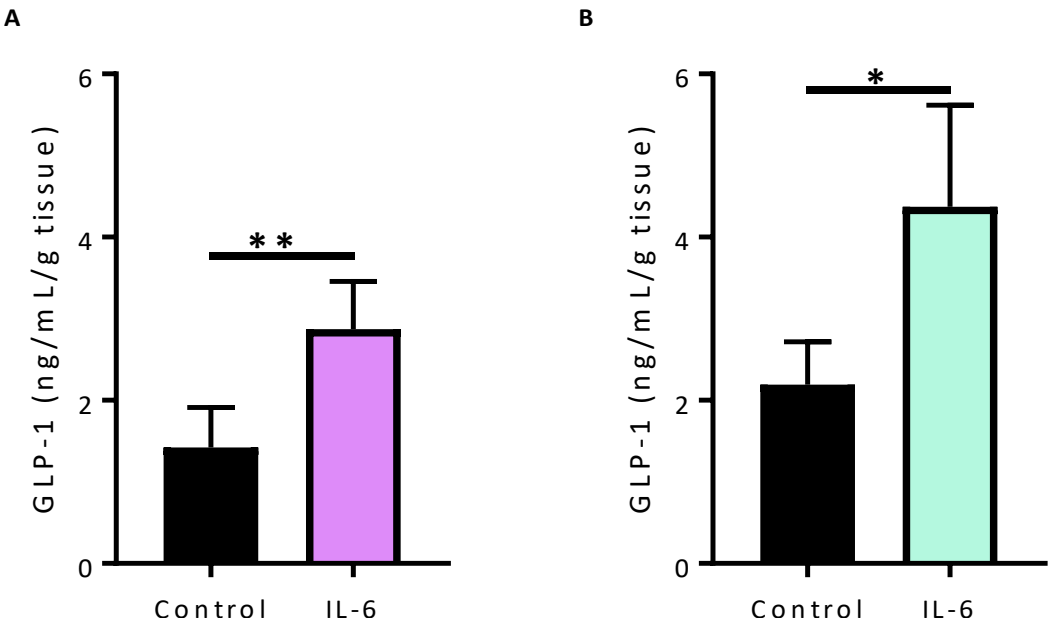


*Figure 3.4.1 100 ng/mL IL-6 significantly induced GLP-1 secretion from (A) ileum (n = 8; **p < 0.01) and (B) colon (n = 14; *p < 0.05)*

In line with results discussed in previous chapter, the increase in GLP-1 secretion from the colon was also accompanied by a significant increase in PYY secretion (Figure 3.4.2).

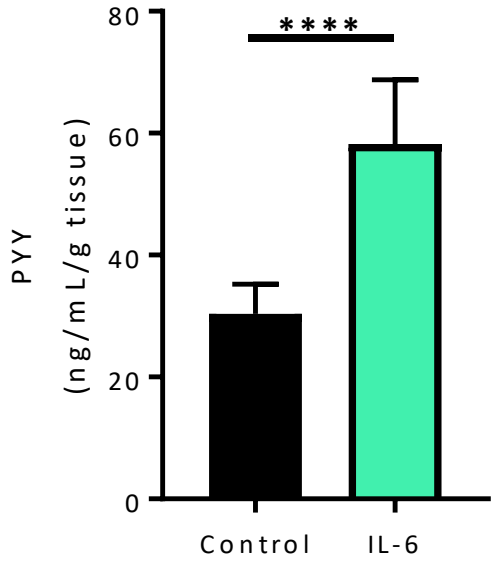


*Figure 3.4.2 100 ng/mL IL-6 triggered significant PYY secretion from human colonic epithelial preparations (n = 19; ****p < 0.0001).*

Acute exposure to various concentrations of IC7 (1 ng/mL to 1 µg/mL) triggered significant increases in intact GLP-1 levels when compared with control by at least 1.5 fold (Figure 3.4.3).

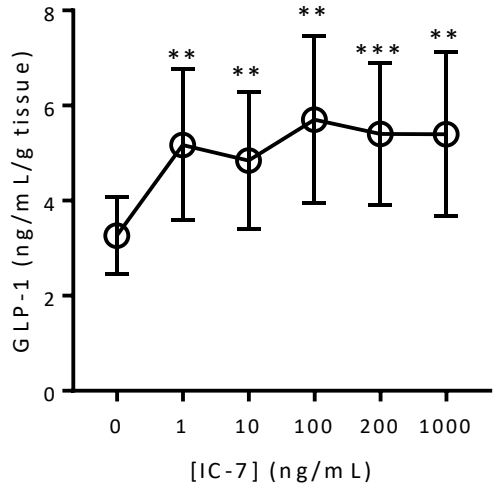


*Figure 3.4.3 The CNTF-IL-6 chimeric protein stimulated significant GLP-1 secretion from human colonic epithelial preparations at all concentrations tested (n = 10; **p < 0.05, ***p < 0.01).*

3.4.4 Discussion and Conclusion

This is the first demonstration of the stimulatory effect of IL-6 on GLP-1 secretion from the human intestine. Previous work demonstrated that IL-6 is a GLP-1 secretagogue in mice[95] and that GLP-1 levels are positively associated with circulating IL-6 levels in critically ill patients[927] or patients undergoing cardiac surgeries[928].

The fact that both IL-6 and the CNTF/IL-6 chimeric protein, IC7, are potent GLP secretagogues suggest that IL-6R is expressed in the gut epithelium, possibly on L cells. Although this study did not seek to investigate the underlying mechanisms of IL-6-induced GLP-1 secretion from the human gut epithelium, IL-6 induced AMPK activation could be implicated as it mediates the enhanced fatty acid oxidation and glucose uptake in skeletal muscles triggered by both IL-6[918,954] and CNTF[948]. As it has been demonstrated in section 3.3, AMPK activation by metformin triggered GLP-1 secretion from the human gut epithelia and thus it is reasonable to speculate that AMPK activation is implicated in IL-6-induced GLP-1 secretion in humans. Immunolabelling of IL-6R would be an appropriate next step to determine if GLP-1-secreting cells express IL-6R. The underlying mechanism of IL-6-induced GLP-1 secretion can be further interrogated with AMPK inhibitors, such as dorsomorphin.

Although exercise acutely increases GLP-1[955-957] and IL-6[912,913] levels in humans, results from this experiment provide direct evidence that IL-6 causes GLP-1 secretion in humans. The concentration of IL-6 used in this study resembles that observed during exercise or sepsis, instead of that observed in a chronic low-grade inflammation setting. This leads to the question of what could be the physiological functions of IL-6 induced GLP-1 secretion in humans under such scenarios. Since insulin secretion is suppressed and glucagon secretion is increased during exercise[955], it is unlikely for IL-6 induced GLP-1 to exert any effects through its insulinotropic and glucagonostatic actions. GLP-1 causes rapid microvascular recruitment in human skeletal muscles[958-960] and could increase GLUT4 translocation to enhance glucose uptake in skeletal muscles[958,961], although the latter finding remains controversial[960]. Nonetheless, glucose uptake into skeletal muscle during exercise is believed to be partly mediated by insulin-independent pathways[962,963]. As such, it is possible that in the face of intramyocellular glycogen depletion during exercise, skeletal muscles release IL-6 to increase glucose uptake, partly through the action of GLP-1.

GLP-1 could also serve as a regulator of IL-6 secretion and as a modulator of its downstream effects. IL-6 secretion in response to a lipopolysaccharide (LPS) challenge is significantly exaggerated in *Glp1r*$^{-/-}$ mice, in comparison with wildtype controls[964]. The notion of a potential IL-6-GLP-1 feedback loop is further supported by the report that a seven-day treatment of the GLP-1R agonist, liraglutide in T2D patients significantly reduced circulating IL-6 levels[965]. IL-6 levels are markedly elevated during extreme physiological stress such as septic shock, cardiovascular events or invasive surgeries. While a powerful inflammatory response is warranted in these scenarios to ensure survival, mechanisms need to be in place to limit any collateral damages caused by elevated levels of pro-inflammatory cytokines such as endothelial damage[966]. GLP-1R agonists exert protective effects on endothelial cells from pro-inflammatory cytokine-induced oxidative stress[967,968]. Indeed, GLP-1 infusions have been demonstrated to significantly improve cardiovascular outcomes in post-

myocardial infarction patients[969,970], likely through increased vasodilation and coronary blood flow[971]. GLP-1 also acts directly on immune cells to attenuate inflammatory responses[972-974], with pro-inflammatory cytokine secretion from human invariant natural killer T cells being dose-dependently suppressed by GLP-1[974]. Altogether, these reports support the view that IL-6-induced GLP-1 secretion under physiological stress could be an intrinsic protective mechanism against the inflammatory actions of IL-6.

IC7 is an effective anti-obesity tool in various animal models[975,976], which is consistent with the actions of its parent moleculte[952], CNTF[942-949]. The rationally designed IL-6/CNTF chimeric protein demonstrated low immunogenicity in an array of human cell-based assays[975], which makes it a more attractive drug candidate than other CNTF analogues that had previously failed clinical trials. IC7 also has a higher affinity for IL-6R than endogenous CNTF such it can exert a CNTFR-like intracellular signalling cascade through membrane-bound IL-6R[934,936] at a much lower concentration than that required of endogenous CNTF, further reducing the likelihood of immunogenic reactions to the chimeric protein. The fact that IC7 significantly triggered GLP-1 secretion implies that the IL-6R/gp130Rβ/LIFR complex is capable of causing GLP-1 release. This result indicates that IL-6 induced GLP-1 secretion does not depend of IL-6R *trans*-signalling as IC7 does not signal through sIL-6R[977]. The profound pro-inflammatory effects of IL-6 are believed to be mainly mediated by *trans*-signalling through sIL-6R[935].

The distinction between exercise-induced cytokine release and elevated cytokine levels secondary to chronic low-grade inflammation is that the former is a transient surge of cytokines (IL-6 levels increase by up to 100-fold in some cases[978]) while the latter is characterized by a prolonged, 2-3 fold elevation of cytokine levels. In addition, the types of cytokines that are upregulated in the two scenarios are profoundly different; IL-6 is by far the most prominent exercise-induced cytokine while TNF-α and IL-1β, the pro-inflammatory cytokines that are characteristic of low grade inflammations, generally do not increase in response to exercise[979]. Given the positive effect of IL-6 on metabolism such as enhanced insulin sensitivity and increased fatty acid oxidation in skeletal muscle[979], it is possible that elevated levels of the cytokines observed in obese and T2D individuals could be an adaptive response to the metabolic challenge of overnutrition. Enhancing GLP-1 secretion could be a way for IL-6 to restore metabolic homeostasis as GLP-1 has trophic effects on pancreatic β cells and central anorectic effects.

4 The role of dynamin in mediating L cell secretion in the human colon

4.1 Introduction

As discussed in Chapter 1, metabolic gains from bariatric surgeries have been partly attributed to enhanced postprandial levels of the secretory products of the enteroendocrine L cell, GLP-1 and PYY[314,390]. Targeting the individual pathways has shown promising results with liraglutide, for example, now an established T2D treatment and has recently been approved as an anti-obesity treatment. However, the magnitude of weight-loss achieved is modest compared to that achieved through surgery[389]. The efficacy of PYY in appetite suppression has also been clearly demonstrated[631,632] but dose-limiting gastrointestinal side effects[980,981] and formulation challenges[804] have significantly lessened the appeal of PYY alone as a therapeutic target. Interestingly, GLP-1 and PYY act synergistically to suppress appetite in humans[982,983]. GLP-1 and PYY are co-localized in L cells[107,620] and in a previous chapter, I demonstrated that the release of the two hormones is strongly correlated. Thus, development of an L cell secretagogue that is capable of triggering the co-release of these hormones such that they could act synergistically is of strong clinical interest. The pursuit to develop orally-available, small molecule L cell-specific secretagogues has been met with limited success. Agonists for the long chain fatty acid receptor GPR119 agonists had demonstrated impressive efficacy in pre-clinical models[674,751,984] but were ineffective in humans[752,985], potentially due to substantial species differences[620]. Although macronutrients are potent L cell secretagogues[986], the caloric content of nutrients would offset the appetite suppressing effects of these hormones. Importantly, glucose[113,115,116] and lipids[117] also stimulate the release of other gut hormones such as serotonin, which has obesogenic and diabetogenic potency[121,123,132]. Therefore, identification of an L cell-specific secretory pathway is crucial.

One of the major obstacles that hinders the development of effective L cell secretagogues is our lack of knowledge of the underlying molecular mechanisms governing the secretory process of human enteroendocrine L cells, due to a lack of highly translatable *in vitro* models. While it has been established that L cells are electrically-excitable[692,709] and that exocytosis of GLP-1 and PYY-containing vesicles is triggered by increased intracellular calcium[692], the molecular machinery governing the exocytotic process in L cells remains largely unknown. Exocytosis involves the translocation of vesicles from the cytoplasm towards the target plasma membrane, followed by tethering and docking of the vesicles at the target membrane, which are then primed for release upon stimulation[987]. The SNARE (soluble *N*-ethyl-maleimide-sensitive fusion protein attachment

protein receptor) hypothesis was proposed over two decades ago[988] and remains the most widely accepted working model of cellular exocytosis[989]. Simplistically, this model proposes that the v-SNARE proteins (synaptobrevin) embedded within the **v**esicular membrane interact with the t-SNARE proteins (SNAP25 and syntaxin) on the **t**arget plasma membrane to form the SNARE complex, which is stabilized by the calcium-sensor, synaptotagmin to prevent uncoordinated membrane fusion. Ca^{2+}-binding results in a conformational change in synaptotagmin that allows the SNARE complex to zipper, thereby bringing together the vesicular and target plasma membranes to increase the curvature and lateral tension of these membranes, eventually causing the fusion of the two membranes. This results in the formation of a fusion pore, from which vesicular contents such as neurotransmitters and hormones are released into extracellular space.

Substantial parts of our knowledge concerning the exocytotic process are derived from studies using adrenal chromaffin cells as the prototype secretory cell[990], given the relative ease to obtain cultures of high purity and that their relatively slow rate of exocytosis allows for detailed interrogations[991]. Moreover, catecholamines are oxidizable, which permits precise quantification of transmitter released per exocytotic event by electrophysiological techniques such as carbon fibre amperometry[992]. However, significant differences do exist between different secretory cell types. Work from our laboratory has demonstrated that adrenal chromaffin cells release over 70 times more catecholamines per exocytotic event than serotonin-secreting enterochromaffin cells do from vesicles that are of comparable size[109], likely due to markedly smaller fusion pores formed in the latter[993]. Thus, extrapolations of results derived from adrenal chromaffin cells to enteroendocrine cells onto other cell types should be made with caution.

Detailed characterization of the mechanisms underlying the exocytotic process of enteroendocrine L cells specifically remains of high importance to broaden our understanding of L cell physiology. Unlike enterochromaffin cells, which make up half of the enteroendocrine cell population and can be isolated as a highly-enriched culture using a Percoll density gradient, similar approach to obtain viable L cell culture were not successful, as discussed in detailed in chapter 2. In addition, secretory products of L cells are peptides that are not readily oxidizable, in contrast to serotonin released by enterochromaffin cells. As a result, single-cell electrophysiological techniques such as patch-clamping and amperometry cannot be used to interrogate the mechanisms underlying the secretory process of human enteroendocrine L cells. Nonetheless, knowledge concerning the exocytotic process in other neuroendocrine cell types has been invaluable as they formed the basis of several recent studies that investigated the physiology of L cell exocytosis. These were based on the knowledge that the calcium sensor synaptotagmin-7 plays a crucial role in mediating exocytosis in

both pancreatic α[994] and β[995] cells and the same protein was identified as a positive regulator of GLP-1 secretion from enteroendocrine L cells[996]. Synpatotagmin-7 deficiency impaired oral glucose-induced GLP-1 secretion in mice by approximately 50% and whole-cell patch-clamping experiments in GLUTag cells indicated that the impairment lies downstream of the calcium signal[996]. The fact that synaptotagmin-7 knockout did not completely abolish GLP-1 secretion, unlike that observed for glucagon secretion[994], suggests that other members of the synaptotagmin family are likely to be involved in the exocytosis process in L cells. Recently, the t-SNARE protein, syntaxin 1A was shown to be regulate L cell exocytosis, although similar to synpatotagmin-7, intestine-specific knockout of syntaxin 1A did not completely abolish GLP-1 secretion, indicating this protein is involved in, but not crucial in mediating L cell exocytosis[997].

Dynamin is a 100 kDa GTPase mechanochemical enzyme that is encoded by three *DNM* genes in the mammalian genome. There are distinct differences in the expression patterns of the three dynamin isoforms: dynamin-1 is selectively expressed in neuronal cells[998], dynamin-2 is ubiquitously expressed in all cell types[999] while dynamin-3 is expressed primarily in the brain, testis[1000,1001] and lungs[1002]. Dynamin has a well-established role in mediating clathrin-mediated endocytosis, a pivotal cellular process that is closely coupled with exocytosis[1003]. Without effective endocytosis, vesicle recycling within the cell is impaired, which would compromise subsequent exocytotic processes[1004]. Insulin secretion from pancreatic β cells that are deficient in *Dnm2* is impaired due to compromised vesicle recycling[1005]. Moreover, the insertion of the vesicular membrane into the plasma membrane from an exocytotic event causes a net expansion of the plasma membrane and therefore, lowers the membrane tension. Thus, prompt membrane retrieval via endocytosis is required to return the plasma membrane tension to homeostatic set point[1006]. The GTPase activity of dynamin is critical in endocytosis as it provides the mechanical force that elongates the neck of a budding endocytic vesicle and causes its subsequent fission from the plasma membrane. Dynamin is also involved in the maturation of endocytic vesicles by promoting the recruitment of an array of endocytic proteins, many of which directly bind to dynamin[1007].

In addition to its critical role in mediating endocytosis, there is an increasing appreciation for dynamin's role in regulating the exocytosis process. Fusion pore expansion during exocytosis is under the control of dynamin[1008,1009], which in turns governs the amount of vesicular content released. The rate of fusion pore expansion and quantal release are significantly reduced when the dynamin's action is disrupted[1008-1010]. Recent work from our laboratory demonstrated that the small molecule dynamin activator, Ryngo 1-23[1011], significantly increased the amount of catecholamine release in the murine chromaffin cells by promoting dynamin oligomerization, a process that is critical to stimulate the GTPase activity essential for its downstream mechanical actions[1012]. It is

believed Ryngo 1-23 does so by stabilizing the ring structure of the dynamin oligomer[1013], thereby stabilizing the opening of the fusion pore to enable catecholamine release. The aim of this study is to determine whether the release of GLP-1 and PYY from human colonic L cells could be triggered by activating dynamin with Ryngo 1-23 and other related dynamin activators. I hypothesize that dynamin is implicated in regulating the secretory process of enteroendocrine L cells. Previous work from our laboratory also showed that the positive effects of Ryngo 1-23 on murine adrenal chromaffin cells were completely abolished by disrupting F-actin polymerization or myosin II action with latrunculin B and blebbistatin, respectively[1012]. As actin and myosin II are major binding partners of dynamin, their roles in L cells secretion were also investigated.

4.2 Methods

4.2.1 Human tissue collection and *ex vivo* secretion experiments

Human colonic mucosae were obtained and prepared as described in previous sections. The tissue pieces were pre-treated with test reagents in a microfuge tube for 15 minutes at room temperature before they were transferred to a 96-well plate containing 250 µL pre-warmed Krebs buffer (control) or with test reagents for a 15 minute incubation at 37°C in 95%/5% O_2/CO_2 as previously described. High K^+ Krebs was used as positive control (in mM, NaCl 68, KCl 74.5, $CaCl_2$ 2.6, $NaHCO_3$ 4.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, HEPES 10, Glucose 5, 1 µM Sitagliptin, 0.1 % BSA). Active GLP-1 and total PYY levels were quantitated separately using commercially available ELISA kits as described in previous chapters.

4.2.2 Materials

Ryngo 1-23, Ryngo 3-37, CR5026A, CR6034B (all obtained from Prof. Phil Robinson, University of Sydney), Dynole 34-2 (abcam, ab120463), blebbistatin (Sigma Aldrich, B0560), latrunculin B (Sigma Aldrich, L5288) were all dissolved in DMSO at concentrations such that the final concentration of DMSO in any working solution did not exceed 0.1%. Stock solutions were stored at -20°C as single-use aliquots.

4.2.3 Statistical analysis

All statistical analysis was conducted as paired analyses, comparing response in tissues obtained from the same individual to relevant control conditions. A paired ratio Student's *t*-test was used for single comparisons. As secretion experiments did not always include a full sequence of all conditions used in this study, a paired one-way ANOVA with Fisher Least Significant Difference post-hoc test was used for multiple comparisons. Statistical significance was set at $p < 0.05$. All data are shown as mean ± SEM, unless stated otherwise.

4.3 Results

4.3.1 Dynamin activation significantly increased PYY and GLP-1 secretion from human colon

Incubation of human colonic mucosae with 1 µM Ryngo 1-23 significantly triggered GLP-1 and PYY release within 15 minutes (Figure 4.3.1: GLP-1: 2.68 ± 0.76 vs 8.62 ± 2.5 ng/mL per g tissue, n = 6, $p < 0.01$; PYY: 39.05 ± 4.92 vs 71.83 ± 13.87 ng/mL per g tissue, n = 13, $p < 0.05$). The concentration 1 µM was chosen based on *in vitro* results that showed this concentration was effective in increasing catecholamine release from murine chromaffin cells.

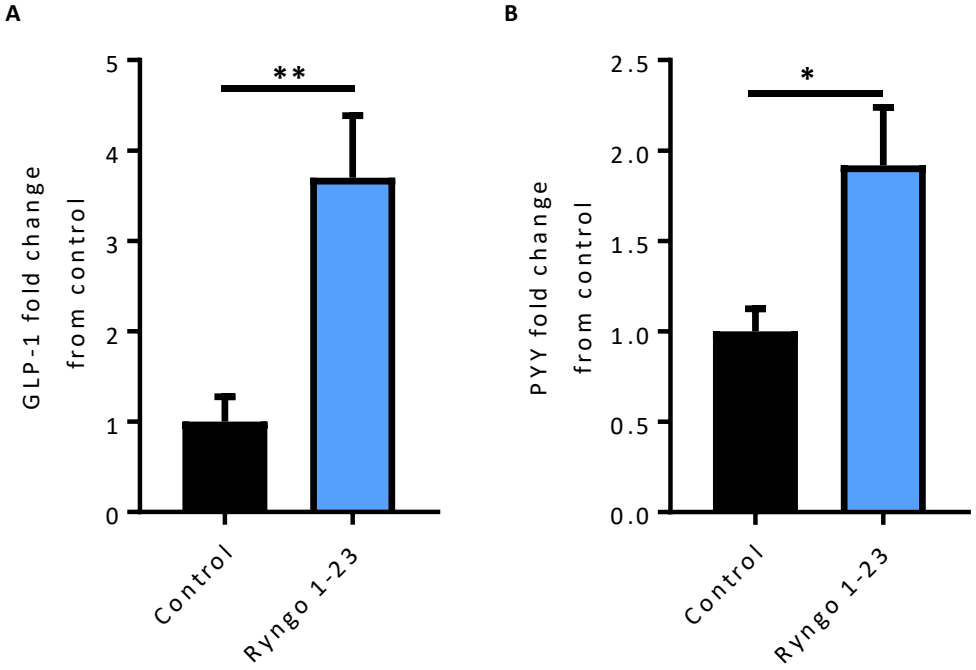


*Figure 4.3.1 1 µM Ryngo 1-23 significantly stimulated (A) GLP-1 (n = 13) and (B) PYY (n = 6) secretion from human colonic mucosae. *p < 0.05, **p < 0.01*

4.3.2 The effects of modulating dynamin, actin and myosin activity on PYY secretion from human colon

I next sought to elucidate the underlying mechanism of Ryngo 1-23-induced L cell secretion using a range of pharmacological tools. As illustrated in previous chapters, GLP-1 and PYY secretions are closely correlated, with PYY being a superior marker for colonic L ell secretion, the following experiments only assayed for PYY levels.

The effect of dynamin inhibition on basal and stimulated PYY secretion was investigated using the validated small molecule dynamin inhibitor, Dynole 3-42 (Figure 4.3.2.A). High potassium significantly triggered PYY release from basal levels (Control vs 70 mM K^+: 35.99 ± 5.26 vs 55.04 ± 6.91 ng/mL per g tissue, n = 7, $p < 0.001$). Dynole 3-42 had no effect on basal PYY release (1 µM

Dynole 3-42: 40.56 ± 10.73 ng/mL per g tissue, n = 7, $p > 0.05$ vs control) but significantly attenuated high K^+-stimulated PYY release (70 mM K^+ + 1 µM Dynole 3-42: 36.19 ± 8.94 ng/mL per g tissue, n = 7, $p < 0.05$ vs 70 mM K^+).

I next sought to investigate the mechanisms underlying Ryngo 1-23-induced PYY secretion. Surprisingly, Ryngo 1-23 did not increase PYY secretion from basal levels in the majority of the subsequent samples tested, despite the fact that all these samples responded to the positive control in this subsequent set of experiment (n = 7 out of 10, data not shown). Only three Ryngo 1-23 responders were therefore included in this mechanistic analysis relating to actin and myosin in dynamin-related L cell secretion.

There was a trend for Ryngo 1-23 to stimulate PYY secretion from basal level (Figure 4.3.2.B, Control vs 1 µM Ryngo 1-23: 23.74 ± 8.7 vs 27.12 ± 12.87 ng/mL per g tissue, n = 3, $p = 0.08$). Inhibiting actin polymerization with 200 nM latrunculin B in the presence of Ryngo 1-23 did not affect PYY release (25.49 ± 8.27 ng/mL per g tissue, n = 3, $p > 0.05$ vs 1 µM Ryngo 1-23), nor did myosin II inhibition with blebbistatin (1 µM Ryngo 1-23 + 10 µM Blebbistatin: 22.28 ± 5.70 ng/mL per g tissue, n = 3, $p > 0.05$ vs 1 µM Ryngo 1-23).

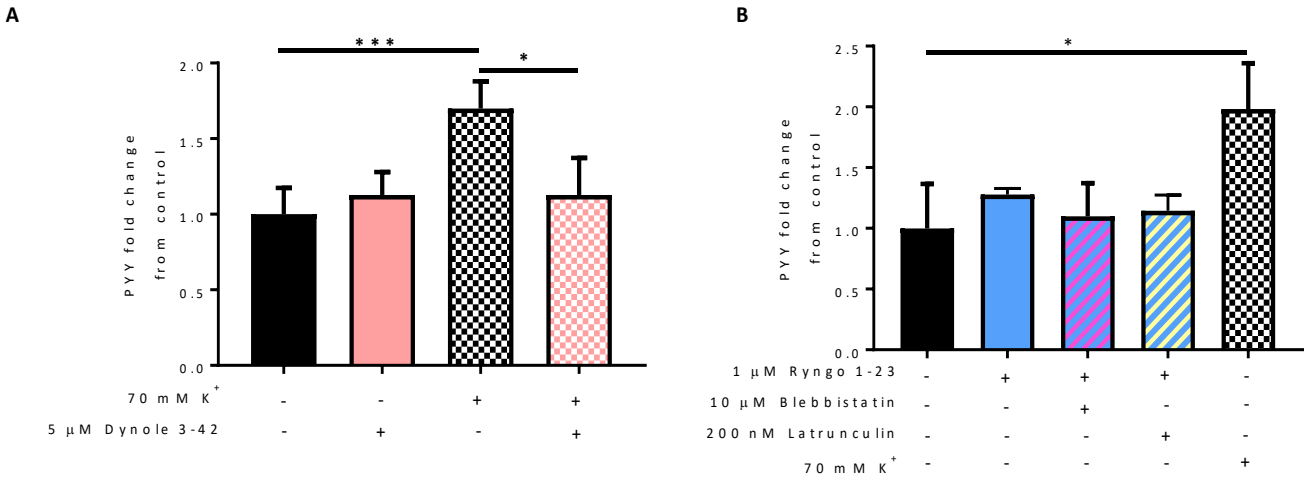


*Figure 4.3.2 The effects of inhibiting dynamin and its binding partners on PYY secretion from the human colon. (A) Dynamin inhibition with 5 µM Dynole 3-42 significantly attenuated high K^+-stimulated PYY secretion (n = 7 for all groups, *p < 0.05, ***p < 0.001); (B) Inhibition of actin polymerization with 200 nM latrunculin B and inhibition of myosin II with 10 µM blebbistatin (n = 3 for all groups, *p < 0.05).*

4.3.3 The effects of the newer generations of dynamin activators on PYY secretion from human colon

I next tested Ryngo 3-37 (Figure 4.3.3), a new generation of Ryngo compound that is more stable than Ryngo 1-23 at physiological temperature (37℃) (Prof. Phil Robinson, unpublished data and

personal communication). Ryngo 3-37 had a modest, but significant stimulatory effect on basal PYY secretion (Figure 4.3.5.A Control vs 5 µM Ryngo 3-37: 42.34 ± 6.05 vs 49.41 ± 7.44 ng/mL per g tissue, n = 13, $p < 0.05$). Ryngo 3-37 had no effect on high potassium-stimulated PYY secretion (70 mM K^+ vs 70 mM K^+ + 5 µM Ryngo 3-37: 67.1 ± 10.24 vs 65.12 ± 13.93 pg/mL per mg tissue, n = 13).

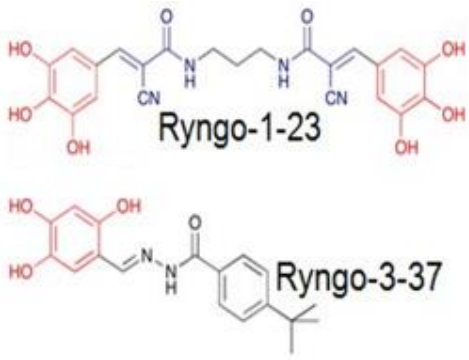


Figure 4.3.3 Chemical structures of Ryngo 1-23 and Ryngo 3-37.

I finally tested two other newly-developed dynamin activators, CR5026A and CR6034B (Professor Phil Robinson, University of Sydney). Both CR5026A and CR6034B have superior stability to the original Ryngo compounds (Prof. Phil Robinson, unpublished data and personal communication). 100 nM CR6034B significantly increased PYY secretion from basal levels (Control vs 100 nM CR6034B: 61.39 ± 12.01 vs 109.6 ± 35.0 ng/mL per g tissue, n = 5, $p < 0.05$). There is a trend for Ryngo 1-23, CR5026A, and other concentrations of CR6034B to increase PYY secretion from basal levels but these did not reach statistical significance for any of the concentrations tested.

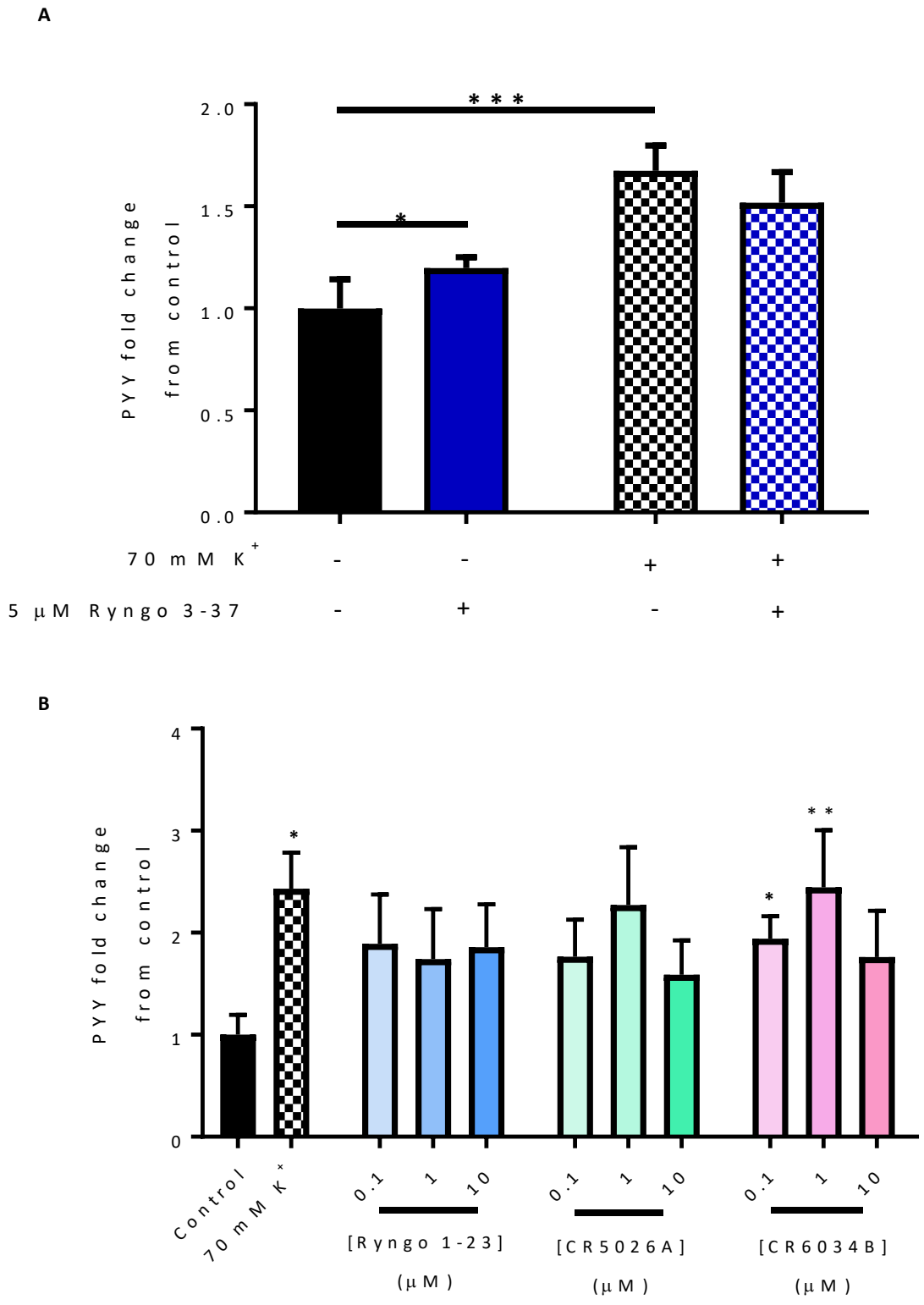


*Figure 4.3.4 The effects of new generation of dynamin activators on PYY secretion from the human colon. (A) 5 µM Ryngo 3-37 modestly increased PYY secretion from basal but not with high K^+ stimulation (n = 13); (B) There is a trend for a new batch of Ryngo 1-23 to increase PYY secretion from basal (n = 5). The new generation dynamin activator CR6034B significantly increased PYY secretion from basal at 10 nM and 1 µM but increases at 10 µM did not reach statistical significance (n = 5). There is also a trend for CR5026A to increase PYY secretion from basal at all concentrations tested (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001.*

4.4 Discussion and conclusion

Dynamin has emerged as an important regulator of exocytosis[1009,1012,1014], in addition to its well-known role in clathrin-mediated[1015] and clathrin-independent endocytosis[1016]. In this set of experiments, I first demonstrated that activating dynamin using Ryngo 1-23 significantly triggered L cell secretion. Exposure of human colonic mucosae to the drug for 15 minutes resulted in increased PYY and GLP-1 secretion by approximately two- and four-fold, respectively. This stimulatory effect was also observed with the new generations of dynamin activators, Ryngo 3-37 and CR6034B, which have superior *in vitro* stability to Ryngo 1-23 (Prof. Phil Robinson, unpublished data and personal communication). Moreover, the fact that high-K^+ stimulated PYY secretion is inhibited by Dynole 34-2, which inhibits dynamin GTPase activity, further supports the role of dynamin in regulating L cell secretion from human colonic mucosae. This is consistent with my original hypothesis and previous work from our laboratory, which clearly showed that the activation of dynamin significantly augments stimulated catecholamine release from murine adrenal chromaffin cells[1012]. However, one major limitation with the *ex vivo* static incubation approach is that it could not determine whether Ryngo 1-23 directly acts on L cells. Immunohistochemistry should be performed in subsequent studies to determine if L cells express dynamin 1 or other dynamin isoforms. Testing the effects of dynamin modulators on cell lines such as GLUTag, or FACS-purified murine L cells, would be informative.

Although dynamin is crucial in clarthrin-mediated endocytosis[1004], it is unlikely that Ryngo 1-23 increased L cell secretion by promoting vesicle recycling as dynamin activation by Ryngo 1-23 does not affect clarthrin-mediated endocytosis[1017]. Ryngo 1-23 promotes dynamin oligomerization and thus, stimulates its GTPase activity[1018], which stabilizes the fusion pore[1009] and slows fusion pore expansion[1012]. As such, the fusion pore is thought to be opened for longer, promoting the increased expulsion of vesicular contents into the extracellular space and thus, increased quantal release of L cell hormones[1012]. This notion is supported by the observation that dynamin activation with Ryngo 1-23 increases the amount of transmitter release per exocytotic event in murine adrenal chromaffin cells without affecting the frequency of these events, indicating a shift from “kiss and run” events[1012], a mode of exocytosis in which the secretory vesicle temporarily fuses with the target plasma membrane to release a small portion of its content before it is retrieved into the cytoplasm[1019], to full fusion. Such effects of dynamin activation are likely to be involved in Ryngo 1-23-induced GLP-1 and PYY secretion at basal conditions. Notably, although dynamin activation markedly stabilizes the opening of the fusion pore[1012], its effect on fusion pore size remains unknown. Fusion pore size is one of the parameters that can affect the amount of vesicular content released during an exocytotic event. Indeed, a smaller fusion pore size has been postulated to

underlie the difference in quantal release in enterochromaffin cells and adrenal chromaffin cells[993]. Fusion pore size in enterochromaffin cells is considerably smaller than that of adrenal chromaffin cells[993] and enterochromaffin cells release 70 times less transmitter per exocytotic event[109]. It is possible for dynamin activation to result in increased fusion pore size and thus, increases hormone release.

Contrasting the findings in murine adrenal chromaffin cells[1012], dynamin activation had no effect on high-K^+ stimulated PYY release from human colonic mucosal preparations. Such disparity is likely attributed to experimental approaches used to measure the effect of dynamin activation on hormone release. In previous chromaffin cell experiments, the duration of high K^+ stimulation was limited to 60 seconds[1012]. In contrast, a 15-minute static incubation was used in this current study, which would maximally stimulate cells over the course of incubation. As the maximal stimulation was achieved with high K^+ stimulation alone, it is unlikely for any positive regulators of the secretory process, such as dynamin activation, to exert any further effects on secretion. As maximal GLP-1 secretion invoked by high K^+ from perfused rat small intestine was achieved after 5 minutes and prolonged perfusion of the high K^+ solution did not further increase GLP-1 secretion[733], future experiments investigating the effect of Ryngo 1-23 on stimulated PYY secretion should be carried out with an incubation time under 5 minutes. It is also worth noting that L cells have a relatively high amount of secretion at baseline compared to chromaffin cells, and that the size of the respective vesicle contents being measured are vastly different. Small signalling molecules such as catecholamines are able to escape through a small, transiently flickering fusion pore, while the release of larger peptides require expanded fusion pores[1020]. To allow the relatively large peptide hormones PYY and GLP-1 to escape from secretory vesicles, expanded fusion pore width must be occurring in L cells at basal conditions, which could be induced by dynamin activators. During high K^+-stimulation, full fusion events dominate and thus, fusion pore expansion occurs regardless of the presence of dynamin activators, dwarfing any potential fusion pore expanding effects of dynamin activators.

Dynamin inhibition with Dynole 34-2 had no effect on basal PYY secretion but significantly attenuated high K^+-stimulated PYY secretion. Dynole 34-2 inhibits dynamin by inhibiting its GTPase activity[1021] and unlike Ryngo 1-23, exerts potent inhibitory effects on clathrin-mediated endocytosis[1017,1021]. It remains to be determined how much of this attenuation is attributed to impaired vesicle recycling and how much is due to its effects on fusion pore dynamics. Dynole 34-2 treatment significantly attenuated catecholamine release from high K^+-stimulated murine adrenal chromaffin cells by reducing the number of exocytotic events and quantal release per event[1012]. The former could be attributed to impaired vesicle recycling but the latter is likely accounted for by

changes in fusion pore dynamics. Therefore, it is plausible that Dynole 34-2 inhibits stimulated PYY release from human colon in a similar fashion.

The role of actin in mediating exocytosis has been widely studied. Trafficking of secretory vesicles from the reserve pool towards the plasma membrane is dependent on an intact actin cytoskeletal network. Exocytosis requires the partial depolymerization of the cortical actin network to enable the secretory vesicles that are "trapped" within the cortical actin meshwork to translocate towards the target plasma membrane[1022-1024]. Disruption of actin polymerization with cytochalasin D has been demonstrated to augment exocytosis[1025] while latrunculin B-induced F-actin depolymerization significantly increased insulin-induced GLP-1 secretion in GLUTag and NCI-H716 cells[1026]. However, emerging evidence supports a more dynamic role for actin in mediating exocytosis, in a dynamin-dependent manner[1027]. Contrary to its role in vesicle trafficking, it appears fusion pore expansion requires *de novo* polymerization of actin monomers[1028,1029], which is promoted by dynamin oligomerization. Conversely, F-actin polymerization also enhances dynamin oligomerization[1018,1030]. Indeed, inhibition of actin polymerization with cytochalasin B or latrunculin B completely abolished the effect of Ryngo 1-23 in augmenting catecholamine release from murine adrenal chromaffin cells[1012]. Thus, I sought to investigate whether interfering actin polymerization with latrunculin B would affect PYY secretion from human colonic mucosae. Although I did not observe any impact of latrunculin B treatment on basal PYY secretion in the presence of Ryngo 1-23, the role of actin-dynamin interaction in L cell exocytosis should not be discounted as the current study is very underpowered due to the low small number of Ryngo 1-23 responder. Disruption of *de novo* F-actin polymerization could contribute to destabilization of the fusion pore, which would oppose the fusion pore-stabilizing effect of dynamin activation. Indeed, Latrunculin B has been demonstrated to promote fusion pore closure in pancreatic acinar cells[1031]. Further studies are warranted to investigate how is the actin-dynamin interaction implicated in L cell hormone release.

The motor protein myosin II is a major binding partner of actin and its role in vesicle translocation along the actin cytoskeleton is well established. Disruption of myosin II function markedly reduce vesicle mobility and thus severely compromise the replenishment of vesicles in the readily releasable pool[1032,1033]. There is now an increasing appreciation for its role in controlling fusion pore dynamics[1034-1036], in addition to its well established role in vesicle trafficking. However, the present study is hugely underpowered to detect any effect of myosin II inhibition on Ryngo 1-23 induced PYY secretion. Results from the three Ryngo 1-23 responders suggest myosin II could be implicated as Ryngo-induced PYY release shows a trend to be reduced by blebbstatin. Upon membrane fusion, an actin-myosin II complex forms a coat around the fused vesicle to aid the expulsion of granule content into the extracellular space[1037,1038], although such mechanism may not relevant to all

secretory systems. For example, blebbistatin did not reduce quantal release per exocytotic event of catecholamines from adrenal chromaffin cells[1036] and serotonin from thrombin-stimulated platelets[1039]. Nonetheless, the function of actin-myosin coating is likely to be more relevant for high molecular weight vesicular cargoes, or larger vesicles[1027] where diffusion alone may not be sufficient for the released transmitter/hormone molecules to have diffused far enough away from the secreting cell before they are recaptured by rapid endocytosis following exocytosis. Results from a recent study using the *Gcg*-Venus transgenic mouse model suggests that compound exocytosis is the predominant mode of exocytosis in primary murine L cells[997]. In this mode of exocytosis, secretory vesicles fuse with another vesicle that is already fused with the plasma membrane (although fusion of multiple vesicles intracellularly has also been described)[1040]. Thus, the size of secretory vesicles in L cells is likely to be larger than those of chromaffin cells, despite comparable size prior to any fusion events[107,1041,1042]. Coupled with the fact that large peptides diffuse much more slowly than small molecules such as adrenaline or serotonin, expulsion of PYY and GLP-1 from L cell secretory granules may require additional mechanical force from the actin-myosin II complex. Indeed, the actin-myosin II complex has been implicated in the secretion of tear proteins from lacrimal acinar epithelial cells, a process that is also predominated by compound exocytosis[1038].

L cell hormones play important roles in maintaining metabolic homeostasis. The fact that dynamin activation by Ryngo 1-23 significantly increases L cell secretion makes it an attractive candidate for potential clinical development as treatment for diabetes and obesity. However, this prospect is significantly hampered by the susceptibility of Ryngo 1-23 to degradation in solution at physiological temperature (Prof. Phil Robinson, unpublished data and personal communication). Nonetheless, new generations of dynamin activators with improved stability profiles have been developed to overcome this problem. Crucially, the stimulatory effects on L cell were preserved in these new dynamin activators. Ryngo 3-37, a new generation dynamin activator, modestly increased basal PYY secretion. I also showed that CRs, which belong to the newest generation of dynamin activators that have even better stability than Ryngo 3-37, tended to increase basal PYY secretion. However, as the heterogeneity nature of human specimens significantly increases the sample size required to attain adequate statistical power, follow up experiments with larger sample sizes should be undertaken to confirm these findings. If these compounds are found to reliably trigger L cell secretion, subsequent *in vivo* testing of these drugs can be carried out in rodent models of obesity and diabetes to investigate if dynamin activation is beneficial in correcting dysregulated metabolism. Such *in vivo* experiments would also provide valuable insights into potential off-target effects of dynamin activation, such as its effect on the secretion of other gut hormones. While preliminary data from our laboratory suggests that serotonin secretion by enterochromaffin cells is not affected by

dynamin activation (Keating *et al.*, unpublished data), its effect on other enteroendocrine cell types is unknown.

In summary, this study demonstrated that dynamin activation could increase L cell hormone release and actin and myosin II are likely to be implicated in the process. However, the precise roles of these proteins in regulating L cell exocytosis warrants further investigations as the current study is vastly underpowered.

5 Gut-derived glucagon in human

5.1 Introduction

Fully-processed glucagon has recently been detected in the plasma of pancreatectomized patients and such extra-pancreatic source(s) of glucagon could only be triggered by enteral, but not parenteral glucose[463]. Results from this study, combined with early findings of glucagon-like immunoreactivity in the human gut epithelium[1043-1045], suggest that the intestinal epithelium is a source of glucagon that is identical to that of pancreatic origin. However, due to the high degree of structural similarities between glucagon and other proglucagon-derived peptides (PGDPs) produced by enteroendocrine L cells, concerns over the specificity of immunochemical detection methods such as radioimmunoassays (RIAs) and ELISAs have recently been raised[1046]. This is problematic as oral glucose is a strong stimulus for the secretion of an array of PGDPs that includes oxyntomodulin (OXM)[432], which not only contains the entire sequence of glucagon but also shares the same N-terminus. Several studies showed that commercially available glucagon ELISA and RIA kits suffer from marked cross-reactivity with oxyntomodulin and various forms of glicentin[481,1046-1048]. In fact, the initial findings of the LIBRA trial[1049] could not be replicated when the same samples were re-analyzed using a different glucagon ELISA assay from the original study, both of which are commercially available[1050], thus highlighting the technical difficulties of reliably measuring glucagon levels and its implications. Therefore, immunochemical approaches alone are insufficient to confirm the presence of fully-processed glucagon in the gut as the abundance of these cross-reacting species in gut epithelial tissue lysates may be higher than that of glucagon by several orders of magnitude.

A mass spectrometry (MS)-based approach can circumvent the issue of antibody specificity as the peptide is identified based on its amino acid sequence, rather than the structure of specific epitopes. Thus, structural similarities between closely related peptides do not interfere with peptide detection. However, detection of glucagon using MS is not without major obstacles. The challenges of glucagon detection and quantitation in complex samples employing a peptidomic-based approach has long been acknowledged as sensitivity of instrumentation and the problem of dynamic range markedly hinders detection of low abundance peptides such as glucagon[1051]. Sample preparation for MS-based analysis of glucagon is complicated by the peptide's poor aqueous solubility at physiological pH and susceptibility to degradation extreme pHs[1052,1053]. The fact that both glicentin and oxyntomdulin contain the full 29-amino acid sequence of glucagon renders it a poor candidate for peptidase digestion upstream of MS detection. Furthermore, ionization of the glucagon peptide typically results in at least three molecular ion species[1054], which further reduces sensitivity of MS-

based methods. Notably, upstream immunoenrichment has been shown to considerably enhance the sensitivity of MS-based assays[1055]. Indeed, a MS-based glucagon assay for analyzing plasma samples was recently developed using such an approach[1056]. Therefore, the first aim of this study was to detect the presence of glucagon from the human gut epithelial extract, a considerably more complex sample than plasma, using a MS-based method with the aid of immunoenrichment.

The second aim of this study was to investigate what triggers the secretion of glucagon from the gut. Since the release of extra-pancreatic glucagon in pancreatectomized patients is triggered by the enteral, but not parenteral glucose[463], I hypothesize that the gut epithelium is a source of glucagon and its release can be triggered by exposure to high glucose. Moreover, since amino acids are potent stimuli for glucagon secretion[1057,1058], I hypothesize that the amino acid, arginine, could trigger the release of glucagon from the gut.

5.2 Materials and methods

5.2.1 Materials

Acetonitrile, pure formic acid and 0.1% formic acid, all MS-grade, were purchased from Sigma Aldrich. Sitagliptin was provided by Pfizer Inc. Recombinant human glucagon (Glucagen®, Novo Nordisk) was purchased from Flinders Medical Centre pharmacy, reconstituted in deionized water to make 1 mg/mL stock and stored in aliquots at -20°C. Oxyntomodulin (OXM) was purchased from Phoenix Peptides (Catalogue no. 028-22).

5.2.2 Human tissue collection

Consented surgical specimens were obtained from the Flinders Medical Centre and Flinders Private Hospital colorectal unit. Ileum and colon specimens were collected from patients undergoing bowel resection for cancer or stoma reversal. In the case of resection specimens, samples were obtained from sites there were at least 10 cm away from tumour location. Specimens from patients that had any form of inflammatory bowel diseases were excluded from this study. The specimens were immediately placed in iced-cold Krebs buffer and transported to the laboratory for dissection within 15 minutes as described previously. The specimens were rinsed with iced-cold Krebs buffer to remove any luminal contents and blood clots and dissected clear of adipose, muscular and connective tissue. The mucosae were separated from the submucosae with stainless steel spring scissors, cut into pieces of approximately 80 - 100 mg for secretion studies or snap frozen in liquid nitrogen and stored at -80°C until analysis.

5.2.3 Sample preparation for LC-MS

The sample preparation procedure was adapted from the methods reported by Kuhre *et al*.[241]. Snap frozen tissue was weighed and transferred to a 2 mL Eppendorf SafeLock tube (Eppendorf, Germany), to which lysis buffer (1 % formic acid, supplemented with 100 µM sitagliptin and 1:100 protease inhibitor cocktail (T8340, Sigma Aldrich)) was added at a ratio of 1 mL/100 mg tissue. The tissue was homogenized using a 5 mm stainless steel bead and bead mill (TissueLyzer, Qiagen) at 30 Hz for 20 minutes. The lysate was left to stand at room temperature (RT) for 1 hour before it was centrifuged to remove debris (3300 x *g* for 15 minutes at RT) and the pH of the supernatant was adjusted to pH 3 with NaOH. 300 µL of the pH-adjusted supernatant was added to commercially available ELISA plate pre-coated with monoclonal antibody directed at the N-terminus of glucagon (10-1271-01, Mercodia), left to incubate at RT overnight on a plate shaker at 600 rpm. The supernatants were subsequently aspirated and the wells washed three times with 1X PBS (Life Technologies). Bound peptides were eluted by adding 100 µL of 70 % acetonitrile in 0.1 % formic acid incubated at RT for 15 minutes on a plate shaker at 600 rpm. The eluents were then transferred to MS autosampler vials (Thermo Pierce) and the ACN was completely evaporated in a vacuum concentrator (Martin Christ, RVC2-33). The samples were then placed into the autosampler for LC-MS analysis.

5.2.4 Liquid chromatography-mass spectrometry (LC-MS)

Analysis of peptides was carried out using a TripleTOF™ 5600$^+$ mass spectrometer (AB Sciex) coupled to an Eksigent nanoLC 400 nano HPLC. Samples were applied to a 10mm x 300 µm ProteCol™ trap column (C18, 120 Å 3 µm, SGE Analytical Science) and eluted onto a 15 cm C18 120 Å 5 µm analytical column (Nikkyos Technos) using a 2 to 40% acetonitrile gradient over 42 min. The instrument was operated in high sensitivity positive ion mode, employing dynamic accumulation and rolling collision energy. MS scans covering the 350 to 1600 m/z mass range were performed using a 50 ms accumulation time. MS/MS scans were performed employing a 150 ms maximum accumulation time over a mass range of 100 to 1500 m/z. Ions were selected for fragmentation via the following criteria: charge state > 2; intensity > 400 counts per second (cps); mass tolerance 20 mDa. LC-MS results were searched against human Uniprot database using ProteinPilot 4.0 (AB Sciex) with a no-digest setting.

5.2.5 Secretion experiments:

Mucosal pieces were incubated with 200 µL of buffer (control) or buffer containing test agents in a 24-well plate for 15 minutes. The buffer was a modified Krebs buffer described previously with the addition of sitagliptin 1 µM and 0.1 % BSA (A1595, Sigma Aldrich, Australia) at pH 7.4. Following

incubation at 37°C in 95% O_2/5% CO_2, supernatants were collected and stored in aliquots at -20°C. Glucagon levels were quantitated using a validated commercially available sandwich ELISA, according to manufacturer's instructions (10-1271-01, Mercodia, Uppsala, Sweden). Oxyntomodulin levels were quantitated using a prototype sandwich ELISA[432] (Mercodia, Uppsala, Sweden) by Dr. Nicolai Wewer Albrechtsen at the University of Copenhagen, Denmark.

5.2.6 Statistical analysis:

All statistical analysis was conducted as paired analyses, comparing responses in tissues obtained from the same individual. A paired-ratio Student's t-test was used for single comparisons. Statistical significance was $p < 0.05$. All data are shown as mean ± SEM.

5.3 Results

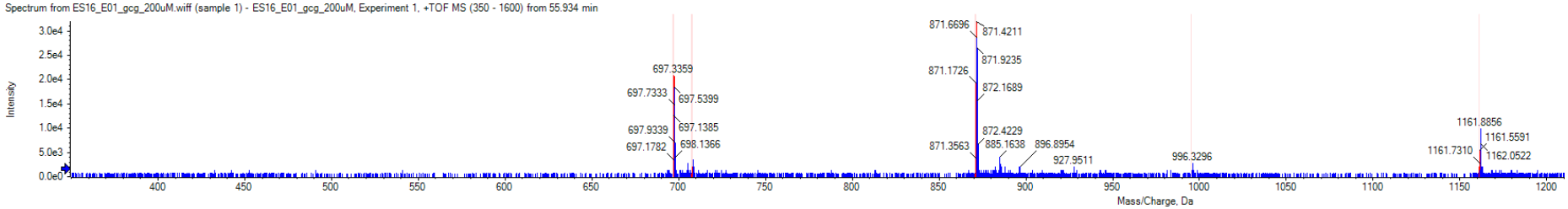
5.3.1 LC-MS analysis of recombinant glucagon

200 µM recombinant human glucagon was subjected to LC-MS and the elution time of glucagon using this gradient was 55.94 min. Electrospray ionization produced three distinct ion species (Figure 5.3.1.A): $[M+5H^+]^{5+}$ of 697.1 m/z, $[M+4H^+]^{4+}$ of 871.1 m/z and $[M+3H^+]^{3+}$ of 1161.1 m/z. Further fragmentation of all three parent ion species were achieved and produced sufficient fragment ions (Figure 5.3.1.B,C). The MS-MS spectra of these fragment ions were analysed the Analyst software. Detected peptide fragments generated from the 4-charge parent ion ($[M+4H^+]^{4+}$) is depicted in Figure 5.3.1.D. These results indicate that the intact glucagon can be adequately ionized and satisfactory fragmentations can be achieved without upstream enzyme digestion.

5.3.2 Fully-processed glucagon in human ileal mucosal lysate was detected by LC-MS

A very low level of glucagon was detected in the immune-enriched human ileal mucosal extract, as indicated by the low intensity. Due to a much higher sample complexity, glucagon in the extract eluted later at 58.1 minute (Figure 5.3.2.A). Only 2 ion species ($[M+5H^+]^{5+}$ and $[M+4H^+]^{4+}$) were reliably detected (Figure 5.3.2.B and C, respectively). Of the two parent ions, only the 5-charge ion was fragmented in subsequent MS/MS experiment (Figure 5.3.2.D). Initial analysis using the Analyst® software identified 9 peptide fragments that matched the glucagon sequence (Figure 5.3.2.E, m/z of these fragment ions are indicated in bold red). Subsequent analysis using the ProteinPilot® software identified 11 more peptide fragments that matched to glucagon, providing a total sequence coverage of 69%.

A


Spectrum from ES16_E01_gcg_200uM.wiff (sample 1) - ES16_E01_gcg_200uM, Experiment 1, +TOF MS (350 - 1600) from 55.934 min
Intensity
Mass/Charge, Da
697.3359
697.7333
697.5399
697.9339
697.1385
697.1782
698.1366
871.6696
871.4211
871.9235
871.1726
872.1689
872.4229
871.3563
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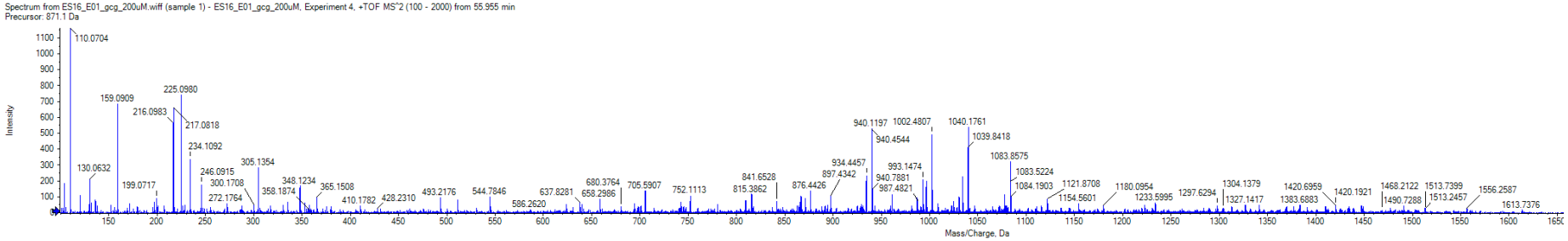
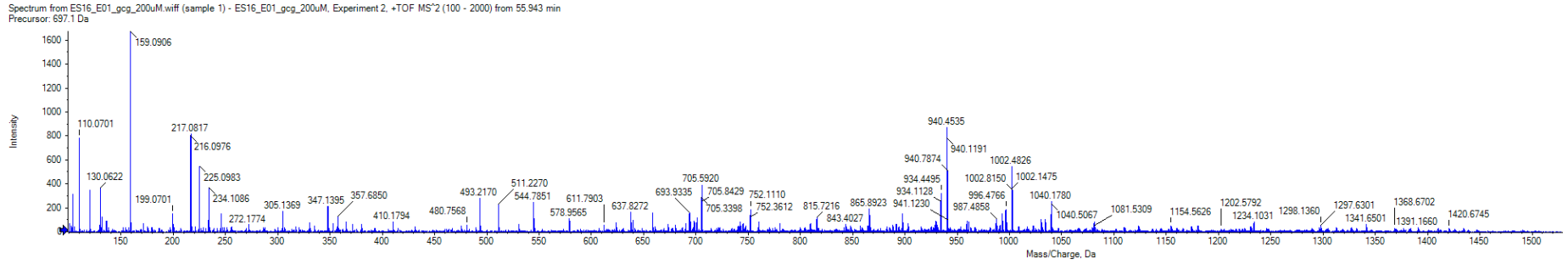
1161.5591
1161.7310
1162.0522

B


Spectrum from ES16_E01_gcg_200uM.wiff (sample 1) - ES16_E01_gcg_200uM, Experiment 4, +TOF MS^2 (100 - 2000) from 55.955 min
Precursor: 871.1 Da
Intensity
Mass/Charge, Da
110.0704
159.0909
225.0980
216.0983
217.0818
234.1092
130.0632
246.0915
305.1354
199.0717
300.1708
348.1234
365.1508
272.1764
358.1874
410.1782
428.2310
493.2176
544.7846
637.8281
586.2620
680.3764
658.2986
705.5907
752.1113
841.6528
815.3862
876.4426
934.4457
897.4342
940.1197
1002.4807
940.4544
993.1474
940.7881
987.4821
1040.1761
1039.8418
1083.8575
1083.5224
1084.1903
1121.8708
1154.5601
1180.0954
1233.5995
1297.6294
1304.1379
1327.1417
1383.6883
1420.6959
1420.1921
1468.2122
1490.7288
1513.7399
1513.2457
1556.2587
1613.7376

C


Spectrum from ES16_E01_gcg_200uM.wiff (sample 1) - ES16_E01_gcg_200uM, Experiment 2, +TOF MS^2 (100 - 2000) from 55.943 min
Precursor: 697.1 Da
Intensity
Mass/Charge, Da
159.0906
110.0701
217.0817
216.0976
130.0622
225.0983
234.1086
199.0701
305.1369
347.1395
357.6850
272.1774
410.1794
480.7568
493.2170
511.2270
544.7851
611.7903
578.9565
637.8272
693.9335
705.5920
705.8429
705.3398
752.1110
752.3612
815.7216
865.8923
843.4027
940.4535
940.1191
940.7874
934.4495
934.1128
941.1230
1002.4826
1002.8150
1002.1475
996.4766
987.4858
1040.1780
1040.5067
1081.5309
1154.5626
1202.5792
1234.1031
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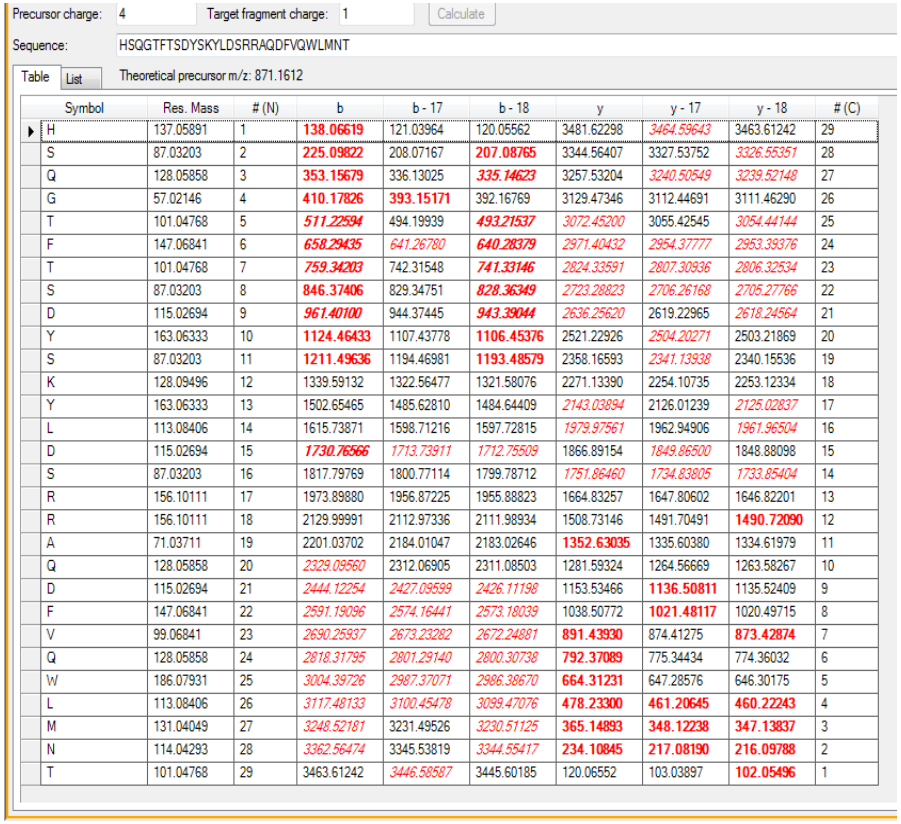
1368.6702
1391.1660
1420.6745

D



Precursor charge: 4 Target fragment charge: 1 Calculate

Sequence: HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Table List Theoretical precursor m/z: 871.1612

Symbol	Res. Mass	# (N)	b	b - 17	b - 18	y	y - 17	y - 18	# (C)
H	137.05891	1	**138.06619**	121.03964	120.05562	3481.62298	*3464.59643*	3463.61242	29
S	87.03203	2	**225.09822**	208.07167	**207.08765**	3344.56407	3327.53752	*3326.55351*	28
Q	128.05858	3	**353.15679**	336.13025	***335.14623***	3257.53204	*3240.50549*	*3239.52148*	27
G	57.02146	4	**410.17826**	**393.15171**	392.16769	3129.47346	3112.44691	3111.46290	26
T	101.04768	5	***511.22594***	494.19939	***493.21537***	*3072.45200*	3055.42545	*3054.44144*	25
F	147.06841	6	***658.29435***	*641.26780*	***640.28379***	*2971.40432*	*2954.37777*	*2953.39376*	24
T	101.04768	7	***759.34203***	742.31548	***741.33146***	*2824.33591*	*2807.30936*	*2806.32534*	23
S	87.03203	8	**846.37406**	829.34751	***828.36349***	*2723.28823*	*2706.26168*	*2705.27766*	22
D	115.02694	9	***961.40100***	944.37445	***943.39044***	*2636.25620*	2619.22965	*2618.24564*	21
Y	163.06333	10	**1124.46433**	1107.43778	**1106.45376**	2521.22926	*2504.20271*	2503.21869	20
S	87.03203	11	**1211.49636**	1194.46981	**1193.48579**	2358.16593	*2341.13938*	2340.15536	19
K	128.09496	12	1339.59132	1322.56477	1321.58076	2271.13390	2254.10735	2253.12334	18
Y	163.06333	13	1502.65465	1485.62810	1484.64409	*2143.03894*	2126.01239	*2125.02837*	17
L	113.08406	14	1615.73871	1598.71216	1597.72815	*1979.97561*	1962.94906	*1961.96504*	16
D	115.02694	15	***1730.76566***	*1713.73911*	*1712.75509*	1866.89154	*1849.86500*	1848.88098	15
S	87.03203	16	1817.79769	1800.77114	1799.78712	*1751.86460*	*1734.83805*	*1733.85404*	14
R	156.10111	17	1973.89880	1956.87225	1955.88823	1664.83257	1647.80602	1646.82201	13
R	156.10111	18	2129.99991	2112.97336	2111.98934	1508.73146	1491.70491	**1490.72090**	12
A	71.03711	19	2201.03702	2184.01047	2183.02646	**1352.63035**	1335.60380	1334.61979	11
Q	128.05858	20	*2329.09560*	2312.06905	2311.08503	1281.59324	1264.56669	1263.58267	10
D	115.02694	21	*2444.12254*	*2427.09599*	*2426.11198*	1153.53466	**1136.50811**	1135.52409	9
F	147.06841	22	*2591.19096*	*2574.16441*	*2573.18039*	1038.50772	**1021.48117**	1020.49715	8
V	99.06841	23	*2690.25937*	*2673.23282*	*2672.24881*	**891.43930**	874.41275	**873.42874**	7
Q	128.05858	24	*2818.31795*	*2801.29140*	*2800.30738*	**792.37089**	775.34434	774.36032	6
W	186.07931	25	*3004.39726*	*2987.37071*	*2986.38670*	**664.31231**	647.28576	646.30175	5
L	113.08406	26	*3117.48133*	*3100.45478*	*3099.47076*	**478.23300**	**461.20645**	**460.22243**	4
M	131.04049	27	*3248.52181*	3231.49526	*3230.51125*	**365.14893**	**348.12238**	**347.13837**	3
N	114.04293	28	*3362.56474*	3345.53819	*3344.55417*	**234.10845**	**217.08190**	**216.09788**	2
T	101.04768	29	3463.61242	*3446.58587*	3445.60185	120.06552	103.03897	**102.05496**	1

HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (Glucagon amino acid sequence)

H
HS
HSQ
HSQG
HSQGT
HSQGTF
HSQGTFT
HSQGTFTS
HSQGTFTSD
HSQGTFTSDY
HSQGTFTSDYS

HSQGTFTSDYSKYLD

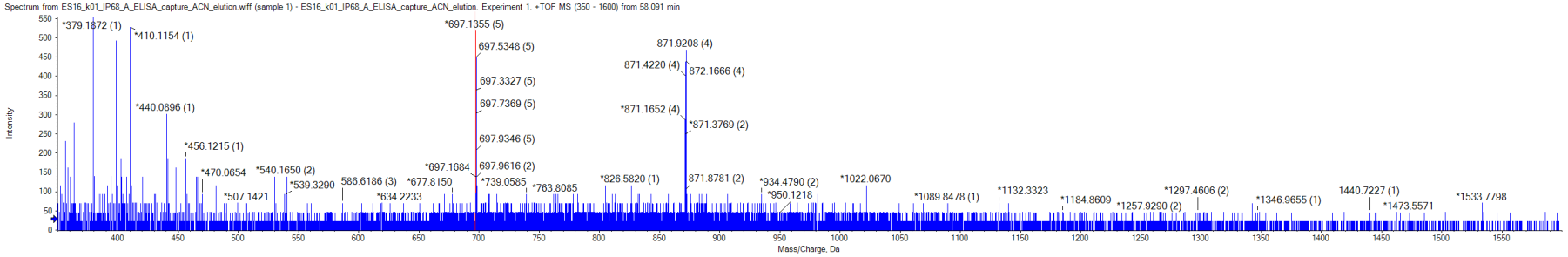
RAQDFVQWLMNT
AQDFVQWLMNT

DFVQWLMNT
FVQWLMNT
VQWLMNT
QWLMNT
WLMNT
LMNT
MNT
NT
T

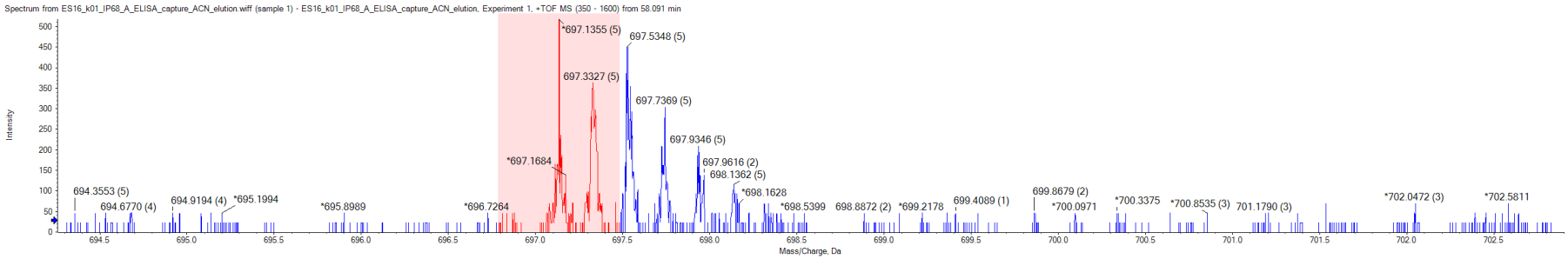
Glucagon peptide fragments from the 4-charge parent ion (m/z 871.1) detected by MS

Figure 5.3.1 Product ion spectra of recombinant human glucagon (Glucagen®). (A) A full scan MS spectrum showing glucagon was eluted at 55.94 min and formed three ion species: 697.1 m/z, 871.1 m/z and 1161.1 m/z. (B) MS/MS spectrum of $[M+4H^+]^{4+}$ ion series (871.1 m/z). (C) MS/MS spectrum of $[M+5H^+]^{5+}$ ion series (697.1 m/z). (D) m/z of peptide fragments generated from the 4-charge parent ion that matched the glucagon amino acid sequence are indicated in bold red. b: b-ion series (N-terminus), b-17: b ion with the loss of ammonia during fragmentation (-17 Da), b-18: b ion with a loss of water during fragmentation (-18 Da). y: y-ion series (C-termins), y-17: y ion with the loss of ammonia during fragmentation (-17 Da), y-18: y ion with the loss of water during fragmentation (-18 Da).

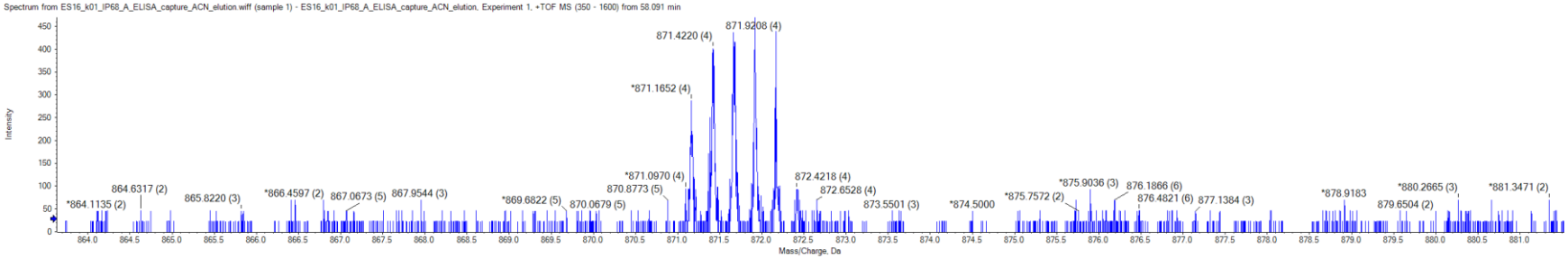
A


Spectrum from ES16_k01_IP68_A_ELISA_capture_ACN_elution.wiff (sample 1) - ES16_k01_IP68_A_ELISA_capture_ACN_elution, Experiment 1, +TOF MS (350 - 1600) from 58.091 min
Intensity
Mass/Charge, Da
*379.1372 (1)
*410.1154 (1)
*440.0896 (1)
*456.1215 (1)
*470.0654
*507.1421
*540.1650 (2)
*539.3290
586.6186 (3)
*634.2233
*677.8150
*697.1684
*697.1355 (5)
697.5348 (5)
697.3327 (5)
697.7369 (5)
697.9346 (5)
697.9616 (2)
*739.0585
*763.8085
*826.5820 (1)
871.9208 (4)
871.4220 (4)
872.1666 (4)
*871.1652 (4)
*871.3769 (2)
871.8781 (2)
*934.4790 (2)
*950.1218
*1022.0670
*1089.8478 (1)
*1132.3323
*1184.8609
*1257.9290 (2)
*1297.4606 (2)
*1346.9655 (1)
1440.7227 (1)
*1473.5571
*1533.7798

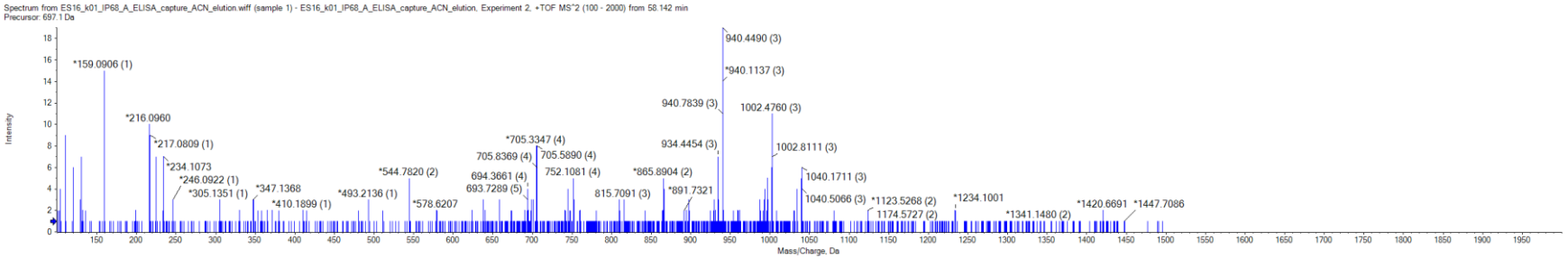
B


Spectrum from ES16_k01_IP68_A_ELISA_capture_ACN_elution.wiff (sample 1) - ES16_k01_IP68_A_ELISA_capture_ACN_elution, Experiment 1, +TOF MS (350 - 1600) from 58.091 min
Intensity
Mass/Charge, Da
694.3553 (5)
694.6770 (4)
694.9194 (4)
*695.1994
*695.8989
*696.7264
*697.1684
*697.1355 (5)
697.5348 (5)
697.3327 (5)
697.7369 (5)
697.9346 (5)
697.9616 (2)
698.1362 (5)
*698.1628
*698.5399
698.8872 (2)
*699.2178
699.4089 (1)
699.8679 (2)
*700.0971
*700.3375
*700.8535 (3)
701.1790 (3)
*702.0472 (3)
*702.5811

C


Spectrum from ES16_k01_IP68_A_ELISA_capture_ACN_elution.wiff (sample 1) - ES16_k01_IP68_A_ELISA_capture_ACN_elution, Experiment 1, +TOF MS (350 - 1600) from 58.091 min
Intensity
Mass/Charge, Da
871.9208 (4)
871.4220 (4)
*871.1652 (4)
*871.0970 (4)
870.8773 (5)
872.4218 (4)
872.6528 (4)
*864.1135 (2)
864.6317 (2)
865.8220 (3)
*866.4597 (2)
867.0673 (5)
867.9544 (3)
*869.6822 (5)
870.0679 (5)
873.5501 (3)
*874.5000
*875.7572 (2)
*875.9036 (3)
876.1866 (6)
876.4821 (6)
877.1384 (3)
*878.9183
879.6504 (2)
*880.2665 (3)
*881.3471 (2)

D


Spectrum from ES16_k01_IP68_A_ELISA_capture_ACN_elution.wiff (sample 1) - ES16_k01_IP68_A_ELISA_capture_ACN_elution, Experiment 2, +TOF MS^2 (100 - 2000) from 58.142 min
Precursor: 697.1 Da
Intensity
Mass/Charge, Da
940.4490 (3)
*940.1137 (3)
940.7839 (3)
1002.4760 (3)
*159.0906 (1)
*216.0960
*217.0809 (1)
*705.3347 (4)
934.4454 (3)
1002.8111 (3)
*234.1073
705.8369 (4)
705.5890 (4)
*544.7820 (2)
694.3661 (4)
752.1081 (4)
*865.8904 (2)
1040.1711 (3)
*246.0922 (1)
693.7289 (5)
*305.1351 (1)
*347.1368
*493.2136 (1)
815.7091 (3)
*891.7321
1040.5066 (3)
*1123.5268 (2)
*1234.1001
*410.1899 (1)
*578.6207
1174.5727 (2)
*1341.1480 (2)
*1420.6691
*1447.7086

E

Precursor charge: 5 Target fragment charge: 1 Calculate

Sequence: HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Table | List | Theoretical precursor m/z: 697.1304

	Symbol	Res. Mass	# (N)	b	b - 17	b - 18	y	y - 17	y - 18	# (C)
▶	H	137.05891	1	138.06619	121.03964	120.05562	3481.62298	*3464.59643*	*3463.61242*	29
	S	87.03203	2	**225.09822**	208.07167	207.08765	3344.56407	3327.53752	3326.55351	28
	Q	128.05858	3	**353.15679**	336.13025	335.14623	3257.53204	3240.50549	3239.52148	27
	G	57.02146	4	410.17826	393.15171	392.16769	3129.47346	3112.44691	3111.46290	26
	T	101.04768	5	**511.22594**	494.19939	**493.21537**	3072.45200	3055.42545	3054.44144	25
	F	147.06841	6	**658.29435**	641.26780	**640.28379**	*2971.40432*	2954.37777	2953.39376	24
	T	101.04768	7	*759.34203*	742.31548	***741.33146***	2824.33591	2807.30936	2806.32534	23
	S	87.03203	8	846.37406	829.34751	828.36349	2723.28823	2706.26168	2705.27766	22
	D	115.02694	9	**961.40100**	944.37445	943.39044	2636.25620	2619.22965	2618.24564	21
	Y	163.06333	10	1124.46433	1107.43778	1106.45376	2521.22926	2504.20271	2503.21869	20
	S	87.03203	11	1211.49636	1194.46981	1193.48579	2358.16593	2341.13938	2340.15536	19
	K	128.09496	12	1339.59132	1322.56477	1321.58076	2271.13390	2254.10735	2253.12334	18
	Y	163.06333	13	1502.65465	1485.62810	1484.64409	2143.03894	2126.01239	2125.02837	17
	L	113.08406	14	1615.73871	1598.71216	1597.72815	1979.97561	1962.94906	1961.96504	16
	D	115.02694	15	*1730.76566*	1713.73911	1712.75509	1866.89154	1849.86500	1848.88098	15
	S	87.03203	16	1817.79769	1800.77114	1799.78712	1751.86460	*1734.83805*	*1733.85404*	14
	R	156.10111	17	*1973.89880*	1956.87225	1955.88823	1664.83257	1647.80602	1646.82201	13
	R	156.10111	18	2129.99991	2112.97336	2111.98934	1508.73146	1491.70491	1490.72090	12
	A	71.03711	19	2201.03702	2184.01047	2183.02646	1352.63035	1335.60380	1334.61979	11
	Q	128.05858	20	2329.09560	2312.06905	2311.08503	1281.59324	1264.56669	1263.58267	10
	D	115.02694	21	*2444.12254*	*2427.09599*	*2426.11198*	1153.53466	1136.50811	1135.52409	9
	F	147.06841	22	*2591.19096*	2574.16441	2573.18039	1038.50772	1021.48117	1020.49715	8
	V	99.06841	23	*2690.25937*	*2673.23282*	2672.24881	891.43930	874.41275	873.42874	7
	Q	128.05858	24	*2818.31795*	*2801.29140*	*2800.30738*	792.37089	775.34434	774.36032	6
	W	186.07931	25	*3004.39726*	*2987.37071*	*2986.38670*	664.31231	647.28576	646.30175	5
	L	113.08406	26	*3117.48133*	*3100.45478*	*3099.47076*	478.23300	461.20645	460.22243	4
	M	131.04049	27	3248.52181	3231.49526	3230.51125	**365.14893**	**348.12238**	**347.13837**	3
	N	114.04293	28	*3362.56474*	3345.53819	3344.55417	**234.10845**	**217.08190**	**216.09788**	2
	T	101.04768	29	*3463.61242*	3446.58587	3445.60185	**120.06552**	103.03897	**102.05496**	1

HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (Glucagon amino acid sequence)

HS
HSQ

HSQGT
HSQGTF
HSQGTFT

HSQGTFTSD

MNT
NT
T

Glucagon peptide fragments from the 3-charge parent ion (m/z 697.1) detected by MS

F

Fragmentation Evidence for Peptide

HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Residue	b	b+2	y	y+2
H	138.0662	69.5367	3481.6230	1741.3151
S	**225.0982**	113.0527	3344.5641	1672.7857
Q	**353.1568**	177.0820	3257.5320	1629.2697
G	**410.1783**	205.5928	3129.4735	1565.2404
T	**511.2259**	**256.1166**	3072.4520	1536.7296
F	**658.2944**	329.6508	2971.4043	1486.2058
T	**759.3420**	**380.1747**	2824.3359	1412.6716
S	846.3741	423.6907	2723.2882	1362.1478
D	**961.4010**	**481.2041**	2636.2562	1318.6317
Y	**1124.4643**	562.7358	2521.2293	1261.1183
S	1211.4964	606.2518	2358.1659	1179.5866
K	1339.5913	670.2993	2271.1339	1136.0706
Y	1502.6546	**751.8310**	2143.0389	1072.0231
L	1615.7387	808.3730	1979.9756	**990.4914**
D	1730.7657	**865.8865**	1866.8915	933.9494
S	1817.7977	909.4025	1751.8646	**876.4359**
R	1973.8988	**987.4530**	1664.8326	832.9199
R	2129.9999	**1065.5036**	1508.7315	754.8694
A	2201.0370	1101.0221	1352.6303	676.8188
Q	2329.0956	1165.0514	1281.5932	641.3003
D	2444.1225	1222.5649	1153.5347	577.2710
F	2591.1910	1296.0991	1038.5077	519.7575
V	2690.2594	1345.6333	**891.4393**	446.2233
Q	2818.3179	**1409.6626**	792.3709	396.6891
W	3004.3973	1502.7023	664.3123	332.6598
L	3117.4813	1559.2443	478.2330	239.6201
M	3248.5218	1624.7645	**365.1489**	183.0781
N	3362.5647	1681.7860	**234.1084**	117.5579
T	3463.6124	1732.3098	**120.0655**	60.5364

HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (Glucagon amino acid sequence)

HSQG

HSQGTFT

HSQGTFTSDY

HSQGTFTSDYSKY

LDSRRAQDFVQWLMNT

HSQGTFTSDYSKYLD

SRRAQDFVQWLMNT

HSQGTFTSDYSKYLDSR

HSQGTFTSDYSKYLDSRR

VQWLMNT

HSQGTFTSDYSKYLDSRRAQ

Glucagon peptide fragments from the 3-charge parent ion (m/z 697.1) identified by ProteinPilot® that were not identified in the preliminary analysis by Analyst®.

Figure 5.3.2 Full scan and product ion scan spectra of human ileal mucosal lysate after immunoenrichment (A) Mucosal lysate full scan MS spectrum. Glucagon was eluted at 58.1 minutes, the $[M+5H^+]^{5+}$ ion was detected (as indicated in red line) and selected for fragmentation. (B) A mass window of 694-701m/z was isolated showing the 5+ charge state ion series selected for fragmentation (red shade). (C) A mass window of 864-875 m/z was isolated showing the 4+ charge state ion series. (D) MS/MS spectrum of the $[M+5H^+]^{5+}$ ion. (E) m/z of peptide fragments generated from the 5-charge parent ion that matched the glucagon amino acid sequence are indicated in bold red using Analyst®. b: b-ion series (N-terminus), b-17: b ion with the loss of ammonia during fragmentation (-17 Da), b-18: b ion with a loss of water during fragmentation (-18 Da). y: y-ion series (C-terminus), y-17: y ion with the loss of ammonia during fragmentation (-17 Da), y-18: y ion with the loss of water during fragmentation (-18 Da). (E) Analysis with ProteinPilot® software identified further peptide fragments matching the glucagon amino acid sequence.

5.3.3 Glucagon is released from ex vivo preparations of human ileal and colonic epithelia

Glucagon was readily detectable in *ex vivo* secretion assay supernatants from human ileal epithelia at basal levels but its release did not appear to be triggered by any of the stimulants tested (Figure 5.3.3.A). Glucagon release from human colonic epithelia was detectable at basal (Figure 5.3.3.B, Control: 0.611 ± 0.17 ng/mL/g tissue, n = 8) and was reliably triggered by IBMX/FSK (IBMX/FSK: 1.30 ± 0.55 ng/mL/g tissue, n = 8, $p < 0.05$), high potassium (70 mM K^+: 0.85 ± 0.17 ng/mL/g tissue, n = 8, $p < 0.05$) and arginine (20 mM Arg: 1.74 ± 0.46 ng/mL/g tissue, n = 6, $p < 0.05$). However, glucagon was not detectable in any of the colonic preparations that were collected from specimens proximal to the sigmoid colon (data not shown).

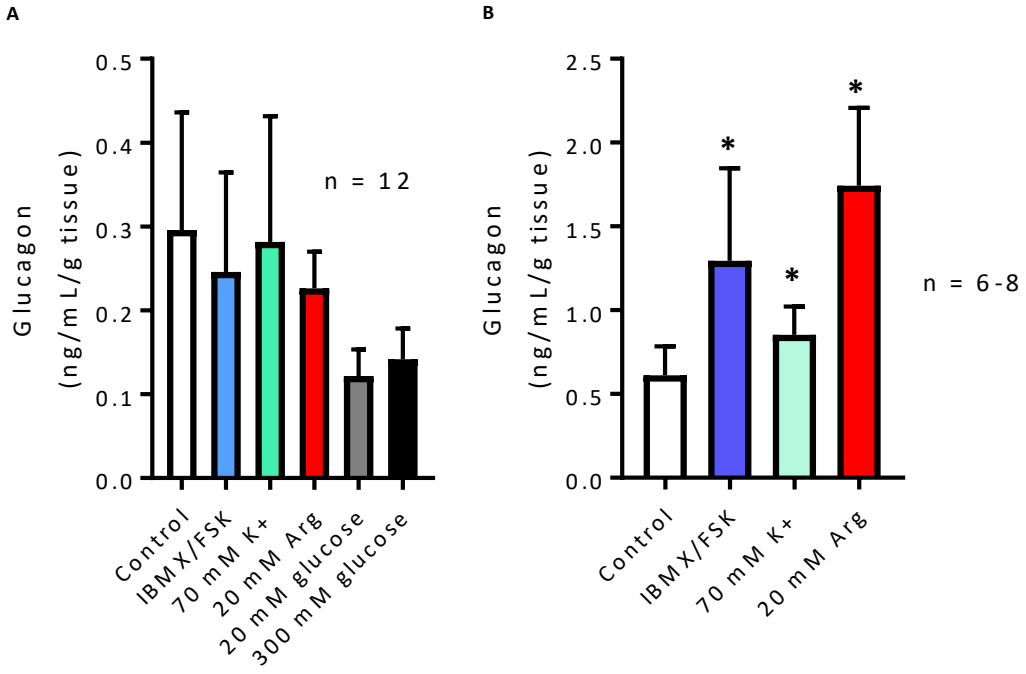


*Figure 5.3.3 In initial experiments, glucagon was readily detectable in secretion assay supertants from human ileal (A) and sigmoid colonic (B) epithelial preparations (*p < 0.05 vs control).*

5.3.4 Glucagon immunoreactivity in secretion is not due to cross-reactivity with oxyntomodulin

Several studies reported the true cross-reactivity of the Mercodia glucagon sandwich ELISA with oxyntomodulin is considerably higher than the reported value of <4.4% by the manufacturer[481,1048]. Thus, it was pertinent to verify the fidelity of the ELISA in my hands. At concentrations below 100 pM, the cross-reactivity of the ELISA with OXM was 5.4%, which is similar to that reported by the manufacturer. However, at 1 nM, the cross-reactivity was much higher at 12.5% (Figure 5.3.4.A), which is similar to that reported by others[481,1048]. Therefore, in order to confirm glucagon detected by the Mercodia sandwich ELISA was not due to potential cross-reactivity with OXM 20 samples

(basal and arginine-stimulated) were assayed for their OXM content using an OXM sandwich ELISA[432]. OXM concentrations in all samples tested were less than 20 pM. As 20 pM OXM could, at most, only account for 2.5 pM of detected glucagon, assuming cross-reactivity is 12.5% (likely to be an overestimate for concentrations less than 100 pM), OXM in these samples could not account for the majority of the detected glucagon immunoreactivity (Figure 5.3.4.B).

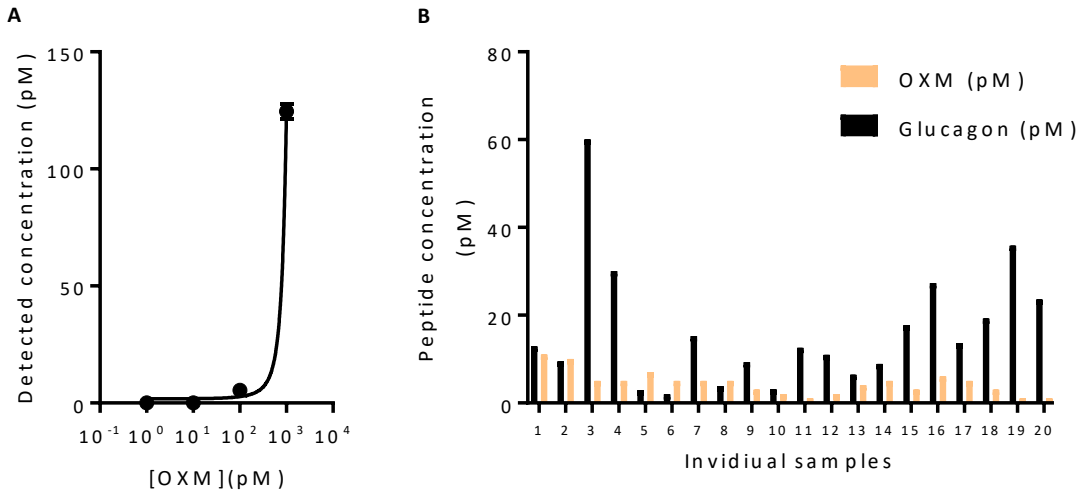


Figure 5.3.4 (A) Cross-reactivity curve for oxyntomodulin (OXM) for Mercodia glucagon sandwich ELISA (n = 3); (B) Glucagon and OXM concentrations in 20 individual samples.

5.3.5 Glucose and arginine stimulate glucagon release from human ileal epithelia

The lack of stimulated glucagon response from ileal preparations was of huge contrast to that of colonic preparations, which gave remarkably consistent responses. Such differences prompted me to question if the lack of response from ileal preparations was due to sample preparation. The mucosa can be easily isolated as an intact sheet from colonic specimens using a stainless-steel spatula in a scraping motion. However, due to the presence of villi on ileal mucosa, the tissue integrity of ileal preparations was significantly compromised when the same technique was employed. Thus, in order to preserve tissue integrity, the mucosa was very carefully dissected from the rest of the specimen using sharp spring scissors instead of being scraped off with a spatula. Nutrient-stimulated experiments were then repeated on samples prepared using this new approach. Arginine significantly triggered glucagon secretion in these preparations (Figure 5.3.5.A: Control vs 20 mM Arg: 0.19 ± 0.03 ng/mL/g tissue vs 0.47 ± 0.10 ng/mL/g tissue, n = 12, $p < 0.0001$). Exposure to 300 mM glucose also significantly increased glucagon release (Figure 5.3.5.B: Control vs 300 mM glucose: 0.31 ± 0.09 ng/mL/g tissue vs 0.90 ± 0.14 ng/mL/g tissue, n = 9, $p < 0.001$) but 20 mM did not significantly trigger release (20 mM glucose: 0.30 ± 0.05 ng/mL/g tissue, n = 9).

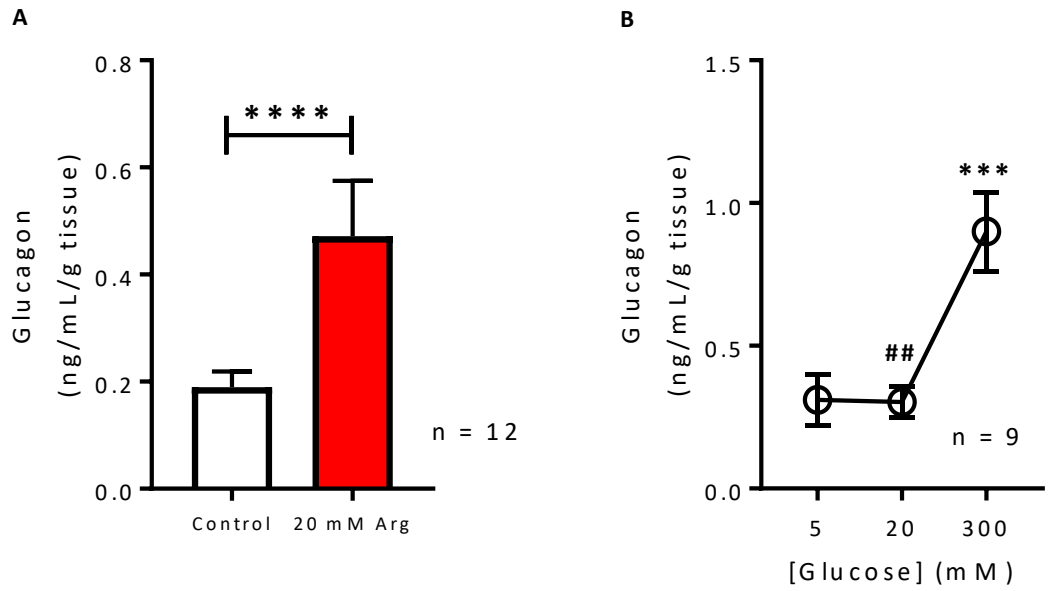


*Figure 5.3.5 Nutrient-induced glucagon secretion from human ileal epithelia. (A) 20 mM Arginine significantly triggered glucagon secretio. (B) Glucose significantly triggered glucagon secretion at 300 mM (***p < 0.001 vs control, n = 9) but not at 20 mM ($^{\#\#}$p < 0.01 vs 300 mM glucose, n = 9).*

5.3.6 Glucose-induced glucagon secretion is not attenuated by SGLT1 inhibition, K_{ATP} channel opening or somatostatin

Since the glucose transporter SGLT1 and K_{ATP} channels are both implicated in glucose-induced GLP-1 secretion[252,733], I sought to investigate if these pathways are also responsible for glucose-induced glucagon secretion in the human ileum. 300 mM glucose significantly increased glucagon secretion (Figure 5.3.6.A: Control vs 300 mM glucose: 0.15 ± 0.03 ng/mL/g tissue vs 0.31 ± 0.04 ng/mL/g tissue, n = 7, $p < 0.01$) but neither SGLT1 blockade with phlorizin (1 mM phlorizin: 0.36 ± 0.05 ng/mL/g tissue, n = 7, $p < 0.01$ vs control) or K_{ATP} channel opening with diazoxide (500 µM diazoxide: 0.46 ± 0.06 ng/mL/g tissue, n = 7, $p < 0.001$ vs control) attenuated glucose-induced glucagon secretion. As it has been postulated that glucose-induced glucagon suppression within pancreatic islets is mediated by somatostatin[1059], I investigated if a 15-minute pre-treatment with somatostatin would attenuate glucose-induced glucagon secretion from the *ex vivo* ileal epithelial preparations. High glucose significantly triggered glucagon secretion (Figure 5.3.6.B: Control vs 300 mM glucose: 0.05 ± 0.01 ng/mL/g tissue vs 0.38 ± 0.15 ng/mL/g tissue, n = 5, $p < 0.05$) and this response was not blocked by somatostatin (10 nM somatostatin: 0.25 ± 0.13 ng/mL/g tissue, n = 5, $p < 0.05$ vs control).

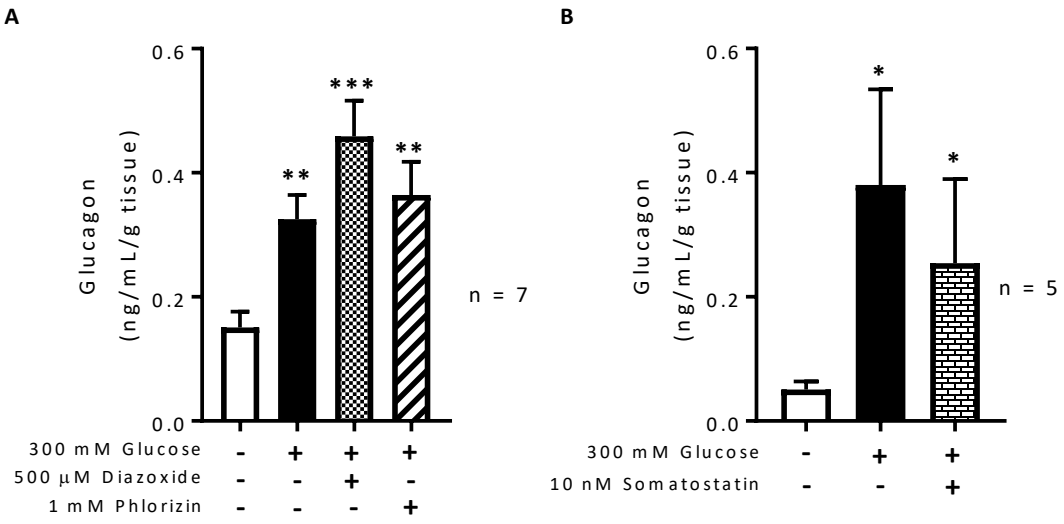


Figure 5.3.6 Glucose-sensing mechanisms of glucagon-secreting cells in human ileal epithelia. (A) Glucose-induced glucagon secretion was not attenuated by the SGLT1 inhibitor, Phlorizin or the K_{ATP} channel open, diazoxide. (B) Somatostatin did not attenuate glucose-induced glucagon secretion.

5.3.7 Glucagon secretion is correlated with GLP-1, but not oxyntomodulin secretion from human ileal epithelia

While there is a strong positive correlation between the glucagon and GLP-1 secretions within the same sample, at baseline and upon stimulation (Figure 5.3.7.A), such a correlation does not exist between glucagon and OXM concentrations (Figure 5.3.7.B), although this could be due to the small number of samples that were assayed for their OXM contents. Nonetheless, contrary to the stimulatory effect of arginine on glucagon secretion, 20 mM arginine significantly attenuated OXM secretion (Figure 5.3.7.C: Control vs 20 mM Arginine: 0.27 ± 0.08 ng/mL/g tissue vs 0.04 ± 0.01 ng/mL/g tissue, n = 5, $p < 0.01$).

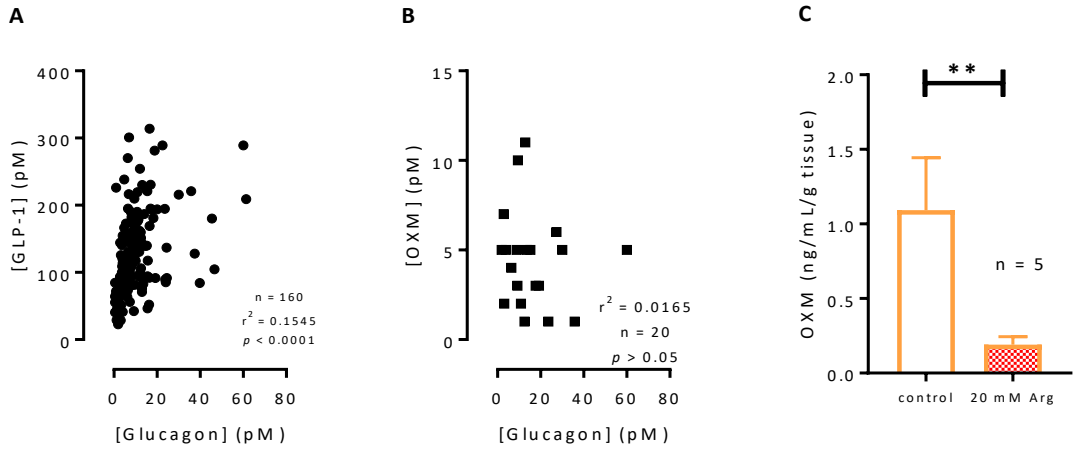


Figure 5.3.7 Correlation of glucagon concentration with other L cell products (A) GLP-1 and (B) OXM. (C) Arginine significantly reduced ileal OXM secretion from basal levels.

5.4 Discussion and Conclusion

5.4.1 The human gut epithelium is a source of fully-processed glucagon

The observation that the release of glucagon was triggered by enteral, but not parenteral glucose in pancreatectomized patients[457,463] strongly suggests that the gut could be a source of extra-pancreatic glucagon, or that gut-based mechanisms regulate the release of extra-pancreatic glucagon in these patients. As such, I hypothesized that the human gut epithelium is a source of fully-processed glucagon. Results from the first part of the current study are the first to provide mass-spectrometry evidence to confirm the long-standing speculation that the human small intestine epithelium is a source of fully processed glucagon, which is consistent with my original hypothesis. While the presence of glucagon-like immunoreactivity in the human epithelium was reported more than three decades ago[1043-1045], peptide immunochemical detection is based on epitope structure, rather than the amino acid sequence of the peptide, as in MS-based approaches. The gut epithelium is a rich source of glicentin and oxyntomodulin, both of which contain the full sequence of glucagon, and thus, the identical epitopes as glucagon, which could give rise to considerable glucagon-immunoreactivity. Although glucagon was detected in the plasmas of pancreatectomized patients using MS[463], the authors could not conclude that the gut epithelium was the source of extra-pancreatic glucagon as they were not able to detect glucagon in endoscopic biopsies of the small intestine using MS despite extensive effort (personal communication, Prof. Filip Knop and Dr. Nicolai Wewer Alberchtsen). As I was able to detect glucagon from terminal ileum specimens, it is likely that glucagon-secreting cells reside in the distal small intestine, rather than the proximal small intestine, from which these endoscopic biopsies were obtained (personal communication, Prof. Filip Knop).

To date, the presence of extra-pancreatic glucagon is only demonstrated in pancreatectomized patients[463], which in itself is an extreme physiological model and raises the important question of whether extra-pancreatic glucagon is present under normal physiological conditions or only arises upon complete elimination of pancreatic α cells as a compensatory response. The fact that none of the specimens used in this study were from pancreatectomized patients and glucagon was reliably detected from secretion supernatants of ileal and colonic mucosal preparations strongly supports the former notion.

In the second part of the study, I first demonstrated glucagon is released from the human gut epithelia. Importantly, the main cross-reacting species, oxyntomodulin, was present at concentrations that would not be sufficient to account for most of the glucagon-immunoreactivity in the samples quantitated, which adds confidence that the results obtained from the assay reflect the true concentrations of glucagon in the samples. I then established that glucagon secretion could be

reliably triggered by high glucose and arginine from human ileal mucosae. I also showed that glucagon is released from the distal colon in response to high potassium, arginine and the combination of IBMX/FSK.

5.4.2 Glucose-induced glucagon secretion

The fact that glucagon is triggered by glucose at concentrations that resemble postprandial luminal, but not vascular, glucose levels indicates that the glucose-sensing machinery of glucagon-secreting cells is likely to be localized to the apical membrane, resembling that of enteroendocrine L cells, which also express *GCG*. This finding is in agreement with an *in vivo* report, which demonstrated that only oral glucose, but not intravenous glucose, could trigger glucagon secretion in pancreatectomized patients[463]. The current finding could also potentially explain the highly prevalent, although often underappreciated, paradoxical phenomenon of postprandial hyperglucagonaemia in gastric-bypass patients[87,257,313,475,1060-1065]. Anatomical rearrangement of the GI tract that increases the delivery of nutrients to the L cell-rich proximal small intestine following meal ingestion stimulates near-pharmacological levels of GLP-1 secretion[19,20], which would result in intra-islet hyperinsulinaemia. As both insulin[1066-1070] and GLP-1[87,298,326] inhibit glucagon secretion, postprandial hyperglucagonaemia due to the hypersecretion of glucagon by pancreatic α cells becomes difficult to reconcile in the presence of these glucagonostatic forces. Instead, the presence of glucagon-secreting cells in the distal small intestine would secrete glucagon in response to nutrient exposure, in a similar manner to that of incretin-secreting cells, potentially provides a logical explanation. The fact that high glucose could trigger the release of glucagon from the gut could also adequately explain the observation that oral glucose is less potent in suppressing glucagon secretion than parenteral glucose in isoglycaemic infusion experiments[1071]. Hyperglycaemia induced by parenteral glucose potently suppresses glucagon secretion from pancreatic α cells without affecting glucagon-secreting cells in the gut, resulting in net reduction in glucagon levels. On the other hand, oral glucose has opposing effects on α cells and glucagon-secreting cells in the gut: hyperglycaemia induced by oral glucose suppresses α cell secretion but luminal glucose exposure stimulates glucagon release from the gut, resulting in an overall reduced magnitude of glucagon suppression. In fact, in healthy individuals, if the oral glucose load is sufficiently large, glucagon levels actually increase[478].

As SGLT1 and K_{ATP} channels are both implicated in glucose-induced GLP-1 secretion from L cells and glucose-induced glucagon suppression from pancreatic α cells, I sought to investigate if glucagon secretion from the human ileum shares these similar pathways. Surprisingly, neither inhibition of SGLT1 and SGLT2 with phlorizin, nor K_{ATP} channel opening with diazoxide, attenuated glucose-induced glucagon secretion from the ileum. Such an observation indicates that it is highly unlikely

that glucagon and GLP-1 originates from the same population of enteroendocrine cells. Therefore, it seems likely that PC1/3 and PC2 are differentially expressed in distinct populations of *GCG*-expressing enteroendocrine cells, with the former releasing GLP-1 and the latter releasing glucagon in response to glucose stimulation.

Two recent clinical case studies reported that somatostatin treatment improved glycaemic control and importantly, markedly attenuated glucagon secretion, in two pancreatectomized patients[1072,1073]. This suggests somatostatin could regulate the release of extra-pancreatic glucagon, in addition to tonically inhibiting GLP-1 release L cells via somatostatin subtype-5 receptor[148,1074]. I therefore investigated if somatostatin treatment could attenuate glucose-induced glucagon secretion from the gut. Contrary to these clinical observations, somatostatin had no significant impact on glucose-induced glucagon secretion from the human ileal mucosal preparations. However, since somatostatin receptors are expressed by enteric neurons[1075], it is possible that somatostatin regulates glucagon secretion indirectly via neural pathways, which were not present in my *ex vivo* preparation.

Since the molar quantity of glucagon released from the gut is remarkably lower than GLP-1 (Fig. 5.4.5 A), the amount of tissue required for glucagon detection in each experimental setup was substantially greater, which markedly limited the number of inhibitors I could test on each specimen. While I demonstrated in a previous chapter that glucose-induced GLP-1 secretion is not caused by osmotic stress, it remains to be determined if glucose-induced glucagon secretion is osmotically driven. In addition, as I, and others[733,769], have demonstrated that the facilitative glucose transporter, GLUT2, plays a pivotal role in glucose-induced GLP-1 secretion, and that GLUT1 is implicated in glucose-sensing in α cells[1076], the role of glucose transporters in mediating glucose-induced glucagon secretion in the gut needs to be investigated. Moreover, although K_{ATP} channel did not appear to mediate the stimulatory effects of glucose on glucagon secretion from the ileum, the importance of glucose metabolism warrants further investigation as there is evidence supporting the role of glucokinase[1077] and sacroplasmatic reticulum Ca^{2+} ATPase[1078] in mediating glucose-sensing in α cells, which could also be present in glucagon-secreting cells in the gut. While the expression of sweet taste receptors has not been reported in α cells, both pancreatic β cells[1079,1080] and enteroendocrine L cells[697,770] express functional sweet taste receptors. Therefore, it is possible that glucagon-secreting cells in the gut sense glucose via sweet taste receptors. There is also the possibility that glucose triggers glucagon secretion from the gut indirectly via the paracrine action of other hormones such as GLP-1 and GLP-2. However, the fact that glucose-induced glucagon secretion was not attenuated by phlorizin or diazoxide strongly refutes such a possibility, as these same compounds significantly dampen glucose-induced GLP-1 release.

5.4.3 Arginine-induced glucagon secretion

Glucagon is a critical regulator of amino acid metabolism[471,1081] and conversely, amino acids are potent α cell secretagogues[1082,1083]. Thus, I sought to determine if the amino acids also stimulate the secretion of glucagon from the gut epithelium. Arginine was chosen as it has been used extensively both *in vitro* and clinically to assess α cell function[458,1057,1067,1082,1084], a concentration of 20 mM was chosen based on that used for static incubation experiments in islets[1085] and pancreas perfusion experiments[1086]. I established that arginine is capable of triggering glucagon secretion from ileal and colonic epithelia, similar to that from pancreatic α cells. While it is beyond the scope of this study to elucidate the underlying mechanisms, it is not unreasonable to speculate that significant overlaps may exist between the amino acid sensing mechanisms in glucagon-secreting cells, other enteroendocrine cells and islet cells. Amino acid-induced exocytosis in enteroendocrine L cells[708] and pancreatic α and β cells[1087,1088] is believed to be primarily driven by electrogenic transport via amino transporters in the solute carrier gene superfamily. Interestingly, glutamine elevates intracellular cAMP and evoke a calcium response in L cells in the absence of extracellular Na^+ or Ca^{2+}, which suggests GPCR-mediated pathways could supplement the predominantly electrogenic response[708]. The identities of such GPCRs remain elusive and could well differ between different populations of enteroendocrine cells. It is possible that similar mechanisms underlie arginine-induced glucagon secretion from the gut epithelium. The fact that high potassium and forskolin/IBMX treatments potently triggered glucagon release from colonic epithelia preparations supports the notion that an electrogenic and a cAMP component are both implicated in the secretion of glucagon in the gut. Although enteroendocrine cells release gut hormones in response to an array of amino acids and oligopeptides *in vitro*[708,756,1089], clinical experiments showed that such stimulatory effects are only apparent with oral administration[1058]. This indicates the amino acid-sensing machinery is localized to the apical membrane. The major limitation of the *ex vivo* experimental setup is that the polarity of the mucosal preparation is not preserved and as such, I cannot confirm if arginine-induced glucagon secretion from the gut epithelium is apically-mediated. However, the fact that immunoreactive glucagon levels remained unaltered upon intravenous arginine infusion in pancreatectomized patients[458] strongly supports the notion that arginine-induced glucagon secretion from the intestine is driven by apical mechanisms.

Given that the small intestine is the primary site of amino acid absorption, the fact that arginine potently stimulated glucagon secretion from colonic mucosal preparation was somewhat unexpected as it suggests the colon epithelium has the capacity to sense amino acids despite a relatively low exposure to them exogenously. Notably, a similar phenomenon has been reported with colonic L cells in which glutamine, and an array of other amino acids, serve as potent GLP-1 and

PYY secretagogues[708] through a calcium-sensing receptor-dependent pathway[1090]. This confirms the amino acid-sensing capacity of some colonic enteroendocrine cells. It is worth noting that although the colonic epithelial cells are not generally exposed to high levels of amino acids derived from ingested proteins as their small intestinal counterparts are, they are exposed to amino acids produced by the gut microbiome[1091]. Whilst *de novo* amino acid biosynthesis by the intestinal flora may not be a major contributor to the overall amino acid intake of the host[1092], it is possible for bacterially-derived amino acids to modulate gut hormone secretion locally within the colonic epithelium, where glucagon-secreting cells reside. The intestinal mucosa plays a prominent role in amino acid metabolism[1093,1094], consistent with the high turnover rate of epithelial cells. Given glucagon's crucial role in regulating hepatic amino acid metabolism[471], it is possible that gut-derived glucagon serves as a local regulator of intestinal amino acid metabolism.

5.4.4 Potential sources of gut-derived glucagon

The source of gut-derived glucagon remains to be determined. Findings from the present study indicate there is a distinct population of *GCG*-expressing enteroendocrine cells in the human small intestine and distal colon that secrete glucagon. Glucagon is the cleavage product of PC2 (encoded by *PCSK2*) while GLP-1 and oxyntomodulin are derived from PC1/3 (encoded by *PCSK1*)[1095] (see Figure 1.3.5). It remains to be determined whether these glucagon-secreting cells belong to a subset of L cells that express both *PCSK1* and *PCSK2,* and are capable of secreting both glucagon and GLP-1, or if they constitute a distinct population that expresses only *PCSK2* and only secrete glucagon. However, such a task remains technically challenging as co-staining for glucagon and GLP-1 is hindered by identical epitopes between glucagon and OXM, GLP-1 and MPGF, meaning that most side-viewing antibodies would not be appropriate for such a purpose. Notably, co-localization of glucagon, PC1/3 and PC2 have been reported in murine embryonic ileal L cells, although the authors reported only very low levels of PC2 staining and that glucagon-positive cells were completely absent in neonatal and adult mice[1096]. Nonetheless, it provides support that the molecular machinery to produce "pancreatic glucagon" does exist in the gut. Moreover, it should be noted that the relatively low levels of glucagon detected in the secretion assay supernatants suggests that glucagon is likely produced in relatively small amount in the gut by a scarce population of cells, further reducing the likelihood of detection with immunohistochemical approaches. Given the remarkable plasticity of the hormonal profile of enteroendocrine cells[102,103,1097,1098], it is possible that *PCSK2* expression is upregulated under conditions of metabolic challenge. Indeed, it has recently been demonstrated that *PCSK2* expression in the small intestine is higher in T2D patients when compared with healthy controls[1099]. Such an observation mirrors that seen in pancreatic α cells, in which *PCSK1* expression is markedly upregulated to increase GLP-1 production by α cells upon

metabolic challenges such as high fat diet[1100], inflammation[95], STZ-induced diabetes[1101] and pregnancy[239]. However, while α cells co-express PC1/3 and PC2[1101] and GLP-1 co-localizes with glucagon granules in individual pancreatic α cells[239], results from the current experiment suggests this is unlikely to be case for GLP-1-secreting enteroendocrine L cells. The differential glucagon and GLP-1 responses to phlorizin and diaxozide in the presence of high glucose suggest the two peptides are unlikely to be co-released from the same cell. The fact that arginine treatment stimulates glucagon secretion and inhibits oxyntomodulin (a PC1/3 cleavage product) secretion supports the notion that these hormones exist in different cells. Moreover, a recent clinical experiment that employed a MS-based multiplex assay to quantitate postprandial PGDPs in healthy individuals reported that although both GLP-1 and glucagon levels increased postprandially, the excursion profile of glucagon substantially differed from that of GLP-1, contrasting oxyntomodulin, which closely tightly matched the postprandial excursion profile of GLP-1. However, it remains perplexing that arginine exerts an inhibitory effect on OXM secretion as clinical experiments showed that arginine, administered orally or intravenously, had no effect on GLP-1 levels[1058]. It is generally assumed that OXM is secreted by GLP-1-secreting cells, considering the two peptides are both products of PC1/3 and that almost all GLP-1 contained OXM vesicles[1102]. Nonetheless, it remains possible that the two peptides are stored in separate vesicles and their exocytosis are differentially regulated. [1103]Together, these observations led the authors to suggest that glucagon and GLP-1 were released postprandially by different cells[1056]. Thus, gut-glucagon is likely the product of a specific population of *GCG*-expressing enteroendocrine cells that expresses only *PCSK2* and not *PCSK1*.

5.4.5 Potential physiological functions of gut-derived glucagon

5.4.5.1 Protection from postprandial hypoglycaemia

It is well-established that oral glucose is less potent at suppressing glucagon secretion[477,478,1104-1106] and reducing hepatic glucose output[1071] than intravenous glucose, despite oral glucose being a more effective stimulus for insulin secretion (due to the incretin effect). However, this paradox may reflect an inherent protective mechanism against postprandial hypoglycaemia, as previously speculated[469]. This is best illustrated in an oral glucose challenge followed by isoglycaemic intravenous glucose infusion (IIGI), in which the glycaemic profile of the IIGI is tightly matched with that from the oral glucose challenge by constantly adjusting the rate of glucose infusion[463,1071]. The amount of circulating glucose in these two scenarios is identical, but oral glucose has a much higher insulinotropic potency due to the incretin effect. Accordingly, the amount of circulating glucose per unit of circulating insulin is much lower after oral glucose. This results in a significantly higher disposal rate of exogenous glucose, presumably because each unit of insulin can direct the uptake of

a definitive amount of glucose by insulin-sensitive tissues before hepatic extraction. Therefore the amount of circulating glucose remaining after insulin-mediated glucose disposal would be lower in the case of oral glucose compared with intravenous glucose infusion. Thus, the risk of hypoglycaemia is higher if no counter-regulatory mechanisms are in place. Maintaining an adequate level of hepatic glucose output with a modest amount of glucagon therefore minimises the risk of postprandial hypoglycaemia. Indeed, there is evidence suggesting the lack of postprandial glucagon response could be a major contributing factor of postprandial reactive hypoglycaemia[1107]. Although postprandial hypoglycaemia has not been reported in animal models in which GCGR signalling was compromised, clinical studies in bariatric patients that were symptomatic for postprandial hypoglycaemia evidenced the importance of maintaining an adequate insulin: glucagon ratio in preventing postprandial hypoglycaemia[1108]. In bariatric patients with symptomatic postprandial hypoglycaemia, both insulin and C-peptide: glucose molar ratios were significantly higher than in non-symptomatic bariatric patients, and while postprandial glucagon levels were not different between the two groups, such an exaggerated insulin: glucagon may be the underlying cause of reactive hypoglycaemia[1108].

Since incretin secretion and subsequent insulin secretion are proportional to the oral glucose load[204,359,1104,1109-1112], glucagon-secreting cells in the gut would be well-situated to respond to this load. Additionally, incretins could modulate glucagon secretion from the gut via paracrine signalling and direct glucagon-secreting cells in the gut to secrete adequate amounts of glucagon to counteract the facilitated insulin response. Importantly, glucagon that is secreted postprandially should not affect insulin-mediated glucose clearance as the primary site for postprandial glucose disposal is skeletal muscles[1113], which do not appear to express GCGR[1114]. However, without detailed characterization of the distribution of GCGR in humans, it will be difficult to elucidate the exact role of extra-pancreatic glucagon.

5.4.5.2 Nutrient sensor and satiety signals

Glucagon-secreting cells in the gut may act as nutrient sensors and convey satiety signals alongside other gut hormones such as GLP-1, PYY and CCK. Peripherally administered glucagon activates neurons in the brainstem and amygdala, increasing c-Fos immunoreactivity in similar regions to that following GLP-1-induced activation[388]. This adds support that the two proglucagon-derived peptides share the same CNS targets to induce satiety and affect food intake, and is likely mediated by a direct activation of GCGR-expressing vagal afferent nerve terminals[585] since glucagon has limited access at the blood brain barrier[586,587]. Gut-derived glucagon may also modulate feeding by promoting intestinal gluconeogenesis[470]. The small intestine expresses gluconeogenic enzymes[1115,1116] and is capable of gluconeogenesis[1117]. This substantially increases portal glucose

concentrations, which subsequently activates glucose sensors along the portal vein and relays the signal to hypothalamic nuclei involved in appetite regulation to reduce food intake[594-596]. A protein rich meal is a potent stimulant of glucagon secretion *in vivo*[479,597,598] and dietary protein is also a strong activator of intestinal gluconeogenesis[595,599]. Therefore, it is plausible that glucagon-secreting cells in the gut epithelium are strongly activated by ingested protein, and release glucagon to promote intestinal gluconeogenesis. Such a mechanism is proposed to underlie the satiating effects of dietary proteins[595]. Moreover, it is possible for dietary protein to directly stimulate glucagon-secreting cells in the gut to secrete glucagon, which subsequently drives postprandial amino acid disposal[1118,1119] by promoting ureagenesis in the liver to prevent accumulation of ammonia[471].

5.4.5.3 Regulator of intestinal motility

Glucagon is a powerful inhibitor of GI motility, intestinal contractions were inhibited typically within the first minute upon administration of glucagon[1120-1122]. However, such inhibitory effect was only apparent when plasma glucagon levels exceeds 800 pg/mL[1123], which is more than ten-fold of physiological levels. Therefore, it is highly unlikely for glucagon originating from pancreatic α cells to mediate such effect in an endocrine fashion. Although potential neural involvements cannot be discounted, paracrine signalling between glucagon cells within the gut epithelium and intestinal smooth muscle is highly plausible. As such, intestinal motility is reduced upon luminal nutrient exposure, which enables optimal nutrient absorption and serves as a satiety signal.

5.4.6 Conclusion

The aim of this study was to confirm the presence of, and to elucidate the factors that regulate the secretion of, glucagon from the gut. As such, I established that the ileal epithelium releases glucagon in response to glucose and arginine stimulation. This result could serve to explain postprandial hyperglucagonaemia in post-bariatric patients, in which surgical manipulations of their gut anatomy resulted in accelerated nutrient delivery to their distal small intestine, and subsequently markedly increases post-prandial levels of most proglucagon-derived peptides, including glucagon[432,463,1046]. However, the underlying mechanisms of nutrient-induced glucagon secretion remain to be determined. Glucose-induced glucagon secretion was not attenuated by the blockade of SGLT1 and SGLT2, nor was it affected by the opening of K_{ATP} channels, indicating that glucagon and GLP-1 do not originate from the same cells. Moreover, the entero-glucagonotropic potential of other amino acids and nutrients warrant further investigation. It is worth noting that plasma glucagon levels markedly increased upon enteral[1124], but not parenteral lipid administration[1125], which suggests glucagon-secreting cells in the gut could be implicated. This further adds support to the notion that one of the major physiological functions of gut-derived glucagon is to counteract the insulinotropic actions of

incretins as plasma levels of GIP and GLP-1 increase considerably upon oral lipid ingestion[1125,1126]. Altogether, the results from this study will form the basis for future experiments to explore the regulatory factors controlling the secretion of glucagon from the gut.

6 Summary and future directions

The primary aim of this project was to elucidate the mechanism underlying the secretory response of proglucagon-expressing enteroendocrine cells in humans. Collectively, enteroendocrine cells make up the largest endocrine organ in the body[760]. In order to study the physiology of these endocrine cells in detail, it is necessary to obtain these cells in highly enriched primary culture. However, in contrast to most other endocrine organs where hormone-secreting cells are found in distinct clusters, either as a homogenous population such as chromaffin cells of the adrenal glands, or in heterogenous clusters such as the pancreatic islet cells, where α, β, δ and PP cells are found juxtaposed to each other, enteroendocrine cells are sparsely distributed along the entire length of the gastrointestinal tract[97]. Indeed, two enteroendocrine cells are rarely found adjacent to one and other due to the inhibitory notch-signalling during the differentiation process of gut epithelial cells[100]. Moreover, there is a high degree of diversity in the hormone expression profiles of these cells, which gives rise to different subtypes of enteroendocrine cells[98]. Altogether, to be able to isolate a specific subtype of enteroendocrine cells and to study them in pure primary culture has proved to be extremely difficult. Thus, research on L cell physiology has been heavily-reliant on cell lines such as GLUTag and NCI-H716 cells. The single cell clones permit analysis of direct effects on the cell through the use of techniques such as calcium-imagining and patch-clamping[657,658,668,1127]. Cell lines also have the advantage of being readily transfected[1128]. Altogether, despite some major disparities from native L cells, they remain the model of choice for high throughput experiments. The generation of transgenic mice with specific hormone promoter-driven expression of fluorescent proteins has enabled several groups of obtain GIP-secreting K cells[706] and GLP-1-secreting L cells[692] in pure cultures using FACS-based methods. However, it is generally accepted that FACS is a highly traumatic process for primary secretory cells. Indeed, Reimann *et al.* reported that most of the FACS-sorted L cells were not viable[692] and thus, could not be used for functional experiments such as patch clamping or calcium-imaging. In addition, such fluorescent protein-based method could not be used to obtain pure cultures of human enteroendocrine cells. Pioneering work from our laboratory has enabled the isolation and purification of the most abundant enteroendocrine cells, the serotonin-secreting enterochromaffin cells from mice[113,115], guinea pigs[116,993], and humans[109,127] using a Percoll density gradient. Importantly, these pure primary enterochromaffin cell cultures could be kept viable for long enough to allow for functional experiments to be conducted. The first aim of this project was to develop a method to isolate L cells from human gut epithelia using a similar approach. Despite relentless effort, it was concluded that the Percoll-density gradient-based method was not suitable as L cell-enriched mixed cell cultures obtained using this method did not display secretory

responses that were consistent with that reported in the literature. It is well-established the short chain fatty acids are potent L cell stimulants *in vitro*[710], *ex vivo*[1129,1130] and *in vivo*[761], yet all three short chain fatty acids tested had inhibitory, rather than stimulatory effects on GLP-1 secretion. In addition, the mixed cell culture obtained using the Percoll-density gradient did not respond to the combined stimulatory actions of forskolin and IBMX, an adenylyl cyclase activator and a phosphodiesterase inhibitor, respectively, contrasting to results reported by Habib *et al.*, in which such combination was used as a positive control for GLP-1 secretory response from primary human mixed colonic epithelial cell culture[620]. Hence, I sought to develop a different platform to study the secretory function of human enteroendocrine L cells.

6.1 Development of an *ex vivo* platform to study hormone secretion from human gut epithelial tissue

Symonds *et al.* reported that a stimulatory GLP-1 response could be reliably triggered from human colonic epithelial biopsies with long chain fatty acids, indicating an *ex vivo* approach could be more suitable for studying the secretory function of GLP-1-secreting cells in humans[117]. Therefore, I sought to develop an *ex vivo* static incubation secretion assay using human gut epithelial tissue obtained from fresh surgical specimens. Panaro *et al.* reported that gene expression *Mc4r*, which encodes for the melanocortin 4 receptor (MC4R), is highly enriched in murine L cells[762]. Moreover, MC4R agonists significantly triggered GLP-1 and PYY secretion in mice *in vivo* and *ex vivo* in an Ussing chamber setup[762]. Therefore, a range of MC4R agonists were used as stimulants for GLP-1 and PYY secretion from human colonic epithelia to validate this *ex vivo* secretion assay. Initially, the composition of the secretion assay buffer resembled that of cell culture media and the incubation time of two hours was chosen based on the duration for most GLP-1 secretion experiments reported in the literature[95,117,186,620,660,692,708,710,756,762,768]. Under these parameters, none of the MC4R agonists, nor did the combination of forskolin and IBMX, significantly triggered GLP-1 or PYY secretion from basal levels after two hours, as was expected based on results reported by Panaro *et al.*[762]. However, I later noted that although MC4R agonists significantly trigger GLP-1 secretion from a rat intestine perfusion model, the effect was relatively modest and occurred within the first 5-10 minutes of stimulation and gradually returned to baseline despite continual stimulations[762]. Thus, a two-hour incubation was likely to hinder the detection of any stimulatory responses from my assay. Therefore, I tested the feasibility of shorter incubation times. It was later determined that 15 minutes was the optimal incubation time to detect any stimulatory response.

6.2 Glucose-induced GLP-1 secretion in humans is driven by SGLT1, GLUT2 and mitochondrial metabolism

Using this *ex vivo* gut epithelial secretion assay platform, I elucidated the molecular mechanism of glucose-induced GLP-1 secretion from the human small intestine. Exposure to postprandial luminal, but not vascular, glucose concentrations, potently triggered GLP-1 release from human duodenal and ileal, but not colonic mucosae. I then showed that this stimulatory response is independent of osmotic influences and requires the delivery of glucose via GLUT2 and mitochondrial metabolism. The requirement of the activation of voltage-gated Na^+ and Ca^{2+} channels indicates that GLP-1 release occurs in response to membrane depolarization. This is predominantly driven by the electrogenic activity of sodium glucose co-transporter, SGLT1, as glucose-induced GLP-1 was blocked by the SGLT1 inhibitor phlorizin or by the replacement of extracellular Na^+ with NMDG. On the other hand, K_{ATP} channel closure alone is insufficient to trigger depolarization as tolbutamide treatment did not trigger GLP-1 release, consistent with the reported inability of sulfonylureas to trigger GLP-1 release in humans[774].

6.3 Metformin exposure causes L cell secretion in an AMPK-dependent manner

Metformin is the first line T2D treatment. While it has clear clinical anti-diabetic effects, its mechanism of action remains uncertain[778,790]. The increased efficacy of delayed release metformin over intravenous metformin[794] indicates that a lower bowel-mediated mechanism may underlie some of its anti-diabetic action. Therefore, I tested whether metformin directly stimulate the secretion of GLP-1 and PYY from human L cells. I found that acute exposure to 10 μM of metformin significantly triggered GLP-1 and PYY release, and this stimulatory effect was preserved in T2D patients and was not correlated with BMI of the specimen donors. I further demonstrated that AMPK inhibition blocked the L cell response to metformin, as did antagonists of PMAT and SERT, membrane transporters associated with metformin internalisation. Thus, it is likely that upon oral administration, metformin is internalised in L cells and activates AMPK to trigger GLP-1 and PYY secretion. This mechanism may subserve weight loss and glycaemia benefits of metformin and are in-line with the growing acceptance that the gastrointestinal tract is a primary site of metformin action.

6.4 Melanocortin 4 receptor activation in the gut triggers L cell secretion in humans

The central melanocortin system is a key regulator of energy homeostasis[408]. Loss-of-function mutations of *MC4R* represents one of the most common monogenic obesity disorders[821]. MC4R is expressed in murine L cells and intraperitoneal administration of MC4R peptide agonists caused significant release GLP-1 and PYY in mice. However, it remains unclear if this pathway exists in human. Thus, I sought to investigate if MC4R activation directly triggers GLP-1 and PYY secretion from the human gut epithelium. I established that MC4R agonists are strong stimulants of GLP-1 and PYY secretion from the human gut, and effect that is blocked by the endogenous MC4R antagonist, AgRP. While further experiments are warranted to decipher to physiological function of MC4R-mediated GLP-1 and PYY secretion, unpublished work from our collaborators showed that POMC-positive cells are highly abundant in the near vicinity of L cells along the human gut (Young *et al.* unpublished data). Therefore, it is possible that a separate melanocortin system distinct from that of the CNS exists in the human gut. Based on these findings, future experiments using an L-cell specific *Mc4r*-knockout mouse models have been planned to determine if MC4R-mediated GLP-1 and PYY secretion are implicated in glucose and/or energy homeostasis.

6.5 Interleukin 6 and IC7 are human L cell secretagogues

The myokine IL-6 is a key driver for many of the metabolic benefits of endurance exercise[922,923]. Rodent studies showed that IL-6 is a potent L cell secretagogue and that some of the metabolic benefits of exercise-induced IL-6 secretion were attenuated in *Glp1r*$^{-/-}$ mice[95], and that IL-6 mediates GIP-induced GLP-1 secretion *in vitro* in rodent and human pancreatic islets[1131], although the effects of IL-6 on human L cells have not been reported. I showed that IL-6 is a potent GLP-1 secretagogue from human ileal and colonic mucosal preparations. The fact that IL-6 is an L cell secretagogue, alongside its other metabolic benefits, makes IL-6R an attractive therapeutic target for the development of anti-diabetic and obesity treatments. However, IL-6R *trans* signalling through the soluble IL-6R extracellular domain is believed to mediate many of the pro-inflammatory effects of IL-6[935,977]. IC7, an IL-6/CNTF chimeric protein developed by Prof. Mark Febbraio and his team, has the capacity to signal through membrane bound, but not soluble, IL-6R, thereby circumventing the undesirable effects of IL-6R *trans* signalling. Preliminary results showed that the protein was effective in reducing weight gain while improving glucose tolerance in DIO mice in a GLP-1R-dependent manner (Allen *et al.* manuscript in review). As part of a collaboration with Prof. Febbraio, I sought to investigate if the IC7 could stimulate GLP-1 release from the human gut, in a similar fashion to that observed with IL-6. I found that IC7 treatment reliably triggered GLP-1 secretion from

the human gut, which adds support to the notion that IL-6-induced GLP-1 secretion is a feasible therapeutic target for treatment against metabolic diseases.

6.6 Dynamin activation augments human L cell secretion

The *ex vivo* secretion platform was later used to investigate if the classic endocytic protein, dynamin, plays a role in mediating L cell exocytosis. Previous work from our laboratory demonstrated that dynamin, in addition to its well-defined role in clathrin-mediated endocytosis, is an important exocytic protein that regulates the secretion of adrenalin from murine adrenal chromaffin cells[1012]. The dynamin activator, Ryngo 1-23[1011], showed potential as a reliable L cell secretagogue. The stimulatory effect of dynamin activation was also preserved in newer, more stable dynamin activators. The inhibition of dynamin inhibitor, dynole 34-2[1021], significantly attenuated high-K^+ stimulated PYY secretion, indicating dynamin activation is implicated regulating the exocytotic process of human L cells, potentially through changing fusion pore dynamics such that it favours increased hormone secretion. I sought to determine if actin and myosin II, binding partners of dynamin[1027,1030], are implicated in the stimulatory effects of dynamin activation. However, these experiments were vastly underpowered as the majority of specimens tested did not respond to the stimulatory effect of Ryngo 1-23. Thus, the mechanisms underlying the stimulatory effect of dynamin activation in human L cell remains to be characterized.

6.7 The human ileal epithelium releases glucagon upon arginine or high glucose stimulation

Lund *et al.* recently confirmed that pancreatectomized patients have detectable levels of pancreatic glucagon in their circulation, in contrast to insulin and C-peptide, neither of which were detectable after surgery[463]. Based on the finding that the release of this extra-pancreatic glucagon was potently triggered by enteral, but not parenteral glucose in these pancreatectomized patients[463], I hypothesized that the human gut epithelium could be a source of this fully processed glucagon. Indeed, findings from my mass spectrometry that confirmed the presence of glucagon in human ileal mucosal extract supported this hypothesis. I next hypothesized that direct exposure of the small intestinal mucosa to high glucose could stimulate the release of gut-derived glucagon, potentially in a similar fashion as glucose-induced GLP-1 release from the small intestine. While the first part of the hypothesis was confirmed, glucose-stimulated glucagon release from the gut could be not reliably blocked by phlorizin, an SGLT1 and SGLT2 antagonist, or diazoxide, the K_{ATP} channel opener. As somatostatin has been suggested to mediate glucose-induced glucagon suppression in pancreatic α cells[1059,1085], I hypothesized that somatostatin pre-treatment could attenuate glucose-induced

glucagon secretion from the gut epithelia. Surprisingly, somatostatin also failed to block the glucagon response from high glucose stimulation. Therefore, the glucose-sensing pathways of glucagon-secreting cells in the gut remain to be investigated. Given the multifaceted role of glucagon in metabolism, it is now urgent to define the physiological functions of such gut-derived glucagon and importantly, to elucidate the underlying mechanisms that regulate its secretion. The attenuation, or the lack, of prandial glucose-induced glucagon suppression has been classically associated with decreased insulin sensitivity[1132], in line with the established deleterious effects of excess glucagon on glucose homeostasis. The contribution of gut-derived glucagon to such phenotype should be considered. Furthermore, it is important to recognise the potential metabolic effects of gut-derived glucagon, especially in the absence of insulin, such as in pancreatectomized patients. These include beneficial contributions to protection from hypoglycaemia and aiding in the removal of ammonia, via ureagenesis, during protein and amino acid metabolism[1118,1119]. Moreover, it could have significant pathophysiological implications in postprandial hyperglucagonaemia and hyperglycaemia in diabetic patients. Thus, the mechanisms that regulate the secretory response of glucagon-secreting cells from the gut warrants further investigation. It would also be interesting to investigate if *GCG*-expressing cells in the gut are as plastic as pancreatic α cells, and can increase glucagon secretion in response to metabolic challenges in a way that is similar to increased GLP-1 production by α cells in response to STZ treatment[1133] or IL-6 exposure[95]. Notably, α cell-derived GLP-1 plays a crucial role in maintaining glucose homeostasis through its paracrine action within the islets[312], despite this source of GLP-1 being unlikely to account for much of the circulating GLP-1. Thus, gut-derived glucagon could be of high functional importance in a similar manner as that of α cell-derived GLP-1.

In summary, I have developed a high throughput experimental platform that is suitable to study GLP-1 and PYY secretion from the human gut. My results confirmed many of mechanisms that have been shown to regulate GLP-1 and PYY secretion in *in vitro* and *in vivo* in rodents. Indeed, this *ex vivo* platform can serve as an intermediary step between *in vivo* rodent experiments and expensive clinical trials, allowing one to confirm that pathways of interest do exist in human at the level of the gut before clinical trials are conducted. Moreover, this platform is highly versatile as it can be modified to study the secretion of other hormones, as evident by my findings showing ileal mucosae released glucagon in response to glucose stimulation. However, it should be acknowledged that the current model has several major limitations. Firstly, it does not permit determination of direct effects on L cells as they are not studied in isolation. Moreover, while the *ex vivo* platform provides a physiologically-relevant model to study gut hormones secretion in human, its *ad hoc* nature and polymorphisms in specimen donors markedly limit its throughput. Contrasting human intestinal

organoids, this current method using *ex vivo* human gut tissue does not allow for transfection and thus, is heavily reliant on pharmacological tools, which could be limited by physical and chemical properties of these compounds. Nonetheless, many of the findings from this current project have formed the basis of some of the experiments that are currently being undertaken by others in the laboratory, in addition to many that are currently being planned.

7 References

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