Gastrointestinal motor and sensory function, and hormone secretion – implications for postprandial blood glucose regulation in type 2 diabetes mellitus

A thesis submitted by
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B Med (Clin), M Med (Clin)

For the degree of
Doctor in Philosophy

Discipline of Medicine
University of Adelaide

October 2013
To my dearest parents, Junbao and Cui’e, and wife Lifang

Thank you for your selfless love and support,

I couldn’t have done this without you.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>THESIS SUMMARY</td>
<td>XI</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>XIV</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>XVI</td>
</tr>
<tr>
<td>PUBLICATIONS ARISING FROM THE THESIS</td>
<td>XIX</td>
</tr>
<tr>
<td>STATEMENT OF AUTHORSHIP</td>
<td>XXI</td>
</tr>
<tr>
<td>CHAPTER 1. GUT MOTILITY AND ENTEROENDOCRINE SECRETION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Physiology of GI motility</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Impact of GI motility on enteroendocrine secretion</td>
<td>3</td>
</tr>
<tr>
<td>1.3.1 Interdigestive motility</td>
<td>3</td>
</tr>
<tr>
<td>1.3.2 Postprandial state</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Regulation of GI motility – the role of enteroendocrine secretion</td>
<td>7</td>
</tr>
<tr>
<td>1.4.1 Interdigestive motility – roles of motilin and ghrelin</td>
<td>7</td>
</tr>
<tr>
<td>1.4.1.1 Motilin</td>
<td>7</td>
</tr>
<tr>
<td>1.4.1.2 Ghrelin</td>
<td>7</td>
</tr>
<tr>
<td>1.4.2 Postprandial GI motility – roles of CCK, GIP, GLP-1 and PYY</td>
<td>8</td>
</tr>
<tr>
<td>1.4.2.1 CCK</td>
<td>8</td>
</tr>
<tr>
<td>1.4.2.2 GIP</td>
<td>9</td>
</tr>
<tr>
<td>1.4.2.3 GLP-1</td>
<td>10</td>
</tr>
<tr>
<td>1.4.2.4 PYY</td>
<td>11</td>
</tr>
</tbody>
</table>
## 1.5 GI motor and enteroendocrine function and glycaemia in diabetes

1.5.1 Interdigestive GI motility and enteroendocrine hormones 11
1.5.2 Postprandial GI motor and enteroendocrine function 12
    1.5.2.1 Postprandial GI motility 12
    1.5.2.2 Postprandial enteroendocrine hormones 13

## 1.6 Conclusions

### CHAPTER 2: DIETARY EFFECTS ON INCRETIN HORMONE SECRETION

#### 2.1 Introduction

2.2 Physiology of the incretin hormones

2.2.1 Biological actions of GLP-1 19
2.2.2 Biological actions of GIP 21

2.3 Dietary influence on incretin hormone secretion

2.3.1 Effects of carbohydrates on incretin hormone release 23
2.3.2 Effect of fat on incretin release 26
2.3.3 Effect of protein on incretin release 27

2.4 Mechanisms by which nutrients stimulate incretin release

2.4.1 Mechanisms underlying glucose sensing 29
    2.4.1.1 K\(_{ATP}\) channel 30
    2.4.1.2 Sodium-glucose cotransporter 30
    2.4.1.3 Sweet taste receptor 31
2.4.2 Mechanisms underlying fat sensing 32
2.4.3 Mechanisms underlying protein sensing 34

2.4 Incretin responses in obesity and diabetes

2.5 Therapeutic implications

2.5.1 Modifying macronutrient composition 37
2.5.2 The ‘preload’ concept 38
2.5.3 Targeting GIP 40

2.6 Conclusions 40

CHAPTER 3: METHODOLOGIES 49

3.1 Introduction 49
3.2 Ethical approval 49
3.3 Recruitment of study subjects 50
3.4 Gastrointestinal symptom questionnaires 50
3.5 Autonomic nerve function 50
3.6 Glycaemic clamp 51
3.7 Gastric emptying 52
   3.7.1 Stable isotope breath tests 52
   3.7.2 APD motility 53
3.8 Unsedated endoscopic biopsy sampling and assessment of intestinal STRs 54
3.9 Appetite perception and energy intake 56
   3.9.1 Visual analogue scales (VAS) 56
   3.9.2 Buffet meal 56
3.10 Assessment of intestinal absorption 57
   3.10.1 3-O-methylglucose (3-OMG) 57
   3.10.2 Breath hydrogen test 57
3.11 Intraluminal infusion 58
   3.11.1 Intraduodenal infusion 58
CHAPTER 4. EFFECTS OF DIFFERENT SWEET PRELOADS ON INCRETIN HORMONE SECRETION, GASTRIC EMPTYING, AND POSTPRANDIAL GLYCAEMIA IN HEALTHY HUMANS

4.1 Summary

4.2 Introduction

4.3 Subjects and methods

4.4 Results

4.4.1 Blood glucose concentrations

4.4.2 Serum insulin concentrations

4.4.3 Plasma GLP-1 concentrations
4.4.4 Plasma GIP concentrations 74
4.4.5 Gastric emptying (T50) 75
4.4.6 Appetite sensations 75

4.5 Discussion 75

CHAPTER 5. EFFECTS OF A D-XYLOSE PRELOAD, WITH OR WITHOUT SITAGLIPTIN, ON GASTRIC EMPTYING, GLUCAGON-LIKE PEPTIDE-1, AND POSTPRANDIAL GLYCAEMIA IN TYPE 2 DIABETES 86

5.1 Summary 86

5.2 Introduction 87

5.3 Research design and methods 90
  5.3.1 Subjects 90
  5.3.2 Protocol 90
  5.3.3 Measurements 92
    5.3.3.1 Blood glucose, serum insulin and intact GLP-1 92
    5.3.3.2 Gastric emptying 92
    5.3.3.3 Breath hydrogen 93
  5.3.4 Statistical analysis 93

5.4 Results 94
  5.4.1 Blood glucose concentrations 94
  5.4.2 Serum insulin 95
  5.4.3 Plasma intact GLP-1 95
  5.4.4 Breath hydrogen production 96
  5.4.5 Gastric emptying 96
  5.4.6 Relationships between blood glucose and gastric emptying, plasma intact GLP-1 and breath hydrogen production 97

5.5 Conclusions 97
CHAPTER 6. EFFECTS OF ENTERIC-COATED LAURIC ACID PELLETS ON POSTPRANDIAL GLYCAEMIA IN LESS WELL-CONTROLLED PATIENTS WITH TYPE 2 DIABETES, WHEN GIVEN CONCURRENTLY WITH SITAGLIPTIN

6.1 Summary 107
6.2 Introduction 108
6.3 Subjects and methods 112
   6.3.1 Subjects 112
   6.3.2 Protocol 112
   6.3.3 Measurements 114
   6.3.4 Statistical analysis 114
6.4 Results 115
   6.4.1 Blood glucose 115
   6.4.2 Gastric emptying 115
   6.4.3 Appetite sensations 115
6.5 Discussion 116

CHAPTER 7. EFFECTS OF SITAGLIPTIN ON GLYCAEMIA, INCRETIN HORMONES, AND ANTROPYLORODUODENAL MOTILITY IN RESPONSE TO INTRADUODENAL GLUCOSE INFUSION IN HEALTHY LEAN AND OBESE HUMANS, AND PATIENTS WITH TYPE 2 DIABETES TREATED WITH OR WITHOUT METFORMIN 123

7.1 Summary 123
7.2 Introduction 124
7.3 Subjects and methods 128
   7.3.1 Subjects 128
8.4.1 Study 1: Administration of TCA in an enema formulation 173
   8.4.1.1 Blood glucose concentrations 173
   8.4.1.2 Plasma total GLP-1 174
   8.4.1.3 Plasma PYY 174
   8.4.1.4 Desire to defecate and frequency of defecation 174
   8.4.1.5 Appetite sensations 175
8.4.2 Study 2: Administration of TCA in a suppository formulation 175
   8.4.2.1 Blood glucose, plasma GLP-1 and PYY 175
   8.4.2.2 Desire to defecate and frequency of defecation 175
   8.4.2.3 Appetite sensations 176

8.5 Discussion 176

CHAPTER 9. EFFECTS OF TAUROCHOLIC ACID ON GLYCAEMIC,
GLUCAGON-LIKE PEPTIDE-1, AND INSULIN RESPONSES TO
SMALL INTESTINAL GLUCOSE INFUSION IN HEALTHY HUMANS. 185

9.1 Summary 185

9.2 Introduction 186

9.3 Materials and methods 188
   9.3.1 Subjects 188
   9.3.2 Materials 188
   9.3.3 Protocol 188
   9.3.4 Measurements 189
   9.3.5 Statistical analysis 190

9.4 Results 191
   9.4.1 Blood glucose 191
   9.4.2 Plasma total GLP-1 191
   9.4.3 Plasma insulin, C-peptide and glucagon 192
9.5 Discussion 192

CHAPTER 10. ARTIFICIAL SWEETENERS HAVE NO EFFECT ON GASTRIC EMPTYING OF, OR THE GLUCAGON-LIKE PEPTIDE-1 AND POSTPRANDIAL GLYCAEMIC RESPONSES TO, ORAL GLUCOSE IN HEALTHY HUMANS 199

10.1 Summary 199

10.2 Introduction 200

10.3 Research design and methods 202
   10.3.1 Subjects 202
   10.3.2 Protocols 203
   10.3.3 Measurements 204
   10.3.4 Statistical analysis 205

10.4 Results 205
   10.4.1 Blood glucose, plasma insulin and total GLP-1 205
   10.4.2 Gastric emptying 206

10.5 Discussion 206

CHAPTER 11. DISORDERED CONTROL OF INTESTINAL SWEET TASTE RECEPTOR EXPRESSION AND GLUCOSE ABSORPTION IN TYPE 2 DIABETES 212

11.1 Summary 212

11.2 Introduction 213

11.3 Methods 216
   11.3.1 Subjects 216
   11.3.2 Screening visit 216
   11.3.3 Endoscopy Protocol 217
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.3.4 Assays</td>
<td>218</td>
</tr>
<tr>
<td>11.3.5 Quantification of gene expression by real time RT-PCR</td>
<td>219</td>
</tr>
<tr>
<td>11.3.6 Immunohistochemistry</td>
<td>220</td>
</tr>
<tr>
<td>11.3.7 Data analysis</td>
<td>220</td>
</tr>
<tr>
<td><strong>11.4 Results</strong></td>
<td></td>
</tr>
<tr>
<td>11.4.1 Baseline STR expression</td>
<td>221</td>
</tr>
<tr>
<td>11.4.2 Effects of acute changes in glycaemia on STR expression</td>
<td>222</td>
</tr>
<tr>
<td>11.4.3 Effects of luminal glucose on duodenal STR expression</td>
<td>222</td>
</tr>
<tr>
<td>11.4.4 Plasma hormone concentrations</td>
<td>223</td>
</tr>
<tr>
<td>11.4.5 Serum 3-OMG concentrations</td>
<td>224</td>
</tr>
<tr>
<td>11.4.6 Phenotype of human intestinal sweet taste cells</td>
<td>224</td>
</tr>
<tr>
<td>11.4.7 Relationships between variables</td>
<td>225</td>
</tr>
<tr>
<td><strong>11.5 Discussion</strong></td>
<td></td>
</tr>
<tr>
<td><strong>CHAPTER 12: CONCLUSIONS</strong></td>
<td>242</td>
</tr>
<tr>
<td><strong>BIBLIOGRAPHY</strong></td>
<td>250</td>
</tr>
</tbody>
</table>
THESIS SUMMARY

This thesis focuses on the role of gastrointestinal motor and sensory function, and gut hormone secretion, in postprandial blood glucose regulation in health and type 2 diabetes. The key themes relate to: 1) evaluation of the effects of potential dietary and/or pharmacological strategies on gastric emptying, secretion of ‘incretin’ hormones (i.e. glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP)), and postprandial glycaemia, 2) effects of intraluminal bile acids on GLP-1 secretion and blood glucose homeostasis, and 3) the role of the sweet taste sensing pathway in gastrointestinal motor, secretory, and absorptive function in health and type 2 diabetes.

Macronutrient ‘preloads’ taken before a meal can stimulate endogenous GIP from the proximal gut, and GLP-1 from the distal gut, slow gastric emptying, and reduce postprandial glycaemic excursions, but entail additional energy intake. The study reported in Chapter 4 evaluates the effects of 1) 3-O-methylglucose (a non-metabolised substrate of sodium glucose co-transporter-1) and 2) a mixture of poorly absorbed tagatose and isomalt, when given as preloads in healthy humans. Since the incretin hormones are rapidly degraded by the enzyme, dipeptidyl peptidase 4 (DPP-4), the study in Chapter 5 evaluates whether the effects of a D-xylose preload (a poorly absorbed, low-energy pentose) could be further optimised by concurrent DPP-4 inhibition with sitagliptin, in patients with type 2 diabetes.
It was recently established that a small dose of lauric acid, delivered to a long segment of distal gut via enteric-coated pellets, can stimulate GLP-1 and attenuate postprandial glycaemia in well controlled type 2 patients. The study reported in Chapter 6 evaluates the glucose-lowering effect of these pellets in less well-controlled type 2 patients, when given concurrently with sitagliptin.

The effects of DPP-4 inhibition on the incretin hormone, glycaemic, and gastrointestinal motor responses to intraluminal glucose have not been well characterised in obesity and type 2 diabetes. It has been suggested that metformin has the capacity to augment plasma GLP-1 concentrations, and may synergise with DPP-4 inhibitors to improve glycaemia in type 2 diabetes. The study described in Chapter 7 examines the effects of sitagliptin on glycaemia and antropyloroduodenal motility in response to intraduodenal glucose infusion in health, obesity, and type 2 diabetes treated with or without metformin.

It is emerging that bile acids function as important signalling molecules, and are essential in blood glucose regulation. In animal models, intraluminal bile acids have been shown to stimulate GLP-1 and peptide YY (PYY) via activation of the TGR5 receptor. The study reported in Chapter 8 evaluates the effects of rectally administered taurocholic acid (TCA) on the release of GLP-1 and PYY in healthy humans. In Chapter 9, the effects of intrajejunal TCA on blood
glucose, GLP-1 and insulin responses to intrajejunal glucose infusion are evaluated.

The mechanisms underlying nutrient detection in the small intestine and consequent stimulation of incretin hormone release are poorly understood. Emerging data support the involvement of intestinal sweet taste receptors (STR) in carbohydrate sensing. In rodents, intestinal STR transcript and protein levels are rapidly down-regulated upon acute luminal exposure to glucose or artificial sweeteners. In Chapter 10, the capacity for the non-caloric artificial sweeteners, sucralose and acesulfame potassium, to stimulate GLP-1 release, slow gastric emptying, and modify postprandial glycaemia when given with oral glucose is evaluated. In Chapter 11, the modulation of duodenal STR expression in response to acute changes in luminal and systemic glucose exposure in healthy humans is assessed, and comparison is made to patients with type 2 diabetes. Furthermore, relationships between STR expression, glucose absorption and gut hormone secretion are examined in both groups.
DECLARATION

Name: Tongzhi Wu  Program: Doctor of Philosophy

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ACKNOWLEDGEMENTS

This thesis represents the scientific work that I have accomplished during my 4-year PhD journey. It would not have been possible without the support and encouragement I received from many people.

Firstly, I would like to say a sincere “thank you” to all my supervisors, Associate Professor Chris Rayner, Professor Michael Horowitz and Professor Karen Jones, who are kind, approachable, patient and inspirational. Their subtle guidance and on-going encouragement have greatly stimulated my scientific interests and made my PhD study manageable and enjoyable. Their generous support for my attendance at major national and international conferences has provided me with great opportunities to yield insights into the beauty of science. What I have learnt from three such great supervisors is tremendous, not only in professional growth, but also in personal development. My special thank you must go to my principal supervisor Associate Professor Chris Rayner, who has dedicated so much of his precious time and energy to helping and guiding me, and has always made himself available whenever I needed any help or advice.

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I gratefully acknowledge the excellent collaborative work in studying expression of human duodenal sweet taste receptors undertaken by Dr Richard Young and Ms Bridgette Chia in Nerve Gut Laboratory at Royal Adelaide Hospital, who provided me with a great opportunity to link clinical research to basic science. My deep appreciation also goes to Mr Max Bellon in Nuclear Medicine at Royal Adelaide Hospital, for spending so much precious time analysing breath test samples for my studies, and to the friendly staff on Ward Q7 at Royal Adelaide Hospital for assistance in arranging endoscopic procedures.

Many thanks go out to Professor James Meyer, Professor Michael Nauck, Professor Juris Meier, Professor Daniel Drucker, and Professor Gerald Watts,
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A special “thank you” goes to my Master supervisor, Professor Zilin Sun, who convinced me with a great deal of encouragement during my Masters study that I should pursue a doctoral degree and who made it possible for me to obtain postgraduate scholarship from the China Scholarship Council (CSC).

I would like to express my deepest gratitude to my parents, Junbao and Cui’e, who have always been proud of me, believing in me, and supporting me in every way.

And finally, a big “thank you” to my wife Lifang, who has been my driving force for academic advancement since high school. Her love and comfort accompanied my 7-year medical study, and carried me through the most challenging period of my life. She has continually been supporting my PhD study with her wholehearted love, understanding and encouragement.

Now looking back at my PhD journey, I can see that it has been a great time in my life that is filled with support, encouragement, inspiration, friendship and love. The 4-year study experience in Adelaide is my invaluable life treasure.
PUBLICATIONS ARISING FROM THE THESIS


Publications arising from the thesis


Wu T, Ma J, Bound MJ, Checklin H, Deacon CF, Jones KL, Horowitz M, Rayner CK. Effects of sitagliptin on glycaemia, incretin hormones, and antropyloroduodenal motility in response to intraduodenal glucose infusion in healthy lean and obese humans, and patients with type 2 diabetes treated with or without metformin. (Submitted for publication)
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### Chapter 1.

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Gut motility and enteroendocrine secretion.</th>
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Chapter 2.

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<th>Title of Paper</th>
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Chapter 4.

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<tr>
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CHAPTER 1. GUT MOTILITY AND ENTEROENDOCRINE SECRETION

Adapted from Wu et al., Curr Opin Pharmacol. 2013 Sep 20. [Epub ahead of print]

1.1 Introduction

Enteroendocrine cells – a group of specialised epithelial cells scattered along the gastrointestinal (GI) tract – number less than 1% of the mucosal cell population, yet form the largest endocrine system in the body (Rehfeld, 2012), and account for release of more than 30 known peptides, including motilin and ghrelin during the interdigestive period, and cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) after meals. The latter are key mediators of the shift from an interdigestive to a postprandial GI motor pattern. Conversely, the delivery of luminal contents to be sensed by enteroendocrine cells in various gut regions is dependent on GI motor activity. In humans, the physiology of GI motility and enteroendocrine secretion has mainly been explored by employing exogenous GI hormones at ‘physiological’ doses and/or hormone (receptor) antagonists. This review provides an updated understanding of the inter-relationships between GI motility and enteroendocrine secretion, and their central role in blood glucose regulation (Figure 1).
1.2 Physiology of GI motility

During the interdigestive period, both the stomach and small intestine undergo a
cyclical motor pattern – the ‘migrating motor complex (MMC)’ – consisting of a
quiescent phase (~ 40 min, phase I), a phase of irregular contraction (~ 50 min,
phase II), and a period of maximum contraction (5 ~ 10 min, phase III). The
MMC migrates from the stomach (or proximal small intestine) to the terminal
ileum, and acts to sweep small intestinal contents (including bile, digestive juice
and indigestible debris) towards the large intestine (Camilleri, 2006). Phase III
of the MMC is also associated with spontaneous gallbladder emptying
(Camilleri, 2006).

Meal ingestion converts GI motility into a postprandial pattern that is marked by
proximal gastric relaxation to accommodate an increased volume, and regular
peristaltic contractions of the distal stomach to mix and triturate solids into small
particles (< 2 mm in diameter) prior to their emptying. The latter occurs
predominantly in pulsatile fashion, driven by antral and duodenal contractions
against pyloric resistance. The overall rate of gastric emptying in humans is
dependent on the nature of the meal, including the form (solid, semisolid, or
liquid) and macronutrient composition, and exhibits substantial inter-individual
variation, ranging from 1 to 4 kcal/min (Camilleri, 2006). Solid emptying has an
initial lag phase that relates to food redistribution and trituration, followed by a
linear emptying pattern, while liquid emptying decelerates from an exponential
to a linear pattern as the caloric content increases, and occurs preferentially
before solids (Camilleri, 2006). Synchronous with gastric emptying, the
gallbladder begins to empty in a continuous, and overall exponential fashion,
and the small intestine adopts a pattern of irregular contractions propagated over
short distances which facilitate mixing and absorption. Motor patterns in the
large intestine are less well characterised, but consist predominantly of short
segmental contractions, with a small number of longer propagated contractions.

1.3 Impact of GI motility on enteroendocrine secretion

1.3.1 Interdigestive motility

The cyclical occurrence of MMC activity during the interdigestive state closely
parallels the secretion of motilin, and to a lesser degree, ghrelin. Increases in
plasma motilin concentrations follow immediately each episode of spontaneous
gallbladder emptying, while after phase III there is a decrease in motilin. The
latter might be associated with the relative absence of luminal content due to the
‘house-keeping’ effect of phase III (Deloose et al., 2012). Patients with
gallstones who have defective gallbladder emptying lack the cyclical profile of
motilin concentrations and exhibit a reduced frequency of phase III activity
(Stolk et al., 2001). How bile acids stimulate motilin release is unclear; their
action on the TGR5 receptor of enteroendocrine L-cells to stimulate GLP-1
release will be discussed subsequently (Habib et al., 2013).

The link between MMC activity and ghrelin secretion has not been well
established in humans. A considerable proportion of enteroendocrine cells in the
proximal small intestine co-secrete motilin and ghrelin (Wierup et al., 2007), suggesting a shared pathway for stimulation of these hormones. However, the majority of ghrelin is released from the gastric fundus under tonic control by the vagus nerve and nutritional state (Steinert et al., 2013).

1.3.2 Postprandial state

Meal ingestion is associated with suppression of plasma motilin and ghrelin, other than for a modest, transient, rise in motilin in response to mechanical stretching of the gut by food and the action of fat, possibly by the stimulation of gallbladder emptying. Pancreatic polypeptide (PP), which is released in proportion to energy intake, appears to be responsible for suppressing motilin (Deloose et al., 2012), while the suppression of ghrelin is triggered by exposure of the small intestine to nutrients, even though ghrelin is derived mainly from the stomach (Steinert et al., 2012, Parker et al., 2005). Accordingly, it is not surprising that postprandial plasma ghrelin levels are inversely related to the rate of gastric emptying (Blom et al., 2006), the caloric load of mixed meals (Callahan et al., 2004), and the length of small intestine exposed to nutrient (Little et al., 2006).

Other than for motilin, it remains unclear whether the stomach per se has a physiological role to potentiate enteroendocrine secretion. GLP-1 and PYY secretion are substantially greater in response to intragastric administration of glucose when compared to an intraduodenal infusion delivered at 3 kcal/min
(Steinert et al., 2012). Given that the latter is within the physiological range of gastric emptying, albeit towards the upper end (Camilleri, 2006), it appears that intragastric factors might be important (Steinert et al., 2012). Studies employing direct infusion of nutrients into the small intestine have established that the stimulation of CCK, GIP, GLP-1 and PYY are dependent on the rate of nutrient entry to the small intestine (Ryan et al., 2012, Ma et al., 2012, Pilichiewicz et al., 2007b), together with the region and length of small intestine exposed (Little et al., 2006, Ma et al., 2013). In the case of CCK and GIP, the magnitude of stimulation appears to be proportional to the load of glucose or lipid administered, and peak concentrations occur soon after the onset of the infusion (~15-30 min) with a plateau thereafter (Ma et al., 2012, Pilichiewicz et al., 2007b). In contrast, both GLP-1 and PYY responses are non-linear, being modest at 1-2 kcal/min and substantially greater at 4 kcal/min, and peak hormone levels occur later (Ma et al., 2012, Pilichiewicz et al., 2007b), although transient, early release of GLP-1 is observed during low rates of intraduodenal glucose delivery (Kuo et al., 2008), and might be related to initially rapid transit to the jejunum, or perhaps a proximal-distal neural or hormonal loop. Furthermore, GIP and CCK are strongly stimulated even when exposure to glucose is restricted to the proximal 60 cm of the small intestine, whereas significant GLP-1 secretion is dependent on more distal small intestinal exposure (Little et al., 2006). Moreover, a very small nutrient load, delivered directly to the ileum and colon, induces substantial GLP-1 stimulation, without any effect on GIP (Ma et al., 2013). These observations can be accounted for by differences in the distribution
of the enteroendocrine cells, that is, I- and K-cells predominate in the proximal small intestine, and L-cells in the distal small intestine and colon. Accordingly, stimulation of I- and K-cells is primarily dependent on the rate and duration of entry of nutrients to the proximal small intestine (i.e. gastric emptying), while that of L-cells is determined not only by gastric emptying, but also small intestinal transit and the capacity of unabsorbed nutrients to reach the distal gut. In line with this concept, pharmacological inhibition of proximal small intestinal flow events in healthy humans is associated with attenuated GLP-1 and GIP release (Chaikomin et al., 2007), while malabsorption of sucrose induced by the α-glucosidase inhibitor, acarbose, is associated with enhanced GLP-1 secretion (Qualmann et al., 1995). Not surprisingly, Roux-en-Y gastric bypass surgery, which is used in the management of morbid obesity is associated with abnormally rapid transit of liquid nutrients from the gastric pouch to the jejunum, and is characterised by markedly increased postprandial GLP-1 and PYY secretion (Chronaiou et al., 2012).

There is evidence of reciprocal regulation between enteroendocrine hormones released during the interdigestive and postprandial states. Fasting ghrelin can be suppressed by GLP-1, PYY, and CCK, while ghrelin appears to exert tonic inhibition on GLP-1 and PYY; amongst Roux-en-Y gastric bypass patients, those in whom the gastric fundus has been resected with a consequent reduction in plasma ghrelin, exhibit greater postprandial GLP-1 and PYY responses,
associated with a greater insulin and reduced glycaemic excursion, than those in whom the gastric fundus was retained (Chronaiou et al., 2012).

1.4 Regulation of GI motility – the role of enteroendocrine secretion

The innervation of GI tract does not directly terminate at the luminal surface, but is configured with a wide array of receptors for GI hormones at the basal aspect of the enteroendocrine cells. Enteroendocrine secretion during fasting and postprandial periods, therefore, appears to be fundamental to regulating GI neural and motor activity (Table 1).

1.4.1 Interdigestive motility – roles of motilin and ghrelin

1.4.1.1 Motilin

In humans, it is well established that peak plasma concentrations of endogenous motilin induce antral (but not duodenal) phase III activity (Deloose et al., 2012). Administration of motilin or its agonists also evokes transient, cholinergically-mediated gastric phase III activity (Broad and Sanger, 2013), which is prone to rapid tachyphylaxis via self-regulated receptor desensitisation – a potentially limiting feature for the use of motilin agonists in the clinical setting.

1.4.1.2 Ghrelin

There are substantial inter-species differences in ghrelin biology. In humans and rats, intravenous ghrelin induces premature phase III MMC activity during fasting, without any effect on plasma motilin (Deloose et al., 2012), while in
dogs, exogenous ghrelin suppresses both motilin release and phase III activity (Ogawa et al., 2012). Recent studies on *Suncus murinus*, a shrew with similar MMC patterns and motilin responses to humans and dogs, suggest that ghrelin regulates phase II of the MMC, and potentiates motilin-induced phase III contractions (Mondal et al., 2012). More human studies are required to reveal the physiological relevance of motilin and ghrelin in the regulation of MMC activity.

1.4.2 Postprandial GI motility – roles of CCK, GIP, GLP-1 and PYY

Together with declining levels of the prokinetic hormones motilin and ghrelin, the postprandial state is characterised by the release of multiple enteroendocrine hormones, including CCK, GIP, GLP-1 and PYY, which are key mediators of postprandial GI motility. Although GLP-2 is co-secreted with GLP-1 and PYY after meals, it does not appear to play an important physiological role in modulating GI motility in humans (Meier et al., 2006).

1.4.2.1 CCK

Physiological actions of CCK on GI motility have been explored extensively using either exogenous CCK, or specific CCK antagonists. Intravenous administration of CCK-8 (a major circulating form of CCK) to achieve ‘physiological’ postprandial concentrations induces dose-dependent pyloric contraction, suppresses antral and duodenal motility, stimulates PYY secretion, and suppresses both ghrelin and energy intake (Brennan et al., 2008).
Conversely, dexloxiglumide, a CCK₁ receptor antagonist, accelerates gastric emptying (Little et al., 2010). CCK may initially act on vagal afferent fibres (probably via a paracrine mechanism) to inhibit excitatory vagal efferents supplying the distal stomach, and subsequently to stimulate inhibitory vagal efferents to the proximal stomach via endocrine pathways (Okano-Matsumoto et al., 2011), as evidenced by the amplification of vago-vagal reflex responses in the stomach by intravenous administration of CCK (Viard et al., 2012). On the other hand, small intestinal transit is accelerated by exogenous CCK, and slowed by the CCK₁ receptor antagonist, devazepide (Lin et al., 2002), while CCK also stimulates gallbladder contraction and augments pancreatic enzyme secretion. Despite CCK₁ receptor expression in the human colon, CCK infusion was not found to influence human rectal motor function (van der Schaar et al., 2013).

1.4.2.2 GIP

In dogs, infusion of GIP suppresses motilin-induced ‘spike potentials’ in the antrum and duodenum, and reduces phase III MMC activity (Thor et al., 1987), suggesting a role for GIP in the shift from the interdigestive to postprandial motor pattern. However, gastric emptying is not slowed by exogenous GIP in humans (Asmar et al., 2010), nor does intravenous GIP affect either GI transit or gallbladder motility in animal models. Nevertheless, intravenous infusions do not necessarily mimic the paracrine or portal effects of enteroendocrine hormones, and it was recently reported that intraperitoneal GIP is associated
with inhibition of small intestinal transit and glucose absorption in mice (Ogawa et al., 2011).

1.4.2.3 GLP-1

GLP-1, together with PYY (to be discussed), appears to be an important mediator of the inhibition of upper GI transit observed when the distal gut is exposed to unabsorbed nutrients, a phenomenon known as the ‘ileal brake’ (Camilleri, 2006). The physiological role of GLP-1 in regulating GI motility has been revealed by studies employing the specific GLP-1 antagonist, exendin (9-39), which induces a modest acceleration of gastric emptying in healthy humans (Deane et al., 2010), but may not be evident with small test meals (Witte et al., 2011, Nicolaus et al., 2011) or suboptimal methods to assess gastric emptying (Salehi et al., 2008). GLP-1 appears to slow gastric emptying by suppression of antral contractions and stimulation of phasic and tonic pyloric pressure waves (Schirra et al., 2006). The effects of GLP-1 on intestinal motility in humans are less well characterised, although the improvement in diarrhoea in short bowel syndrome patients treated with the GLP-1 agonist, exenatide (Kunkel et al., 2011), suggests an inhibitory effect on small intestinal transit. However, when treating type 2 diabetes, exenatide frequently causes diarrhoea, which might be associated with stimulation of colonic transit; such an effect is observed in patients with constipation-predominant irritable bowel syndrome treated with the GLP-1 agonist, ROSE-010, without any change in small intestinal transit (Camilleri et al., 2012).
1.4.2.4 PYY

Endogenous PYY circulates as both PYY (1-36) and its product of degradation by dipeptidyl peptidase-4 (DPP-4), PYY (3-36) (Witte et al., 2009). PYY peptides have the capacity to inhibit upper GI motility, as demonstrated in healthy subjects, where PYY (3-36) is more potent (Witte et al., 2009), and in PYY knockout mice, which show accelerated upper GI transit (Tough et al., 2011). In addition, endogenous PYY acts together with neuropeptide Y (NPY) via epithelial Y₁ and neuronal Y₂ receptors to inhibit mucosal fluid and electrolyte secretion, and to inhibit colonic transit in a Y2 receptor-dependent manner (Tough et al., 2011).

1.5 GI motor and enteroendocrine function and glycaemia in diabetes

It has now been recognised that both the GI motor and secreting function are major determinants of blood glucose homeostasis.

1.5.1 Interdigestive GI motility and enteroendocrine hormones

Cyclic MMC activity and associated changes in plasma motilin and ghrelin do not substantially impact on fasting blood glucose concentrations in health and type 2 diabetes (Tong et al., 2013). However, ghrelin, even at physiological doses, suppresses insulin secretion and impairs glucose tolerance by up to one third in response to an intravenous glucose challenge in healthy humans, associated with elevated growth hormone and cortisol levels (Tong et al., 2013).
Conversely, ablation of ghrelin signalling improves insulin sensitivity (Lin et al., 2011). Plasma ghrelin may also exert a tonic suppression on L-cell secretion after a meal (Chronaiou et al., 2012). Therefore, ghrelin antagonism represents a potential therapeutic target for diabetes management.

1.5.2 Postprandial GI motor and enteroendocrine function

1.5.2.1 Postprandial GI motility

Gastric emptying controls the rate of nutrient entry into the small intestine, and accounts for about 35% of the variance in the initial glycaemic response to oral glucose (Rayner et al., 2001). Slowing of gastric emptying is therefore an important mechanism of a number of anti-diabetic medications, including ‘postprandial’ GLP-1 agonists, such as exenatide immediate release (IR) and lixisenatide, although the effect of long-acting GLP-1 agonists (e.g. exenatide extended release (ER) and liraglutide) to slow gastric emptying appears to diminish markedly with sustained use as a result of tachyphylaxis (Horowitz et al., 2013).

Postprandial gallbladder emptying and delivery of bile acids also appear to be important determinants of blood glucose regulation. Cholecystectomised subjects display a substantial reduction in intraluminal bile acids, together with an exaggerated postprandial glycaemic response (Sonne et al., 2013). Moreover, rectal (Wu et al., 2013b, Adrian et al., 2012) or jejunal (Wu et al., 2013c) infusion of the bile acid, taurocholic acid, has recently been shown to stimulate
GLP-1 and PYY secretion, possibly via the G-protein linked TGR-5 receptor found on L-cells. This represents a novel strategy for the management of type 2 diabetes.

1.5.2.2 Postprandial enteroendocrine hormones

GLP-1 and GIP, the two known ‘incretin’ hormones, account for 50-70% of postprandial insulin secretion in health. However, incretin-based therapies for diabetes, e.g. GLP-1 agonists and DPP-4 inhibitors, have hitherto focused on GLP-1, since the insulinotropic action of the GIP is markedly diminished in type 2 diabetes. The stimulation of endogenous GLP-1 represents a promising alternative strategy. For example, a small dose of lauric acid delivered to a long segment of distal gut via enteric coated pellets can stimulate GLP-1 and attenuate postprandial glycaemia in type 2 diabetes (Ma et al., 2013). Alternatively, a small nutrient-based ‘preload’ can be consumed in order to stimulate secretion of enteroendocrine hormones, including GLP-1, in advance of the main meal, with resultant slowing of gastric emptying of the subsequent meal and attenuation of the postprandial glycaemic excursion (Wu et al., 2013d, Wu et al., 2012). The glucose-lowering effect of endogenous GLP-1 secretion can be further optimised by DPP-4 inhibition, as the latter prolongs the half-life of active GLP-1 in the circulation (Wu et al., 2013d).

Other enteroendocrine hormones, such as CCK and PYY, although lacking any significant insulinotropic property, can potently suppress food intake and slow
gastric emptying (Steinert et al., 2013), and therefore also represent promising targets for lowering postprandial glycaemia.

1.6 Conclusions

GI motility has a major impact on enteroendocrine secretion; conversely, enteroendocrine hormones play a pivotal role in the regulation of interdigestive and postprandial GI motility. The significance of these inter-relationships is increasingly recognised as being central to the regulation of postprandial glycaemia. Slowing gastric emptying and intestinal transit, accelerating gallbladder emptying and intestinal exposure to bile acids, and stimulating postprandial enteroendocrine hormones, all represent novel therapeutic approaches for the management of type 2 diabetes.
### Table 1. Main enteroendocrine hormones involved in the regulation of gastrointestinal motility

<table>
<thead>
<tr>
<th>Gut hormone</th>
<th>Site of secretion</th>
<th>Known effects on GI motility</th>
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<td><strong>Interdigestive state</strong></td>
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| Motilin | M-cells, the duodenum and jejunum | Induces gastric origin phase III MMC activity via cholinergic pathways in humans (Deloose et al., 2012)  
Exhibits rapid tachyphylaxis (Broad and Sanger, 2013) |
| Ghrelin | Gr-cells (X/A-cells), mainly gastric fundus, proximal small intestine | Substantial inter-species differences (more human studies required):  
Induces premature phase III MMC activity in humans and rats (Deloose et al., 2012)  
Suppresses motilin release and phase III MMC activity in dogs (Ogawa et al., 2012)  
Regulates phase II, and potentiates motilin-induced phase III MMC contractions in *Suncus murinus* (Mondal et al., 2012) |
| **Postprandial state** | | |
| CCK | I-cells, the duodenum and jejunum | Triggers gallbladder emptying  
Slows gastric emptying (dose-dependently stimulates pyloric, and suppresses antral and duodenal contractions)(Brennan et al., 2008, Little et al., 2010), via integrated paracrine and endocrine pathways (Okano-Matsumoto et al., 2011, Viard et al., 2012)  
Accelerates small intestinal transit (Lin et al., 2002)  
No effect on colonic motility in humans (van der Schaar et al., 2013) |
| GIP | K-cells, the duodenum and jejunum | Reduces phase III MMC activity (Thor et al., 1987)  
Does not slow gastric emptying in humans (Asmar et al., 2010)  
Intraperitoneal administration of GIP is associated with slowing of small intestinal transit in mice (Ogawa et al., 2011) |
| GLP-1 | L-cells, the distal small intestine and colon | ‘Ileal brake’  
Physiological contribution to slow gastric emptying in humans (Deane et al., 2010)  
Probably slows small intestinal transit (Kunkel et al., 2011) and accelerates colonic transit (Camilleri et al., 2012) in humans |
| PYY | L-cells, the distal small intestine and colon | ‘Ileal brake’  
Both PYY (1-36) and PYY (3-36) slow gastric emptying in humans – the latter more potently (Witte et al., 2009)  
Retards small intestinal transit in mice (Tough et al., 2011)  
Inhibition of colonic transit and secretion in mice (Tough et al., 2011) |
Figure 1. Inter-relationships between gastrointestinal (GI) motility and enteroendocrine secretion, and their association with blood glucose homeostasis (graphic abstract). Cyclical release of motilin, and probably ghrelin, regulates interdigestive GI motility. Cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) released after meals suppress motilin and ghrelin, accounting for the shift from an interdigestive, to a postprandial, GI motor pattern. Conversely, GI motor function is a key determinant of enteroendocrine secretion. Both the secretion of enteroendocrine hormones and GI motor activity are central to the regulation of postprandial glycaemia.
CHAPTER 2: DIETARY EFFECTS ON INCRETIN HORMONE SECRETION

Adapted from Wu T et al. Vitam Horm. 2010;84:81-110.

2.1 Introduction

The “incretin effect”, whereby oral glucose elicits a much greater insulin response than an isoglycaemic intravenous glucose infusion, has been recognised for over 40 years (Perley and Kipnis, 1967). To date, the incretin effect has been attributed to two hormones – glucose-dependent insulinotropic polypeptide (GIP, also known as gastric inhibitory polypeptide), first identified in the 1970s in extracts of the upper small intestine (Brown et al., 1975), and glucagon-like peptide-1 (GLP-1), uncovered during the next decade (Drucker et al., 1987). The combined action of these peptides accounts for approximately 50-70% of the total insulin release in healthy humans after ingestion of oral glucose. Furthermore, these two peptides induce glucose-stimulated insulin secretion in a synergetic manner, and appear to contribute approximately equally to the incretin effect in health (Baggio and Drucker, 2007). The delivery of nutrients from the stomach into the duodenum and their subsequent interaction with the small intestine to stimulate incretin hormone release are central determinants of the glycaemic response. An improved understanding of the incretin response to food ingestion is, therefore, of fundamental importance for management of type 2 diabetes. In this review, we summarise current knowledge related to the effects of individual nutrients on incretin
hormone release, and the mechanisms by which nutrients interact with the small intestine to induce GLP-1 and GIP secretion.

2.2 Physiology of the incretin hormones

GLP-1 is released from intestinal L-cells, the majority of which are located in the distal small intestine and colon. In contrast, GIP is secreted by intestinal K-cells, distributed mainly in the upper small intestine (duodenum and proximal jejunum). However, in pigs, rats, and humans, there also exists a large population of ‘K/L-cells’, expressing both GIP and GLP-1, located mainly in the upper small intestine (Theodorakis et al., 2006, Mortensen et al., 2003, Fujita et al., 2008). In response to a variety of stimuli, biologically active GLP-1 is secreted as a 7-37 or 7-36 amide peptide, and GIP as a 42 amino acid sequence. Both incretins are then rapidly inactivated at the site of an alanine residue by an aminopeptidase, dipeptidyl peptidase 4 (DPP-4) (Drucker and Nauck, 2006). More than 50% of GLP-1 is inactivated before it reaches the systemic circulation, and the half-life of circulating GLP-1 is less than 2 minutes (Baggio and Drucker, 2007). Similarly, the half-life of biologically active GIP is short, at approximately 7 and 5 minutes in healthy and type 2 diabetic individuals respectively (Deacon et al., 2000); the decreased half-life in the latter group has been attributed to increased DPP-4 activity (Ryskjaer et al., 2006). Despite their short plasma half-life, the incretin hormones have been found, by the application of GLP-1 and GIP receptor agonists and antagonists and DPP-4 inhibitors, as well as receptor knockout animal models, to mediate a wide range of biological effects via binding to their specific receptors.
2.2.1 Biological actions of GLP-1

GLP-1 contributes to the regulation of glucose homeostasis in a number of ways, including pancreatic and extra-pancreatic actions on insulin secretion, improvement of insulin sensitivity and peripheral glucose disposal, inhibition of gastric emptying, and suppression of glucagon secretion and food intake.

In the pancreas, GLP-1 triggers insulin secretion from β-cells in a glucose-concentration dependent manner (Yu and Jin, 2010). Activation of the GLP-1 receptor (GLP-1R) by exendin-4 enhances β-cell proliferation and neogenesis, and inhibits β-cell apoptosis in a rodent model of diabetes, leading to expansion of β-cell mass and improvement of glucose tolerance (Maida et al., 2009). Furthermore, GLP-1/− mice exhibit defective regeneration of the β-cell, and are more susceptible to streptozotocin-introduced β-cell apoptosis (Maida et al., 2009). In addition, GLP-1 is able to enhance glucose sensitivity by up-regulating the expression of glucose transporters and glucokinases (Holz Iv et al., 1993), thereby improving the capacity of β-cell to respond to glucose.

Rodent studies indicate that GLP-1 mediated stimulation of insulin secretion can occur indirectly via a neural pathway. In the portal vein of rats GLP-1 activates hepatic vagal afferent fibers via the nodose ganglion, which in turn increases the activity of pancreatic vagal efferents leading to glucose-stimulated insulin secretion and glucose disposal (Balkan and Li, 2000). GLP-1 also inhibits hepatic glucose production (Prigeon et al., 2003) and
stimulates glucose uptake in fat and muscle (Villanueva-Penacarrillo et al., 2001).

Among the multiple actions of GLP-1 on glucose homeostasis, inhibition of gastric emptying (the ‘ileal brake’), as demonstrated in studies involving ‘physiological’ infusion of exogenous GLP-1 (Nauck et al., 1997), or the specific GLP-1 antagonist, exendin(9-39) (Deane et al., 2010), is of particular importance, given that it may outweigh the insulinotropic property of GLP-1. This is highlighted by studies demonstrating that exogenous GLP-1 infusion, in the presence of lower blood glucose induced by delayed gastric emptying, leads to less insulin secretion after a meal compared to control (Meier et al., 2005). Slowing of gastric emptying also plays a dominant role in the action of the GLP-1 analog, exenatide, at least acutely, to reduce postprandial glycaemia in type 2 patients (Cervera et al., 2008).

GLP-1 has the capacity to suppress glucagon secretion, when infused in physiological concentrations in patients with type 2 diabetes (Meier et al., 2003a). The glucagonostatic action of GLP-1 is also glucose-dependent, but is not dependent on the stimulation of insulin (Baggio and Drucker, 2007). GLP-1 also appears to regulate feeding behavior – both central and peripheral administration of GLP-1R agonists reduce short-term food and water intake, leading to decreased body weight (Szayna et al., 2000, Turton et al., 1996). That the effects persist after subdiaphragmatic bilateral vagotomy or surgical transaction of the brainstem-hypothalamic pathway indicates the importance of the central actions of GLP-1 (Abbott et al., 2005).
2.2.2 Biological actions of GIP

The actions of GIP on the pancreatic β-cell are similar to those of GLP-1 in health, but diminished in diabetes. In contrast, GIP promotes energy storage via direct action on adipose tissue and is likely to exacerbate insulin resistance.

GIP is able to act synergistically with glucose to stimulate insulin secretion (Baggio and Drucker, 2007), and enhance the survival of pancreatic β-cell lines (Kim et al., 2005). In contrast to the suppression of glucagon seen with GLP-1, GIP stimulates glucagon secretion in healthy humans, at least under euglycaemic conditions (Meier et al., 2003b). Although GIP release is intact, or even increased, in response to nutrient ingestion in type 2 diabetic patients, its insulinotropic effect has been reported to be markedly diminished in this group (Ma et al., 2009b). Some studies have proposed that this impairment of GIP action is attributable to desensitisation and/or down-regulation of GIPR expression during long-standing hyperglycaemia (Nauck et al., 2004a, Meier et al., 2004a, Hojberg et al., 2009), since a period of strict glycaemic control has the capacity to restore the insulinotropic effects of GIP (Hojberg et al., 2009). In contrast to GLP-1, GIP has no effect on gastric emptying (Meier et al., 2004b).

In addition to its insulinotropic properties, GIP exhibits unique physiological actions in extrapancreatic tissues. In adipose tissue, GIP acts in concert with insulin to increase lipoprotein lipase activity and lipogenesis (Irwin and Flatt,
2009). Rodent studies also suggest that GIP has roles in neuroprotection (Nyberg et al., 2005) and bone formation (Ding et al., 2008, Xie et al., 2007). GIPR mRNA is also detectable in the heart, testis, lung, and several other tissues, where its physiologic actions are largely unknown.

**2.3 Dietary influence on incretin hormone secretion**

Plasma concentrations of both GLP-1 and GIP are low (around 10 pmol/L) in the fasting state (Herrmann et al., 1995), but increase approximately 2- to 3-fold after nutrient ingestion (Vilsboll et al., 2001, Elliott et al., 1993, Orskov et al., 1994), with peak values being highly dependent on the rate (Chaikomin et al., 2005) and the load (Pilichiewicz et al., 2007a) of nutrient entry to the small intestine (Fig. 1), the flow patterns within it (Chaikomin et al., 2007), and length of small intestine exposed (Little et al., 2006).

Following meal ingestion, GLP-1 and GIP display distinct secretory profiles – the former characteristically exhibits a biphasic profile with an ‘early’ phase occurring within 15 minutes and lasting for 15-30 minutes, and a ‘late’ phase persisting for 1-2 hours or longer (Herrmann et al., 1995), while secretion of the latter usually occurs with a short time delay, and characteristically remains elevated for several hours (Elliott et al., 1993). These differences are likely to relate, at least in part, to the locations of K-cells and L-cells in the small intestine, as discussed. Although several mechanisms (e.g. nutrient-mediated, neural and hormonal) have been suggested in the regulation of incretin release (Baggio and Drucker, 2007), the direct interaction of luminal nutrients with K-cells and L-cells appears likely to be fundamental, as these enteroendocrine cells are ‘open-type’ and,
therefore, configured to sense the presence of nutrients at the mucosal surface (Dumoulin et al., 1998).

Meal properties, including macronutrient composition, are of central importance to the incretin response. For example, GLP-1 release can be triggered by each of the macronutrients – carbohydrates, fats, and proteins (Baggio and Drucker, 2007) – with fat and carbohydrate generally being the most potent stimuli (Elliott et al., 1993, Herrmann et al., 1995). Similarly, GIP secretion is stimulated strongly by fat and carbohydrate (Meier and Nauck, 2004, Elliott et al., 1993), while protein appears to be less potent (Elliott et al., 1993, Herrmann et al., 1995), although certain amino acids induce GIP release (Thomas et al., 1978). The combination of fat and carbohydrate appears to have an additive effect on incretin secretion, at least in rodent models (Shimotoyodome et al., 2009, Lu et al., 2007).

2.3.1 Effects of carbohydrates on incretin hormone release

Carbohydrates are potent stimuli for both GLP-1 and GIP release, consistent with their role as incretins, and their effect on incretin secretion is dose-dependent. In health, the interaction of nutrients with the small intestine generates feedback to modulate the rate of gastric emptying at about 1-4 kcal per minute (Macdonald, 1996, Brener et al., 1983). Although one study suggested that the stimulation of GLP-1 requires a rate of small intestinal glucose delivery in excess of 1.4 kcal/min (Schirra et al., 1996), we have shown that there is an early, transient rise in GLP-1 in response to intraduodenal glucose at 1 kcal/min (Kuo et al., 2008) (Fig. 2), consistent
with the observation that there is a sufficient density of L-cells in the human duodenum to account for the early phase of GLP-1 secretion (Theodorakis et al., 2006). The transient rise in GLP-1 in response to low glucose loads may also be explained by initial rapid transit of glucose to interact with L-cells in the jejunum, with subsequent inhibition of transit by the release of GLP-1; the latter has been shown to slow small intestinal transit in rats (Tolessa et al., 1998). In health, the magnitude of GLP-1 and GIP responses to intraduodenally infused glucose is dose-dependent – GIP increases in approximately linear fashion with increasing glucose loads, whereas the GLP-1 response is non-linear, being modest at 1-2 kcal/min and substantially greater at 4 kcal/min (Pilichiewicz et al., 2007a). This GLP-1 response to intraduodenal glucose parallels the subsequent reduction in food intake, leading to an improved glycaemic response to the following meal (Pilichiewicz et al., 2007a). Furthermore, the GLP-1 response is greater with an initially rapid and subsequently slower rate of glucose delivery into the small intestine, when compared to delivery of the same total glucose load at a constant infusion rate (O'Donovan et al., 2004b) (Fig. 3).

Both the length and the region of small intestine exposed to carbohydrate are crucial determinants of GLP-1 release; in humans, GLP-1 was stimulated when glucose was allowed to access to the entire small intestine, but not when restricted to the proximal 60 cm (Little et al., 2006) (Fig. 4). Furthermore, secretion of GLP-1 in response to sucrose is increased when malabsorption is induced by the α-glucosidase inhibitor, acarbose (Qualmann et al., 1995), which presumably allows stimulation of a greater
length, or more distal region of the gut by ingested sugar (Gentilcore et al., 2005).

The incretin response profiles vary with different carbohydrates. Observations in vitro and in perfused animal ileum demonstrate that monosaccharides, including glucose, galactose, and 3-O-methylglucose (3OMG), stimulate GLP-1 release, while 2-deoxyglucose does not (Reimann et al., 2008, Ritzel et al., 1997, Sugiyama et al., 1994). A similar pattern of GIP responses to these sugars was observed in ob/ob mice (Flatt et al., 1989). These specificities could relate to glucose transporters (to be discussed later). Fructose stimulates GLP-1 release from both perfused rat ileum (Ritzel et al., 1997) and the small intestine in healthy humans (Rayner et al., 2000a), but does not induce GIP release (Ganda et al., 1979, Tazawa et al., 2005), indicating that signaling pathways other than glucose transporters are likely to be involved in GLP-1 secretion. Whether 3OMG, a non-metabolised substrate for glucose transporters, stimulates release of the incretin hormones in humans is not known.

The glycaemic index (GI) of carbohydrates, a measure of glycaemic response to ingestion of a carbohydrate, when compared to a standard load of oral glucose or white bread, also influences incretin hormone secretion. Diets with a low GI, especially those rich in fibre content (Wolever, 1990), have beneficial effects on long-term glycaemic control in type 2 diabetes (Esposito et al.). It is possible that an increase in GLP-1 contributes to these metabolic improvements, as low-GI carbohydrates exhibit a lower absorption
rate and, therefore, have the potential to give rise to interaction of nutrients with more distal regions of the small intestine. For example, incorporation of indigestible carbohydrates (barley fibre and resistant starch) into an evening meal (white wheat bread) was found to induce greater and more prolonged GLP-1 release and improved glucose tolerance after the following breakfast, when compared to an evening meal with white wheat bread only (Nilsson et al., 2008).

2.3.2 Effect of fat on incretin release

Fats are strong stimuli for both GLP-1 and GIP secretion, although their release is often delayed after fatty meals when compared to carbohydrates (Elliott et al., 1993). Direct fat infusion into the small intestine, bypassing the influence of the stomach in the regulation of nutrient delivery, has established that lipid (e.g. triacylglycerol) is able to induce a prompt GLP-1 response, in both healthy and obese subjects (Feinle-Bisset et al., 2002). The GIP and GLP-1 responses to intraduodenal lipid are load-dependent in rats (Yoder et al., 2009). Whether this is also the case in humans remains to be determined, although this has been shown to be the case in relation to the effects of lipid on peptide YY (PYY) and cholecystokinin (CCK) (Pilichiewicz et al., 2006). In healthy young subjects, incorporation of fat into either a carbohydrate-containing drink (Houghton et al., 1990) or a solid meal (Cunningham and Read, 1989), or direct infusion of lipid into the small intestine (Welch et al., 1987), slows gastric emptying and attenuates the blood glucose and insulin responses, in proportion to the stimulation of incretin hormones, especially GLP-1 (Gentilcore et al., 2006). Slowing of
gastric emptying and stimulation of GLP-1 and GIP are dependent on the digestion of fat to fatty acids. Accordingly, administration of the lipase inhibitor, orlistat, accelerates gastric emptying of high fat carbohydrate-containing meals, attenuates the release of GIP and GLP-1, and exacerbates the glycaemic response in both health and type 2 diabetics (Pilichiewicz et al., 2003, O'Donovan et al., 2004a). Among different fats, olive oil apparently induces a greater GLP-1 response than butter in both healthy subjects (Thomsen et al., 1999) and patients with type 2 diabetes (Thomsen et al., 2003), suggesting that postprandial GLP-1 release might correlate inversely with the saturation of fatty acids. The chain length of fatty acids has also been proposed as a determinant of the GLP-1 response (Feltrin et al., 2004); when fatty acids were introduced intraduodenally in humans, lauric acid (C12) stimulated GLP-1 release, whereas decanoic acid (C10) did not (Feltrin et al., 2004). The droplet size of fat emulsions is another determinant of gut hormone release, at least for CCK and PYY, whose secretion correlates inversely with the droplet size of infused fat emulsions (Seimon et al., 2009). Even though incretin measurements were not reported in the latter study, it seems likely that GLP-1 would be affected similarly to PYY, given that the latter is also released from L-cells.

### 2.3.3 Effect of protein on incretin release

The effects of protein on incretin release are less well studied than those of carbohydrates or fats. Protein appears to require digestion into peptides or amino acids to stimulate gut feedback responses (Meyer et al., 1976). Some studies have indicated that intraduodenal administration of specific amino
acids stimulates GIP release (Thomas et al., 1978), although intact proteins appear to be less effective (Elliott et al., 1993). In healthy humans, the effects of protein (milk and egg) on GLP-1 release were comparable with fat (oleic acid), while GIP levels in the early postprandial period were greater (Carr et al., 2008); while a meal rich in protein (milk and egg) was reported to trigger a greater GLP-1 response than a carbohydrate-rich meal (corn flakes and white bread) (Raben et al., 2003). In contrast, protein (gelatin) supplementation of an oral glucose load in healthy subjects had no effect on the GLP-1 response, and was associated with a reduction in GIP secretion than with glucose alone, but the addition of gelatin was associated with delayed gastric emptying (Karamanlis et al., 2007). Among different protein sources, whey protein has been shown to stimulate GLP-1 and satiety more than casein (Hall et al., 2003). These different effects are likely to related to their differing amino acid profiles (Hall et al., 2003) and rates of absorption (Tessari et al., 2007). We found in type 2 diabetic patients that, when given acutely, whey protein can stimulate GLP-1 and improve postprandial glycaemia (Ma et al., 2009c) (Fig. 5), although the latter was also likely regulated by other mechanisms including slowing of gastric emptying and direct stimulation of insulin release by absorbed amino acids.

2.4 Mechanisms by which nutrients stimulate incretin release

Due to the limited availability of validated experimental models, the mechanisms underlying nutrient detection in the small intestine and consequent stimulation of incretin release are poorly understood. As discussed, the enteroendocrine cells that release GLP-1 and GIP are ‘open-
type’, and are, accordingly, able to sample the luminal nutrients directly. Because of the insulinotropic actions of GLP-1 and GIP and the associated importance of the incretins in glucose homeostasis, much recent research has focused on the mechanisms of carbohydrate sensing by L- and K-cells. However, it is clear that other macronutrients can also stimulate hormone release from these cells, probably via different mechanisms. Carbohydrate, fat and protein are usually consumed together. Therefore, the relative contributions of the mechanisms described below will vary with meal composition.

2.4.1 Mechanisms underlying glucose sensing
A variety of signaling mechanisms have been proposed to explain how enteroendocrine cells might sense glucose, including ATP-sensitive potassium channel closure, sodium glucose cotransporter activity and activation of sweet taste receptors (Tolhurst et al., 2009). It has also been suggested that a proximal-distal neural or hormonal loop might explain how GLP-1 release can occur rapidly after nutrient digestion despite the predominantly distal location of L-cells (Holst, 2007). GIP appears to fulfil this latter function in rodents, but does not stimulate GLP-1 secretion in humans (Nauck et al., 1993, Hansen and Holst, 2002). However, as discussed, it is possible that the density of L-cells in the human duodenum or proximal jejunum may be sufficiently great to account for the early phase of the GLP-1 response (Theodorakis et al., 2006).
2.4.1.1 $K_{\text{ATP}}$ channel

As in the pancreatic beta-cell, the closure of ATP-sensitive $K^+$ channels ($K_{\text{ATP}}$ channels) in enteroendocrine cells occurs in response to glucose exposure, and gives rise to changes in the proportions of ATP and $\text{Mg}^{2+}$-ADP, leading to reduction of background $K^+$ flux and triggering membrane depolarisation. Both the $K_{\text{ATP}}$ channel subunits Kir6.2 and SUR1 are expressed at high levels in purified mouse K- and L-cells (Parker et al., 2009, Reimann et al., 2008). Expression of Kir6.2 and SUR1 is also detectable in human K- and L-cells by immunostaining (Nielsen et al., 2007). Nevertheless, one study in healthy subjects indicated that sulfonylureas, which induce closure of $K_{\text{ATP}}$ channels, do not stimulate GLP-1 or GIP responses to oral glucose (El-Ouaghlidi et al., 2007), suggesting that additional mechanisms are responsible for glucose-mediated incretin release. Furthermore, Kir6.2(-/-) mice exhibit increased, rather than decreased, plasma GIP levels after administration of oral glucose (Miki et al., 2005). Therefore, a role for $K_{\text{ATP}}$ channels in incretin secretion remains uncertain.

2.4.1.2 Sodium-glucose cotransporter

The sodium-glucose cotransporter-1 (SGLT1), encoded by the SLC5A1 gene, is the principle transporter for glucose absorption at the luminal surface (Ritzel et al., 1997), transporting glucose and Na$^+$ in a 1:2 fashion (Wright and Turk, 2004) and, thereby, generating sufficient inward current to excite K- and L-cells (Gribble et al., 2003). Studies in both cell lines and rodent models confirmed the expression of SGLT1 in K- and L-cells and established that monosaccharides that are substrates for SGLT1, regardless
of whether they can be metabolized (e.g. glucose), or not (e.g. 3-OMG), stimulate incretin secretion in the presence of sodium. Phoridzin, which blocks SGLT1, inhibits the release of GLP-1 in response to monosaccharides in animal models (Sugiyama et al., 1994, Moriya et al., 2009). Similarly, in ob/ob mice, GIP is secreted in response to glucose, galactose, and 3OMG (Flatt et al., 1989). SGLT-1 may well be involved in GIP secretion in humans, since glucose and galactose release GIP, while fructose and mannose, which are not SGLT1 substrates, do not (Ganda et al., 1979, Tazawa et al., 2005). Nevertheless, SGLT1 is probably not the only signaling pathway for GLP-1 release, e.g. fructose stimulates GLP-1 release in both perfused rat ileum (Ritzel et al., 1997) and in healthy humans (Rayner et al., 2000a).

### 2.4.1.3 Sweet taste receptor

In the tongue, the molecular mechanism of sweet-taste detection involves activation of a specific sweet taste G-protein coupled receptor (GPCR), a heterodimer of the T1R2 and T1R3 subtypes, located on the apical membranes of taste cells. Activation of the T1R2/T1R3 complex initiates a signal transduction cascade involving the G-protein alpha-gustducin and the transient receptor potential ion channel TRPM5, resulting in cellular depolarization and release of intracellular calcium stores (Nelson et al., 2001). Expression of mRNA for the sweet taste molecules T1R2, T1R3, alpha-gustducin and TRPM5 has been demonstrated in the rodent intestine and in gut enteroendocrine cell lines (Jang et al., 2007), and the same elements were recently observed in the proximal human small intestine.
Chapter 2. Literature Review 2

(Young et al., 2009). Stimulation of sweet taste receptors has been linked to incretin hormone release. For example, gustducin-null mice exhibit a defective GLP-1 response and impaired glucose tolerance after oral glucose administration (Kokrashvili et al., 2009). Stimuli of the sweet taste receptor, including the artificial sweetener sucralose, were reported to release GLP-1 from L cells in vitro (Jang et al., 2007), although sucralose apparently fails to release GLP-1 or GIP in either rodents in vivo (Fujita et al., 2009) or humans (Ma et al., 2009a). Nonetheless, other ligands of the sweet taste receptor could potentially stimulate GLP-1 secretion, e.g. diet soda sweetened with both sucralose and acesulfame potassium was reported to stimulate GLP-1 release synergically with glucose in healthy humans (Brown et al., 2009). Rodent primary K-cells in culture exhibit low levels of gene expression for sweet taste receptors and no responsiveness to sucralose (Parker et al., 2009), indicating that stimulation of sweet taste receptors appears to be of lesser importance for GIP secretion.

2.4.2 Mechanisms underlying fat sensing

Fat is a potent stimulus for both GLP-1 and GIP release. Digestion of fats into fatty acids appears to be essential for stimulating incretin secretion, as demonstrated by the attenuation of GIP and GLP-1 responses to triglyceride in the presence of the lipase inhibitor, orlistat (Pilichiewicz et al., 2003, O'Donovan et al., 2004a, Ellrichmann et al., 2008).

G-protein-coupled receptors (GPRs), including GPR119 (Overton et al., 2006), GPR40 (Edfalk et al., 2008) and GPR120 (Tanaka et al., 2008), have
been identified as important mediators of fatty acid sensing by enteroendocrine cells. The expression of these lipid-sensing receptors has been well established in murine L- and K-cells (Parker et al., 2009, Chu et al., 2009, Lauffer et al., 2009). In humans, one study reported a high density of GPR119 in the human duodenum, ileum, and jejunum when evaluated by quantitative PCR (Chu et al., 2008), while another found relatively high GPR40 mRNA expression in the ileum (Itoh et al., 2003), and co-localization of GPR120 with GLP-1 bearing cells has been demonstrated in the ileum, colon and rectum (Hirasawa et al., 2005). The relevance of these receptors to GLP-1 and GIP secretion has been examined intensively in gene-modified rodent and enteroendocrine cell models. GPR119 is Gs-coupled, and administration of a GPR119 agonist in the rodent model induced prompt elevation of plasma GLP-1 and GIP, and substantial improvements in postprandial glycaemia (Chu et al., 2008). In vitro studies indicate that elevation of intracellular cyclic AMP (cAMP) is fundamental to GPR119-mediated incretin release (Parker et al., 2009, Lauffer et al., 2009). GPR40 and GPR120 are Gq coupled and are proposed as pathways for enhanced GLP-1 and GIP responses to long chain unsaturated fatty acids (Edfalk et al., 2008, Tanaka et al., 2008), possibly via downstream activation of PKC and inositol (1,4,5)-trisphosphate-dependent Ca\textsuperscript{2+} release from intracellular stores (Parker et al., 2010). Consistent with this role, GPR40 knock-out mice exhibit diminished GIP and GLP-1 responses to a high-fat diet (Edfalk et al., 2008), and administration of the GPR120 ligand, \(\alpha\)-linolenic acid, potently elevates plasma GLP-1 levels in rodent models (Tanaka et al., 2008). However, as mentioned previously, species differences
should be taken into consideration and more human evidence is required to determine whether ligands of these receptors will be useful therapeutically.

Recently, another G protein-coupled receptor, TGR5, was identified in the small intestine and is expressed in the enteroendocrine L-cells in rodents (Reimann et al., 2008). Its stimulation by bile acids potentiates GLP-1 release from STC-1 cells in vitro (Katsuma et al., 2005), and in isolated perfused rat colon (Plaisancie et al., 1995). In vivo studies, using either a TGR5 agonist (6alpha-ethyl-23(S)-methyl-cholic acid) or gene knockout/overexpressing rodent models, indicate that TGR5 signaling is able to induce substantial GLP-1 release, leading to enhanced glucose tolerance in mice (Thomas et al., 2009), and suggesting a therapeutic role for the targeting of TGR5 in diabetes management.

2.4.3 Mechanisms underlying protein sensing

The mechanisms mediating protein-induced incretin secretion remain largely unknown. The oligopeptide transporter 1 (PepT1), a proton-coupled di- and tri-peptide transporter, is a candidate for this role, since it is distributed widely on the intestinal surface and is central to absorption of oligopeptides arising from protein digestion (Adibi, 2003). Studies using the enteroendocrine cell lines GLUTag, NCI-H716 and STC-1 have shown that protein hydrolysates (peptones) can release incretins dose-dependently (Cordier-Bussat et al., 1998, Reimer, 2006), and this effect is further enhanced in PepT1-transfected STC-1 cells (Matsumura et al., 2005), but inhibited partially by nifedipine (Matsumura et al., 2005), suggesting a role
of voltage-gated Ca$^{2+}$-channels subsequent to oligopeptide transport by PepT1. In addition, activation of the glycine receptor (a ligand-gated Cl\(^{-}\) channel) by glycine and alanine triggers substantial GLP-1 secretion in GLUTag cells (Gameiro et al., 2005), while glutamine and asparagine trigger GLP-1 release from these cells via Na$^{+}$-coupled electrogenic uptake leading to depolarization (Reimann et al., 2004). These observations suggest that changes in cellular electrical status might be fundamental to protein-induced incretin responses. However, the secretory characteristics of these cell models can differ from the primary cells, not to mention the in vivo responses. For example, in healthy humans, ileal perfusion with peptone induced only a weak plasma GLP-1 response (Layer et al., 1995). Therefore, the mechanisms underlying protein sensing in the gut remain to be clarified.

### 2.4 Incretin responses in obesity and diabetes

Some studies have reported lower postprandial GLP-1 concentrations in patients with type 2 diabetes compared to healthy controls, and that GIP secretion was relatively intact (Toft-Nielsen et al., 2001, Vilsboll et al., 2003), suggesting that impaired secretion of GLP-1 contributes to postprandial hyperglycaemia in type 2 diabetes. Defective GLP-1 secretion is not apparent in relatives of type 2 diabetic patients (Nauck et al., 2004b, Nyholm et al., 1999) and therefore appears to be secondary to the development of diabetes, perhaps due to hyperglycaemia. However, normalization of glycaemia over 4 weeks (i.e. HbA\(_{1c}\) was reduced from 8.0 ± 0.4% to 6.6 ± 0.3%), while improving the insulin response to GLP-1, does not correct the deficiency of GLP-1 release (Hojberg et al., 2008).
Furthermore, obesity is associated with reduced postprandial GLP-1 (Lugari et al., 2004, Naslund et al., 1998), and it has been reported that body mass index and the degree of impaired glucose tolerance act independently, and additively, to reduce GLP-1 secretion (Muscelli et al., 2008). It seems extraordinary that none of these studies has controlled for potential differences in gastric emptying associated with diabetes or obesity. As both type 1 and type 2 diabetic patients are reported a high prevalence of delayed gastric emptying (Horowitz et al., 2002), and obese subjects may have a greater initial rate of gastric emptying (Verdich et al., 2000). By using an intraduodenal glucose infusion, thus bypassing any effect of gastric emptying, we observed that glucose ingestion triggers a similar maximal, but deficient early phase, GLP-1 response in relatively well controlled type 2 diabetic patients when compared to healthy subjects (Ma et al., 2012). Further investigation is needed in these with poor glycaemic control, in order to identify whether GLP-1 is deficient, and if so, why.

Genetic variations are well recognised as important in the pathogenesis of obesity and diabetes. It is interesting to consider whether similar mechanisms underlie impaired incretin secretion and action in these pathophysiological states, although to date, genetic association studies with GLP-1 and GIP levels are relatively few. The diabetes-related transcription factor 7-like 2 (TCF7L2) gene was the first candidate proposed to affect GLP-1 secretion due to its influence on intestinal proglucagon gene expression (Grant et al., 2006), although the principle effect of TCF7L2 is on the pancreatic islets themselves (Schafer et al., 2007). Recently, polymorphisms in KCNQ1,
another gene associated with diabetes, were reported to correlate with the plasma GLP-1 and GIP response to oral glucose (Mussig et al., 2009). Furthermore, incretin release seems to differ between individuals from different racial backgrounds, e.g. African-American children and adolescents exhibit lower fasting and postprandial concentrations of GLP-1 than Caucasians (Velasquez-Mieyer et al., 2008, Higgins et al., 2008).

2.5 Therapeutic implications
A growing number of studies have yielded insights into potential strategies based on the incretin axis for the prevention and management of obesity and diabetes. These approaches have focused on GLP-1, rather than GIP, since, as discussed, the insulinotropic effect of the latter is impaired in type 2 diabetes (Nauck et al., 1993). The concept of stimulating endogenous GLP-1 release, either by modifying macronutrient composition or by changing dietary habits, is an attractive alternative to the use of exogenous GLP-1 analogues as a way of minimising postprandial glucose excursions.

2.5.1 Modifying macronutrient composition
Modifying the macronutrient composition of a meal represents one therapeutic strategy in diabetes prevention and management that potentially impacts on the incretin axis. For example, substitution of low- for high-glycaemic index carbohydrates may result in slowing of small intestinal carbohydrate absorption (Bjorck and Elmstahl, 2003), allowing exposure of the more distal gut to carbohydrate and subsequent stimulation of greater, and more prolonged, GLP-1 release (Little et al., 2006). Similar effects are
Chapter 2.

2.5.1 Literature Review 2

evident with the use of the α-glucosidase inhibitor, acarbose (Qualmann et al., 1995).

Incorporation of fat, particularly monounsaturated fatty acid (Thomsen et al., 2003, Thomsen et al., 1999), into either a carbohydrate-containing drink or a solid meal, slows the rate of gastric emptying and increases incretin hormone levels, especially GLP-1, reducing the postprandial glycaemic excursion (Gentilcore et al., 2006). Nevertheless, diets rich in fat will inevitably increase the overall caloric load and raise a possibility of weight gain in the long term.

Supplementation of a carbohydrate-rich meal (mashed potato) with protein lowers postprandial glucose excursion by slowing the rate of gastric emptying and stimulating incretin hormone secretion (Ma et al., 2009c). A rodent study also suggested that digested whey protein fragments can inhibit the activity of small intestinal DPP-4 (Gunnarsson et al., 2006). A five-week dietary intervention in type 2 diabetic patients, in which the percentage of dietary protein was increased from 15% to 30%, reported a reduction in postprandial glycaemia and a modest decrease in glycated haemoglobin (Gannon et al., 2003).

2.5.2 The ‘preload’ concept

A novel strategy to minimise postprandial glycaemia could be to administer a small load of a macronutrient before a meal, so that the presence of nutrients in the small intestine induces the release of GLP-1 and GIP, and
other gut peptides such as CCK and PYY, to slow gastric emptying, and potentially stimulate insulin secretion in advance of the main nutrient load. For example, giving fat at an interval (e.g. 30min) before the meal appears more efficacious than incorporation of fat into the meal (Gentilcore et al., 2006). Furthermore, the preload may also have the advantage of suppressing appetite and reducing energy intake at the subsequent meal. We recently reported that acute administration of a whey protein preload markedly reduced the postprandial glycaemic excursion (both peak and AUC glucose) in type 2 patients by these mechanisms (Ma et al., 2009c). However, a potential disadvantage of this strategy is the provision of additional energy intake, which might not be compensated for by a reduction in energy intake at the subsequent meal. A preload that stimulates incretin release and induces slowing of gastric emptying, but contributes minimal additional energy intake, would represent a major advance. Non-nutrient ligands of the various receptors involved in incretin detection that were discussed earlier would be obvious targets of such an approach. Nevertheless, there is as yet little published evidence that this goal can be achieved.

Given the short half-life of incretin hormones in the circulation (Baggio and Drucker, 2007) and moderate effect of monotherapy with GLP-1 analogues (e.g. exenatide) on glucose reduction (Moretto et al., 2008), the combination of DPP-4 inhibitors with the dietary strategies discussed above may be a useful manoeuvre to optimise this approach to diabetes management.
2.5.3 Targeting GIP

GIP is associated with fat deposition in adipocytes by acting in concert with insulin to increase lipoprotein lipase activity and lipogenesis (Irwin and Flatt, 2009), and might be an important contributor in the development of obesity. GIPR gene deficient mice are resistant to diet-induced obesity, without reduction of lean mass or food intake when compared with wild type controls (Yamada et al., 2007), indicating an anabolic effect of GIPR signaling in lipid metabolism, and implying that GIP antagonists could be used for the prevention and treatment of obesity. Indeed, in mice, the GIP antagonist (Pro3)GIP effectively decreased body weight, reducing adipose tissue mass and the deposition of triglyceride in liver and muscle and improving insulin sensitivity and glucose tolerance in mice (McClean et al., 2007). As discussed, in humans with type 2 diabetes, the impaired incretin effect is partly attributable to a diminished insulinotropic action of GIP, in addition to impaired GLP-1 secretion, although a period of strict glycaemic control can restore the insulinotropic effect of GIP (Hojberg et al., 2009, Hojberg et al., 2008). It is, therefore, interesting to ask whether the use of GIP agonists in type 2 patients would have an overall beneficial effect by increasing the incretin effect or whether it would drive more fat deposition into adipocytes with a consequent deleterious effect on obesity. Further exploration is required to address this therapeutic issue.

2.6 Conclusions

The macronutrient composition of a meal, together with physical properties of food, influence incretin hormone release, and is accordingly a
fundamental determinant of the postprandial glycaemic response. Recent insights into the mechanisms by which nutrients modulate incretin hormone secretion have lead to the recognition of a number of potential therapeutic strategies that require further evaluation for their use in the management, and potentially prevention, of type 2 diabetes. Incretin-based strategies involving dietary manipulation are promising addition to pharmacological treatments in diabetes management.
Figure 1. Blood glucose (A), plasma insulin (B), GLP-1 (C) and GIP (D) in response to intraduodenal glucose (25%, 1390 mOsmol/L) infused over 120 minutes at rates of 1 (“G1”), 2 (“G2”), or 4 (“G4”) kcal/min, or saline (4.2%, 1390mOsmol/L) control (“C”), in 10 healthy males. (A) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (B) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (C) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (D) * vs. control: $P < 0.01$, # vs. G1: $P < 0.01$. Data are means ± SEM. (Pilichiewicz et al., 2007a)
Figure 2. Blood glucose and plasma insulin, GLP-1 and GIP levels, during intraduodenal infusion of glucose at 1 kcal/min for 120 min. Blood glucose and plasma insulin, GLP-1, and GIP all increased significantly from baseline ($P < 0.001$). For GLP-1, only the values at 15 and 30 min differed significantly from baseline ($*P < 0.001$). Data are means ± SEM. (Kuo et al., 2008)
Figure 3. Effect of initially more rapid intraduodenal glucose infusion (3 kcal/min between $t = 0$ and 15 min and 0.71 kcal/min between $t = 15$ and 120 min) (closed symbols) compared to constant infusion (1 kcal/min between $t = 0$ and 120 min) (open symbols) in healthy subjects (squares) and patients with type 2 diabetes (circles) on blood glucose (A), plasma insulin (B), plasma GLP-1 (C), and plasma GIP (D). Each pair of curves differs between 0 and 30 min for variable vs. constant intraduodenal infusion ($P < 0.05$). (O'Donovan et al., 2004b)
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Figure 4. Blood glucose (A), plasma insulin (B), GIP (C) and GLP-1 (D) concentrations during a 60 min infusion of 1 M glucose to either 1) a 60 cm segment of the proximal small intestine (short-segment infusion) or 2) > 60 cm of the small intestine (long-segment infusion). (A): there was a treatment-by-time interaction for blood glucose ($P < 0.05$). Although blood glucose increased progressively on both days between $t = 15$ and 60 min, the magnitude of the rise was greater during the long-segment infusion between $t = 30$ and 60 min than during the short-segment infusion ($P < 0.05$). (B): there was a treatment-by-time interaction for plasma insulin concentrations ($P < 0.001$). Although plasma insulin concentrations progressively increased from baseline during both treatments between $t = 20$ and 60 min, the magnitude of the rise in plasma insulin was greater during the long-segment infusion between $t = 30$ and 60 min than during the short-segment infusion ($P < 0.05$). (C): there was no effect of treatment on plasma GIP concentrations. (D): there was a treatment-by-time interaction for plasma GLP-1 concentrations ($P < 0.001$). At $t = 20$ and between $t = 45$ and 60 min, plasma GLP-1 was greater during the long- than during the short-segment infusion ($P < 0.01$). Data are means ± SEM; $n = 8$. *Short-segment infusion vs. long-segment infusion, $P < 0.05$. (Little et al., 2006)
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Figure 5. Gastric emptying (A), concentration of blood glucose (B), plasma insulin (C), plasma GLP-1(D), plasma GIP (E), and plasma CCK (F) in response to a mashed potato meal in eight type 2 diabetic patients. On each study, subjects ingested 350 ml beef-flavored soup 30 min before a radiolabeled mashed potato meal; 55g whey protein was added either to the soup (whey preload) or to the potato (whey in meal) or no whey was given (no whey). Data are means ± SEM. *P < 0.05, whey preload vs. whey in meal; # P < 0.05, whey in meal vs. no whey; § P < 0.05, whey preload vs. no whey. (Ma et al., 2009c)
CHAPTER 3: METHODOLOGIES

3.1 Introduction

This chapter provides an overview of the methodologies and techniques employed in this thesis for the assessments of gastric emptying, antropyloroduodenal (APD) motility, intestinal sweet taste receptors (STRs), appetite perception and energy intake, glucose absorption, and glycaemia and gut hormones in response to either oral, intraduodenal, intrajejunal, or intrarectal administration of gastrointestinal stimuli. Employment of the glycaemic clamp technique in healthy subjects and patients with type 2 diabetes allows determination of the effect of acute changes in blood glucose on the function of the gastrointestinal tract. Combined application of these techniques affords comprehensive assessment of the reciprocal relationship between the gut function and glycaemic control in health and type 2 diabetes.

3.2 Ethical approval

All study protocols were approved by the Royal Adelaide Hospital Ethics Committee, and the studies were registered at www.actr.org.au. If a study drug was involved, the Investigational Drug Sub-Committee also gave approval, and a ‘Clinical Trial Notification (CTN) Scheme’ form was lodged with the Australian Government Department of Healthy and Aging, Therapeutic Goods Administration, prior to commencement of the study. Each subject provided informed, written consent before their inclusion. All studies were conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.
3.3 Recruitment of study subjects

Two major groups of subjects, including healthy volunteers and patients with type 2 diabetes, were recruited by advertising in local diabetes magazines and on notice boards in the Royal Adelaide Hospital and local universities, and subjects were screened using inclusion criteria of each study, as described in this thesis, prior to enrolment.

3.4 Gastrointestinal symptom questionnaires

Gastrointestinal symptom questionnaires were used to assess the gastrointestinal wellbeing of all subjects at the screening visit. These consisted of 9 questions, including ‘poor appetite for food’, ‘nausea, or a feeling of sickness’, ‘feeling full after eating only a little food’, ‘discomfort or distension in the upper abdomen’, ‘vomiting’, ‘pain in the abdomen’, ‘difficulty in swallowing’, ‘heartburn’ and ‘acid regurgitation’. These questions were scored from ‘0’ to ‘3’ by the degree that subjects experienced the symptoms in the preceding three months: 0 = none; 1 = mild (the symptom can be ignored); 2 = moderate (the symptom cannot be ignored, but does not influence daily activities); 3 = severe (the symptom affects daily activities) (Horowitz et al., 1991, Jones et al., 2001).

3.5 Autonomic nerve function

Autonomic nerve function was assessed in patients with type 2 diabetes using standardised cardiovascular reflex tests (Ewing and Clarke, 1982, Vinik and Mehrabyan, 2003). This involved assessment of both
parasympathetic function (the variation in R-R interval in response to deep breathing and the change in position from lying to standing (30:15 ratio)) and sympathetic function (systolic blood pressure fall in response to standing), scored according to age-adjusted predefined criteria (Ewing and Clarke, 1982): 0 = normal, 1 = borderline, 2 = abnormal, for a total maximum score of 6. A score of 3 or greater was considered to indicate the presence of autonomic dysfunction (Ewing and Clarke, 1982).

3.6 Glycaemic clamp

A glycaemic clamp was employed in the study reported in Chapter 11 to achieve desired glycaemic conditions (i.e. euglycaemia: ~6 mmol/L, or hyperglycaemia: ~12 mmol/L). An intravenous cannula was inserted into a forearm vein in each arm, one for infusion of insulin and/or glucose, and the other for repeated blood sampling. Blood glucose concentrations were determined by a portable glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA) every 5 min throughout the duration of the glycaemic clamp to guide adjustment for the infusion rate of insulin and/or glucose (Kuo et al., 2010, Rayner et al., 2000b, Jones et al., 1999). On the hyperglycaemic days, a bolus of 50 mL (volume calculated to elevate blood glucose to 12 mmol/L (Ward et al., 1984)) 25% glucose (Baxter Viaflex, Baxter Healthcare, Australia) was infused intravenously over 2 minutes, followed by 25% glucose infusion at a rate adjusted according to blood glucose concentrations measured every 5 minutes. On the euglycaemic days, a bolus of 50 mL 0.9% saline (Baxter Viaflex, Baxter Healthcare, Australia) was infused as a control, followed by 0.9% saline infusion at a
constant rate of 100 mL/h throughout the study. Concurrently, a solution of 100 units of insulin (Actrapid Penfill, 100 IU/mL, Novo Nordisk) made up to 500 mL with 4% succinylated gelatin solution (Gelofusine; B. Braun Australia, Bella Vista, NSW, Australia) that yielded a final insulin concentration at 0.2 IU/mL, was infused intravenously to keep blood glucose at ~ 6 mmol/L, with rates adjusted according to blood glucose concentrations measured.

3.7 Gastric emptying

3.7.1 Stable isotope breath tests

The use of stable isotope breath tests in evaluating gastric emptying is well established (Ghoos et al., 1993) and validated (Chew et al., 2003). It has been developed to assess gastric emptying of solid (Perri et al., 2005), liquid (Kurita et al., 2011), or semi-solid (Cardoso-Junior et al., 2007) meals in humans. Although scintigraphy is considered the gold standard in measuring gastric emptying, it has been limited by concerns of radiation exposure and also requires access to specialised and expensive facilities, whereas stable isotope breath tests are relatively inexpensive, safe, and easily performed in the laboratory. Therefore, gastric emptying was evaluated by isotope breath tests rather than scintigraphy in the studies of this thesis.

In general, $^{13}$C-octanoic acid was used to label the solid and semi-solid meals, and $^{13}$C-acetate to label liquid meals. Following oral ingestion, the emptying of the meal from the stomach into the duodenum is the rate-limiting step. The subsequent absorption and metabolism gives rise to $^{13}$CO$_2$ that can be
measured in the breath. The fitting of $^{13}\text{CO}_2$ excretion curves, i.e. the percentage of dose per hour and cumulative dose per hour, allows calculation of half-emptying time and gastric emptying coefficient (GEC) with established formulas (Ghoos et al., 1993). $^{13}\text{CO}_2$ concentrations in breath samples were measured by an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas chromatographic purification system.

### 3.7.2 APD motility

Manometric measurement of APD motility is a well established technique, which involves intubation of a 16-lumen silicone manometric catheter (diameter 3.5 mm; Densleeve International, Ontario, Canada) through an anesthetised nostril. The 16 lumens (channels) are constantly perfused with 0.9% degassed saline at the rate of 0.15 ml/min and connected to external pressure transducer sensors that are linked to a computer-based program. On the other end, all the channels terminate as side-holes, spaced at 1.5 cm intervals, along the catheter to be positioned in different regions of the APD tract (channels 1 - 7 in the duodenum, a 4.5 cm sleeve sensor (channel 8) and 2 channels on the back of the sleeve (channels 9 and 10) across the pylorus, and channels 11 – 16 in the antrum). In addition, the catheter contains an infusion channel that is 12 cm distal to the end of the sleeve sensor. Any side-hole occlusion by the luminal contraction would generate a back-pressure to the corresponding external sensor; the latter converts this signal into a pressure wave in a computer program (Flexisoft, Oakfield Instruments of Oxford, UK).
The manometric APD catheter was allowed to pass into the small intestine by peristalsis. Its correct positioning was monitored by continuous measurement of the transmucosal potential difference (TMPD) between the most distal antral channel (channel 11) (~ -40 mV) and the most proximal duodenal channel (channel 7) (~ 0 mV), using a reference electrode (a 20-gauge intravenous cannula filled with sterile saline) placed subcutaneously in the left forearm (Heddle et al., 1988b). In the study of Chapter 7, the recorded manometric waves were then analysed using custom-designed software (Prof AJ Smout, Academic Medical Center, Amsterdam, The Netherlands) using accepted definitions (Heddle et al., 1988a, Samsom et al., 1998) to determine the number of isolated pyloric pressure waves (IPPWs), and antral and duodenal pressure waves. The frequency of pressure waves was analysed over successive 15 min periods.

### 3.8 Unsedated endoscopic biopsy sampling and assessment of intestinal STRs

Unsedated endoscopic biopsy sampling, and immunohistostaining and real-time PCR in assessing STRs (i.e. T1R2, T1R3, α-gusducin, and TRPM5) were employed in the study of Chapter 11.

Unsedated endoscopy using a thin endoscope (diameter: 5.3 mm) passed transnasally is well tolerated (Mori et al., 2011, Horiuchi et al., 2009), and associated with a lower risk of respiratory complications (e.g. oxygen desaturation) than endoscopy with sedation (Ciriza de los Rios et al., 2005).
Discomfort in the nose and throat can be minimised by using topical anaesthetics. Potential risks include sore throat and a remote risk of damage to the gastrointestinal mucosa causing bleeding or perforation. Patients at risk of bleeding (i.e. with a history of coagulation defects/use of anticoagulant drugs) were excluded from the study. Unsedated endoscopy was performed by a consultant gastroenterologist (A/Prof CK Rayner; Discipline of Medicine, University of Adelaide, Adelaide, Australia). On each study visit, subjects attended the Gastrointestinal Investigation Unit (Ward Q7, Royal Adelaide Hospital) at approximately 0800h, after 12 hours overnight fast. Local anaesthetic spray was applied to the nose and pharynx, and a small diameter (5.3 mm) Olympus video-endoscope was then passed through the nose, with minimal insufflation of air, into the second part of the duodenum, from which small tissue samples (10 in total per procedure) were taken. In previous studies, biopsy rates up to 38 times per procedure were shown to be safe (Siersema, 2009). The risk of significant bleeding from endoscopic biopsies is extremely small (< 1 in 5,000 per procedure) (Sieg et al., 2001).

Immunohistostaining and quantitative PCR assays for STRs of the biopsy samples were performed by Dr RL Young (Nerve Gut Lab, Royal Adelaide Hospital, Adelaide, Australia) using established methods (Young et al., 2009). Duodenal biopsies were stored immediately in (i) RNAlater and (ii) fixative (4% paraformaldehyde), prior to further processing. RNA was extracted from tissues using an RNeasy Mini kit (Qiagen,) following manufacturer instructions, and RNA yield and quality determined using a
NanoDrop (NanoDrop Technologies, Wilmington, DE). Quantitative real time reverse transcriptase PCR (RT-PCR) was then used to determine the absolute expression of sweet taste molecules. Biopsies stored in fixative were used both to confirm normal duodenal histology and to perform slide-based immunohistochemistry for human T1R2, T1R3, α-gustducin, TRPM5, GLP-1 and GIP.

3.9 Appetite perception and energy intake

3.9.1 Visual analogue scales (VAS)

VAS questionnaires are a commonly used tool to assess appetite perception and gastrointestinal sensations (Parker et al., 2004). Each VAS question evaluated a sensation on a 100 mm horizontal line, where 0 mm represented “sensation not felt at all” and 100 mm represented “sensation felt greatest”. Subjects were asked to indicate how they were feeling at the particular time point by placing a vertical mark on the line. ‘Hunger’, ‘fullness’, ‘desire to eat’ and ‘prospective consumption’ were evaluated over each study period.

3.9.2 Buffet meal

A standardised, cold buffet-style meal, comprising 125 g wholemeal bread, 125 g white bread, 100 g ham, 100 g chicken, 85 g cheese, 100 g tomato, 100 g lettuce, 100 g cucumber, 200 g strawberry yogurt, 140 g fruit salad, 150 g chocolate custard, 170 g apple, 190 g banana, 500 g orange juice, 600 g iced coffee, 600 g water, 20 g margarine and 20 g mayonnaise, was used to evaluate energy intake in the study of Chapter 7, during which, subjects were given 30 min to consume as much as they wished until they felt comfortably
full. The type of food as well as the macronutrient composition and energy content was described in detail previously (Chaikomin et al., 2009, Feltrin et al., 2004). Energy intake was analyzed using the commercially available software (Foodworks 3.01; Xyris Software, Highgate Hill, Queensland, Australia) (Chaikomin et al., 2009, Feltrin et al., 2004).

3.10 Assessment of intestinal absorption

3.10.1 3-O-methylglucose (3-OMG)

Sodium glucose co-transporter 1 (SGLT1) is the main route for the transport of dietary monosaccharides (Shirazi-Beechey et al., 2011b), with a minor role for other transporters (eg. glucose transporter 2) (Shirazi-Beechey et al., 2011b, Ait-Omar et al., 2011). Both glucose and 3-OMG are substrates of SGLT1, absorbed via active transport in the presence of sodium (Uhing and Kimura, 1995). However, ~30% glucose is metabolised in the liver after luminal absorption (Cherrington, 1999), whereas 3-OMG is a non-metabolised glucose analogue. Therefore, measurement of blood 3-OMG represents a more accurate assessment of active glucose uptake in the small intestine (Fordtran et al., 1962a). In the study of Chapter 11, serum 3-OMG concentrations were measured by liquid chromatography/mass sepectrometry (Ma et al., 2010).

3.10.2 Breath hydrogen test

In healthy humans, hydrogen is a product of bacterial fermentation of unabsorbed carbohydrates in the colon (Levitt, 1969). An increase in breath hydrogen reflects enhanced fermentation that may result from small
intestinal malabsorption of carbohydrates and/or small intestinal bacterial overgrowth (Braden, 2009). In the study reported in Chapter 5, breath hydrogen measurement was used to assess small intestinal malabsorption of D-xylose. Hydrogen concentrations in breath samples were measured using Quintron MicroLyzer SC (Quintron Instrument Co Inc., Milwaukee, Wisconsin, USA), and were corrected for CO₂ levels (Jones et al., 2011).

3.11 Intraluminal infusion

3.11.1 Intraduodenal infusion

It is well established that the stimulation of gut hormone secretion is highly dependent on the rate of nutrient entry into the small intestine (ie. the rate of gastric emptying) (Ryan et al., 2012, Ma et al., 2012, Pilichiewicz et al., 2007b). However, there is substantial variation in the normal rate of gastric emptying (1-4 kcal/min) (Brener et al., 1983, Macdonald, 1996). Intraduodenal infusion of nutrients at a controlled rate would allow more precise assessment of gut hormone responses. Intraduodenal infusion of glucose was employed in the studies of Chapter 7 and 11 via a dedicated channel within a multilumen manometric APD catheter and via the endoscopic biopsy channel respectively.

3.11.2 Intrajejunal infusion

Intrajejunal infusion technique was employed in the study of Chapter 9. A customised silicone rubber tube (diameter: 4.2 mm; Dentsleeve International, Ontario, Canada) was positioned transnasally via continuous measurement of TMPD as described above, with the infusion port located 50 cm distal to the
pylorus (in the proximal jejunum), an incorporated balloon (5 cm in length, with a maximum volume of 100 mL) 30 cm distal to the pylorus (to be inflated to occlude the lumen of the duodenum and the jejunum), and an aspiration channel 25 cm distal to the pylorus (to be used to collect proximal gut secretions during the study period). After correct positioning of the catheter, the catheter balloon was inflated with air to block the delivery of duodenal contents into the jejunum. Balloon inflation ceased (~ 35 mL) when subjects reported a sensation of pressure, without discomfort (Little et al., 2006). The complete luminal occlusion was ensured by ~ 30 mmHg intraballoon pressure monitored continuously by a pressure gauge; an intraballoon pressure at ~ 20 mmHg was shown to achieve complete luminal occlusion (von Richter et al., 2001, Little et al., 2006). Thus, intrajejunal infusion excluded the potential influence of duodenal contents on functional changes of the gastrointestinal tract.

3.11.3 Rectal administration

Rectal perfusion of taurocholic acid was performed in the study described in Chapter 8. Subjects were all asked to defecate prior to rectal administration, and then positioned in the left lateral decubitus position during the study. The aqueous gel form of dosage was infused into the rectum using a syringe and soft cannula (i.e. female urinal catheter), without causing any rectal discomfort. The suppository dosage was inserted into the rectum assisted by lubricating gel.
3.12 Biochemical measurements

3.12.1 Blood glucose

The majority of the blood glucose concentrations reported in this thesis were measured by portable glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA) at the bedside. In the study reported in Chapter 7, blood glucose concentrations were measured by using both a YSI analyser (YSI 2300 STAT Plus, Yellow Springs Instruments Corp., Yellow Springs, OH, USA), which uses a glucose oxidase technique, and the Medisense glucometer. The YSI analyser provides comparable accuracy in real time blood glucose assessment with the gold standard device, the Beckman glucose analyser (Chua and Tan, 1978); production of the latter device was discontinued in 2009.

We found that the area under the curve (AUC) for blood glucose showed an excellent correlation between glucometer and YSI analyser ($r = 0.92, P < 0.0001$) (Figure 1), although the absolute values were relatively lower with the latter ($P < 0.001$). The glucose measurement technique had no effect on the statistical outcomes for the effects of sitagliptin and metformin on glycaemia in healthy lean, obese subjects, and patients with type 2 diabetes. Therefore, we regard the glucometer values for blood glucose in the studies reported in Chapter 4, 5, 6, 8, 9, 10 and 11, as acceptably accurate.

3.12.2 Glucagon like-peptide 1 (GLP-1)

In the studies of described in Chapter 4, 8, 9, 10 and 11, plasma total GLP-1 concentrations were measured by radioimmunoassay (GLPIT-36HK; Linco
Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and intra- and inter-assay CVs were 6.8% and 8.5% respectively. In the study described in Chapter 5, plasma intact GLP-1 was measured by radioimmunoassay using a commercially available kit (GLP1A-35HK, Millipore, Billerica, MA), which allows quantification of biologically active forms of GLP-1 (i.e. 7-36 amide and 7-37) in plasma and other biological media. The sensitivity was 3 pmol/L, and intra- and inter-assay CVs were 3.4% and 9.1% respectively.

In the study of Chapter 7, plasma intact GLP-1 was measured using a two-site (sandwich) ELISA, using two monoclonal antibodies: GLP-1F5 as a catching antibody (C-terminally directed) and Mab26.1 as a detecting antibody (N-terminally directed) (Vilsboll et al., 2003), while total GLP-1 was assayed using antiserum 89390, which has an absolute requirement for the intact amidated C-terminus of the molecule, and reacts equally with intact GLP-1 and the primary (N-terminally truncated) metabolite (Orskov et al., 1994). The sensitivity of intact GLP-1 assay was < 0.5 pmol/L, and intra- and inter-assay CVs were 2% and 5% respectively. The sensitivity of total GLP-1 assay was 1 pmol/L, and intra- and inter-assay CVs were < 6% and < 15% respectively.

3.12.3 Glucose-dependent insulinotropic peptide (GIP)

In the study of Chapter 4 and 11, plasma total GIP concentrations were measured by radioimmunoassay (Wishart et al., 1992), with a sensitivity of 2 pmol/L and intra- and inter-assay CVs of 10.2% and 11.2% respectively.
In the study of Chapter 7, plasma intact GIP was analysed with the N-terminally directed antiserum, 98171 (Deacon et al., 2000), with the sensitivity of 5 pmol/L and intra- and inter-assay CVs of < 6% and < 15%, respectively. Plasma total GIP was analysed with the C-terminally directed antiserum, 80867 (Lindgren et al., 2011), with the sensitivity of < 2 pmol/L and intra- and inter-assay CVs of < 6% and < 15%, respectively.

3.12.4 Peptide YY (PYY)

Peptide PYY concentrations were measured by radioimmunoassay using an adaptation of a previously described method (Pilichiewicz et al., 2006). An antiserum (kindly donated by Dr. B Otto, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Munich, Germany), raised against human peptide YY (1-36) (Sigma-Aldrich, St Louis, MO), was employed; the assay does not distinguish between PYY (1-36) and PYY (3-36). This antiserum showed < 0.001% cross reactivity with human pancreatic polypeptide or sulphated cholecystokinin-8 and 0.0025% cross reactivity with human neuropeptide Y. The minimum detectable limit was 1.5 pmol/L, and intra- and inter-assay CVs of 3.5% and 7.9 respectively.

3.12.5 Insulin

Serum insulin concentrations were measured by ELISA immunoassay (10-1113; Mercodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L, and intra- and inter-assay CVs of 2.1% and 6.6% respectively.
3.12.6 C-peptide

C-peptide concentration were measured by ELISA immunoassay (10-1136-01, Mercodia, Uppsala, Sweden). The sensitivity of the assay was 15 pmol/L and the CV was 3.6% within assays and 3.3% between assays.

3.12.7 Glucagon

In the study reported in Chapter 7, plasma glucagon immunoreactivity was determined using the C-terminally directed antiserum 4305, which measures glucagon of pancreatic origin (Orskov et al., 1991). The sensitivity of the assay was ~1 pmol/l and intra- and inter-assay CVs of < 6% and < 15%, respectively. In the study reported in Chapter 9, plasma glucagon was measured by radioimmunoassay (GL-32K, Millipore, Billerica, MA) with sensitivity 20 pg/ml, and intra- and inter-assay CVs of 15 % and 10.5 %.

3.13 Statistical analysis

All analyses were performed using SPSS software (version 17.0 or version 19.0), in consultation with a biomedical statistician (Ms Kylie Lange, Centre of Research Excellence in Translating Nutritional Science to Good Health, Discipline of Medicine, University of Adelaide). Data were presented as mean values ± standard error; $P < 0.05$ was considered statistically significant.

3.14 Conclusions

The methods and techniques employed in this thesis have all been validated and are well tolerated by subjects. They are considered as either the best
available, or the most practical, techniques to address the hypotheses of each study.
Figure 1. The relationship between the area under the curve (AUC) for blood glucose measured by a glucometer was positively related to that with the YSI analyser ($r = 0.92, P < 0.0001$).

**NOTE:**
This publication is included on pages 66-85 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.3945/ajcn.111.021543](http://dx.doi.org/10.3945/ajcn.111.021543)
CHAPTER 5. EFFECTS OF A D-XYLOSE PRELOAD, WITH OR WITHOUT SITAGLIPTIN, ON GASTRIC EMPTYING, GLUCAGON-LIKE PEPTIDE-1, AND POSTPRANDIAL GLYCAEMIA IN TYPE 2 DIABETES


5.1 Summary

Macronutrient ‘preloads’ can reduce postprandial glycaemia by slowing gastric emptying and stimulating glucagon-like peptide-1 (GLP-1) secretion. An ideal preload would entail minimal additional energy intake, and might be optimised by concurrent inhibition of dipeptidyl peptidase-4 (DPP-4). We evaluated the effects of a low energy D-xylose preload, with or without sitagliptin, on gastric emptying, plasma intact GLP-1 concentrations and postprandial glycaemia in type 2 diabetes. 12 type 2 patients were studied on 4 occasions each. After 100 mg sitagliptin (S) or placebo (P) and an overnight fast, patients consumed a preload drink containing either 50 g D-xylose (X), or 80 mg sucralose (control, C), followed after 40min by a mashed potato meal labeled with $^{13}$C-octanoate. Blood was sampled at intervals. Gastric emptying was determined. Both peak blood glucose and the amplitude of glycaemic excursion were lower after ‘PX’ and ‘SC’ than ‘PC’ ($P < 0.01$ for each), and were lowest after ‘SX’ ($P < 0.05$ for each), while overall blood glucose was lower after ‘SX’ than ‘PC’ ($P < 0.05$). The postprandial insulin/glucose ratio was attenuated ($P < 0.05$), and gastric
emptying was slower ($P < 0.01$) after D-xylose, without any effect of sitagliptin. Plasma GLP-1 concentrations were higher after D-xylose than control only before the meal ($P < 0.05$), but were sustained postprandially when combined with sitagliptin ($P < 0.05$). In conclusion, in type 2 diabetes, acute administration of a D-xylose preload reduces postprandial glycaemia, and enhances the effect of a DPP-4 inhibitor.

5.2 Introduction

Therapeutic strategies directed at reducing postprandial glycaemia are of fundamental importance in the management of type 2 diabetes (Inzucchi et al., 2012). For patients with mild to moderate hyperglycaemia, postprandial blood glucose is a better predictor of glycated haemoglobin A1c (HbA1c) than fasting blood glucose (El-Kebbi et al., 2004).

Both gastric emptying, and the action of the ‘incretin’ hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), are major determinants of postprandial glucose excursions (Chaikomin et al., 2006). Gastric emptying determines the rate of nutrient delivery to the small intestine, accounting for about one third of the variation in the initial rise in glycaemia after oral glucose in both health (Horowitz et al., 1993) and type 2 diabetes (Jones et al., 1996). GLP-1 and GIP, released predominantly from the distal and proximal gut respectively, are the known mediators of the ‘incretin’ effect, whereby much more insulin is stimulated by enteral, compared to intravenous,
In type 2 diabetes, the incretin effect is impaired (Nauck et al., 1986), related at least partly to a diminished insulinotropic effect of GIP, while that of GLP-1 is preserved (Nauck et al., 1993). In addition, GLP-1 slows gastric emptying (Deane et al., 2010), suppresses glucagon secretion (Schirra and Goke, 2005), and reduces energy intake (De Silva et al., 2011). Therefore, incretin-based therapies for diabetes have hitherto focused on GLP-1.

One promising strategy to stimulate endogenous GLP-1 is the ‘preload’ concept, which involves administration of a small load of macronutrient at a fixed interval before a meal, so that the presence of nutrients in the small intestine induces the release of gut peptides, including GLP-1, to slow gastric emptying and improve the glycaemic response to the subsequent meal. Fat (Gentilcore et al., 2006) and protein (Ma et al., 2009c) preloads achieve these goals, but entail additional energy intake. We recently demonstrated in healthy subjects the potential for poorly absorbed sweeteners, which yield little energy, to stimulate GLP-1 secretion and slow gastric emptying (Wu et al., 2012).

D-xylose is a pentose sugar, which is incompletely absorbed by passive diffusion in human duodenum and jejunum (Fordtran et al., 1962b), with the remainder delivered to the ileum and the colon, where bacterial fermentation occurs, producing hydrogen that can be detected in the breath (Craig and Ehrenpreis, 1999). We recently showed that oral consumption of D-xylose stimulates GLP-1 secretion to a greater, and more sustained, degree than glucose.
in healthy older subjects (Vanis et al., 2011), consistent with the principle that
the length and region of small intestine exposed to carbohydrate are important
determinants of GLP-1 release (Qualmann et al., 1995). D-xylose also slowed
gastric emptying with similar efficacy to the glucose load when compared to
water (Vanis et al., 2011).

Intact GLP-1 is short-lived in the circulation due largely to rapid degradation by
the enzyme, dipeptidyl peptidase 4 (DPP-4) (Drucker and Nauck, 2006), and
orally administered DPP-4 inhibitors, such as sitagliptin, increase postprandial
plasma concentrations of intact GLP-1 (Ahren, 2007). However, the concept of
stimulating endogenous GLP-1 with enteral nutrients and then optimising its
action with a DPP-4 inhibitor has received little attention. Moreover, little
consideration has been given as to whether the composition of the diet
influences the efficacy of DPP-4 inhibition to lower postprandial blood glucose.

The current study was designed to determine, in patients with type 2 diabetes,
whether a D-xylose preload would slow gastric emptying, stimulate GLP-1
secretion and improve postprandial glycaemia, and whether these effects could
be enhanced by DPP-4 inhibition.
5.3 Research design and methods

5.3.1 Subjects

Twelve patients with type 2 diabetes (9 males and 3 females), managed by diet alone, were studied, after they had provided written, informed consent. The mean age (± standard error) was 66.2 ± 1.4 years; body mass index (BMI) 28.9 ± 1.0 kg/m²; HbA1c 6.6 ± 0.2% (48.9 ± 2.5 mmol/mol); and duration of known diabetes 4.9 ± 1.1 years. None had significant comorbidities, was a smoker, or was taking any medication known to affect gastrointestinal function. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

5.3.2 Protocol

Each subject was studied on four occasions, separated by at least 3 days, in randomised, double-blind, fashion. On the evening before each study day (~1900), each subject consumed a standardised evening meal (McCain’s frozen beef lasagna, 2170 kJ; McCain Foods Proprietary Ltd, Victoria, Australia). At ~2200, subjects took a tablet of either 100 mg sitagliptin (Januvia®; Merck, Sharp and Dohme) or matching placebo, and compliance was reinforced with a reminder phone call that evening.

Subjects then fasted until the following morning, when they attended the laboratory at ~0800 and were seated comfortably for the duration of the study.
An intravenous cannula was inserted into an antecubital vein for repeated blood sampling. On each study day, between t = -40 to -38 min, they consumed a 200 mL preload drink containing either 50 g D-xylose or 80 mg sucralose (a control of equivalent sweetness, which we have shown not to stimulate GLP-1 secretion, or slow gastric emptying in healthy humans (Ma et al., 2009a, Ma et al., 2010)), so that the four treatments were sitagliptin + D-xylose (SX), sitagliptin + control (SC), placebo + D-xylose (PX), and placebo + control (PC). Forty minutes later (between t = 0 to 5 min), they ate a solid meal consisting of 65 g powdered potato (Deb Instant Mashed Potato, Continental, Epping, NSW, Australia) and 20 g glucose, reconstituted with 200 mL water and 1 egg yolk containing 100μL $^{13}$C-octanoic acid. Breath samples were collected immediately before and every 5 min after meal ingestion in the first hour, and every 15 min for a further 3 h for the measurement of gastric emptying. Venous blood samples and additional breath samples, before the preload drink (at t = -40) and at t = -20, 0, 15, 30, 60, 90, 120, 240 min, were taken for the measurements of blood glucose, insulin, intact GLP-1, and breath hydrogen.

Blood samples for insulin were collected in serum tubes. For the measurement of intact GLP-1, venous blood was collected into ice-chilled EDTA tubes containing DPP-4 inhibitor (DPP4-010; Linco Research Inc., St. Charles, MO, USA) (10 μL/mL blood). Samples were mixed six times by gentle inversion, and stored on ice before centrifugation at 3200 rpm for 15 min at 4 degrees Celsius.
within 15 min of collection. Serum and plasma were separated and stored at –70 degrees Celsius for subsequent analysis.

5.3.3 Measurements

5.3.3.1 Blood glucose, serum insulin and intact GLP-1

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). The accuracy of the method has been validated against the hexokinase technique (Horowitz et al., 1993).

Serum insulin was measured by ELISA immunoassay (10-1113; Mercodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L and the coefficient of variation (CV) was 2.1% within assays and 6.6% between assays.

Plasma intact GLP-1 was measured by radioimmunoassay using a commercially available kit (GLP1A-35HK, Millipore, Billerica, MA), which allows quantification of biologically active forms of GLP-1 (i.e. 7-36 amide and 7-37) in plasma and other biological media. The sensitivity was 3 pmol/L, and intra- and inter-assay CVs were 3.4% and 9.1% respectively.

5.3.3.2 Gastric emptying

$^{13}$CO$_2$ concentrations in breath samples were measured by an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas
chromatographic purification system. The half-emptying time (T50) was calculated, using the formula described by Ghoos et al (Ghoos et al., 1993). This method has been validated against scintigraphy for the measurement of gastric emptying (Chew et al., 2003).

5.3.3.3 Breath hydrogen

Hydrogen concentrations in breath samples were measured using Quintron MicroLyzer SC (Quintron Instrument Co Inc., Milwaukee, Wisconsin, USA), and were corrected for CO₂ levels (Jones et al., 2011).

5.3.4 Statistical analysis

The incremental area under the curves (iAUC) were calculated using the trapezoidal rule (Wolever, 2004) for blood glucose, serum insulin, plasma intact GLP-1 and breath hydrogen, and analysed using one-factor repeated-measures ANOVA. These variables were also assessed by repeated-measures ANOVA, with treatment and time as factors. The amplitude of glycaemic excursion (AGE, i.e. postprandial glycaemic peak minus the nadir) and ‘J’-index (J = 0.324 × (Mean Blood Glucose + SD) ²) were calculated as measures of glycaemic variability, as described previously (Standl et al., 2011), and these, together with gastric emptying (T50), were compared using one-factor ANOVA. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni-Holm’s correction, were performed if ANOVAs showed significant effects. Relationships between variables were assessed using linear regression analysis.
Chapter 5. D-xylose preload and sitagliptin

All analyses were performed with SPSS Statistics (version 19.0; IBM Corporation, Armonk, NY, USA). Data are presented as mean values ± standard error; \( P < 0.05 \) was considered statistically significant.

5.4 Results

All subjects tolerated the study well. Three subjects reported mild transient loose stools after completion of the study on the D-xylose days.

5.4.1 Blood glucose concentrations

Fasting blood glucose concentrations did not differ between the four study days (PC: 7.4 ± 0.3 mmol/L; PX: 7.5 ± 0.3 mmol/L; SC: 7.1 ± 0.3 mmol/L; and SX: 7.5 ± 0.4 mmol/L). Before the meal, blood glucose concentrations increased slightly when the D-xylose preload was given (i.e. PX and SX), in contrast to the control days, so that the iAUC (-40 to 0 min) was greater for PX and SX than for PC and SC (\( P < 0.05 \) for each, Table 1). After the meal, blood glucose concentrations increased on each day before returning to baseline. The postprandial glycaemic peak, AGE and ‘J’-index were all lower after PX and SC than PC (\( P < 0.01 \) for each), and were lowest after SX (\( P < 0.05 \) for each, Table 1). There was a significant treatment effect on the overall iAUC for blood glucose (\( P = 0.008 \), Table 1), such that blood glucose was lower after SX when compared to PC (\( P < 0.05 \)) (Figure 1A).
5.4.2 Serum insulin

Fasting serum insulin concentrations did not differ between the four study days. Before the meal, insulin concentrations increased slightly when D-xylose was given (i.e. PX and SX), in contrast to the control days, so that the iAUC (-40 to 0 min) was greater for PX and SX than for PC and SC ($P < 0.05$ for each, Table 1). After the meal, serum insulin concentrations increased on each day, but D-xylose and sitagliptin alone and in combination resulted in a lower postprandial serum insulin than PC ($P = 0.000$ for treatment × time interaction, with significant differences at t = 30, 60 and 90 min for PX vs. PC, and SX vs. PC; t = 90 min for SC vs. PC; and t = 30 min for SX vs. SC, $P < 0.05$ for each). There was also a significant treatment effect on the overall iAUC for serum insulin ($P = 0.009$, Table 1), such that insulin concentrations were lower after SX than PC ($P < 0.05$) (Figure 1B).

Before the meal, the insulin/glucose ratio (Figure 1C) remained unchanged after both D-xylose and control preloads, but the ratio increased after the meal, and was lower from t = 30 to 90 min after the D-xylose preload when compared to control ($P = 0.000$ for a treatment × time interaction: PX vs. PC, and SX vs. SC, $P < 0.05$ for each), without any effect of sitagliptin.

5.4.3 Plasma intact GLP-1

Fasting plasma intact GLP-1 concentrations did not differ between the four study days. Before the meal, GLP-1 concentrations increased when the D-xylose
preload was given, so that the iAUC (-40 to 0) was greater for SX than for PC and SC ($P < 0.05$ for each, Table 1), although the difference between PX and PC was not significant. After the meal, intact GLP-1 increased on the control days, but the combination of the D-xylose preload with sitagliptin resulted in more sustained elevation of plasma intact GLP-1 than on the other days ($P = 0.000$ for a treatment × time interaction, with significant differences at $t = 15, 60, 90$ and $120$ min for SX vs. PC; and during $t = 30$ to $120$ for SX vs. PX, $P < 0.05$ for each). There was also a treatment effect on the overall iAUC for plasma intact GLP-1 ($P = 0.003$, Table 1), such that GLP-1 was greatest after SX (SX vs. PC, PX, and SC, $P < 0.05$ for each) (Figure 1D).

5.4.4 Breath hydrogen production

Fasting breath hydrogen approximated 0 ppm, and did not differ between the four study days. After the D-xylose drink, breath hydrogen increased slightly before the meal ($t = -40$ to 0 min), and continued to rise to a plateau afterwards, while it remained unchanged after the control preload, and was unaffected by sitagliptin ($P = 0.000$ for a treatment × time interaction) (Figure 2).

5.4.5 Gastric emptying

There was a treatment effect for gastric emptying ($P = 0.000$), such that the half-emptying time was greater after D-xylose than control (T50 for PX 238.2 ± 26.4 min and SX 256.9 ± 23.1 min vs. PC 152.3 ± 6.0 min and SC166.3 ± 11.0 min, $P < 0.01$ for each), without any effect of sitagliptin (Figure 3).
5.4.6 Relationships between blood glucose and gastric emptying, plasma intact GLP-1 and breath hydrogen production

When data from the four study visits were pooled, the magnitude of the postprandial rise in blood glucose from baseline at t = 30, 60, and 90 min was inversely related to the T50 ($P < 0.01$ for each), and directly related to the T50 at t = 240 min ($P = 0.001$).

Given that plasma intact GLP-1 would be more sensitive as a measure of GLP-1 secretion when DPP-4 was inhibited, data from the SX day alone were examined for a relationship between breath hydrogen production and GLP-1 secretion. Intact GLP-1 concentrations were found to be related directly to breath hydrogen production (for $iAUC_{40-0}$, $r = 0.72$, $P = 0.009$; for $iAUC_{40-240}$, $r = 0.74$, $P = 0.006$).

5.5 Conclusions

The main observations made in this study of patients with type 2 diabetes managed by diet, were that (i) consumption of the low energy pentose, D-xylose, in advance of a high-carbohydrate meal, attenuates the postprandial glycaemic excursion, in association with stimulation of GLP-1 secretion before the meal and slowing of gastric emptying, (ii) a single dose of the DPP-4 inhibitor, sitagliptin, increases postprandial intact GLP-1 concentrations, and reduces postprandial glycaemia, without slowing gastric emptying or stimulating
postprandial insulin secretion, and (iii) the combination of a D-xylose preload with sitagliptin reduces the postprandial glycaemic excursion more than either treatment alone. The magnitude of the reduction in postprandial blood glucose achieved by the combination of D-xylose preload and sitagliptin in our group of well-controlled type 2 patients was substantial (i.e. reduction in peak blood glucose of ~ 2.5 mmol/L), and moreover, there was a marked reduction in indices of glycaemic variability – the latter is associated with oxidative stress, and may independently increase cardiovascular risk (Standl et al., 2011).

We chose D-xylose as the preload since it is incompletely absorbed and poorly metabolised (Craig and Ehrenpreis, 1999), and, accordingly, additional energy intake is minimised, in contrast to preloads such as fat (Gentilcore et al., 2006) and protein (Ma et al., 2009c). Consistent with our previous report in healthy older subjects (Vanis et al., 2011), ingestion of D-xylose resulted in a modest increase in blood glucose and serum insulin, possibly as a result of enhanced gluconeogenesis. Sucralose was selected as a sweet-tasting negative control, since we have shown that this artificial sweetener when given acutely has no effect on either the secretion of GLP-1 or gastric emptying (Ma et al., 2009a, Ma et al., 2010), and indeed in the current study, there was no increase in intact GLP-1 before the meal on the days when sucralose was given. The osmolarity of the preloads could not readily be matched without changing the taste, and this may have contributed to the effect of the D-xylose preload on gastric emptying.
(Barker et al., 1974), but is unlikely to account for differences in GLP-1 secretion (Ma et al., 2010).

As expected, D-xylose was associated with stimulation of GLP-1 in advance of the meal. It has been postulated that intestinal fermentation to form free fatty acids represents an important mechanism for the stimulation of GLP-1 release (Wu et al., 2010); the latter could be indirectly quantified by resultant hydrogen production in breath samples (Craig and Ehrenpreis, 1999). Our demonstration of a relationship between GLP-1 concentrations and hydrogen production is consistent with this concept. However, the fact that GLP-1 concentrations began to increase within 20 minutes of D-xylose ingestion suggests that other mechanisms, involving for example the passage of D-xylose through the monosaccharide transporters, GLUT2 and GLUT3 (Naula et al., 2010), are likely involved. It may appear surprising that plasma intact GLP-1 levels after the potato meal did not differ between the D-xylose and control preload study days, when sitagliptin was not given. However, the magnitude of GLP-1 secretion is dependent on the rate (Chaikomin et al., 2005) and load (Pilichiewicz et al., 2007a) of nutrient entry to the small intestine, so that the slowing of gastric emptying induced by the D-xylose preload may well have attenuated the component of the postprandial GLP-1 response attributable to the meal.
Chapter 5. D-xylose preload and sitagliptin

We observed that the magnitude of the initial postprandial glycaemic excursion was inversely related to the gastric half-emptying time, consistent with evidence that the rate of gastric emptying is a major determinant of postprandial glycaemia (Horowitz et al., 1993, Jones et al., 1996). In the absence of sitagliptin, the reduction in postprandial glycaemia by D-xylose is probably attributable mainly to the slowing of gastric emptying, since postprandial intact GLP-1 concentrations were no greater than on the control day. The observed decrease in postprandial insulin concentrations after D-xylose in contrast to control, particularly when corrected for differences in blood glucose (i.e. the insulin/glucose ratio), is consistent with what would be expected when gastric emptying of the potato meal is slower (Gentilcore et al., 2006).

The slowing of gastric emptying after the D-xylose preload was associated with stimulation of GLP-1 in advance of the meal, although postprandial intact GLP-1 concentrations were not increased in the absence of sitagliptin. Since endogenous GLP-1 is known to slow gastric emptying (Deane et al., 2010), the elevated GLP-1 may have contributed to the slower gastric emptying after D-xylose, at least during the initial phase. Nevertheless, the combination with sitagliptin, which increased plasma concentrations of intact GLP-1, had no additional effect on the rate of gastric emptying – a finding that is consistent with our recent report of the lack of effect of two days dosing with sitagliptin on gastric emptying (Stevens et al., 2012). This is likely to be because gastric emptying is modulated by multiple mechanisms. For example, acute
hyperglycaemia, even within the physiological range, is known to slow gastric emptying (Schvarcz et al., 1997), and sitagliptin, particularly when combined with D-xylose, potently decreased postprandial glycaemia. Moreover, peptide YY (PYY), which is co-secreted with GLP-1 from enteroendocrine L-cells, has the capacity to slow gastric emptying. PYY 1-36 and 3-36 are the predominant biologically active forms in the circulation; the latter is formed from degradation of PYY 1-36 by DPP-4, and is reportedly more potent at retarding emptying (Witte et al., 2009). Therefore, DPP-4 inhibition might, to some extent, blunt PYY-mediated slowing of gastric emptying.

In contrast, addition of sitagliptin did not affect gastric emptying, but was associated with lowering of postprandial glycaemia. Despite the fact that plasma intact GLP-1 concentrations were increased after sitagliptin, particularly when given in combination with D-xylose, the insulin response to the meal did not increase in parallel. This is partly accounted for by the fact that the insulinotropic effect of GLP-1 is glucose-dependent (Holst and Gromada, 2004), but there is also evidence that mechanisms other than insulin secretion are likely to be important in mediating the glucose-lowering effect of GLP-1; the latter include suppression of both glucagon secretion and endogenous glucose production (Muscelli et al., 2012, Prigeon et al., 2003), and enhancement of peripheral glucose uptake (Edgerton et al., 2009).
It is noteworthy that there has been little consideration of how dietary intake interacts with the actions of DPP-4 inhibitors. The current study is therefore novel in demonstrating how consumption of a specific nutrient can improve the efficacy of a DPP-4 inhibitor for reducing postprandial glycaemia. Although the number of subjects studied was relatively small, the observed effects were consistent between subjects, and were in keeping with previous observations relating to the slowing of gastric emptying by D-xylose (Vanis et al., 2011) and glucose lowering by sitagliptin (Ahren, 2007). Our study represents an acute intervention in a relatively well-controlled group of patients with type 2 diabetes managed by diet alone. In view of the positive outcomes, it would be of particular interest to investigate this approach in type 2 patients taking metformin, given the synergistic effect of DPP-4 inhibitors with metformin for increasing intact GLP-1 and improving glycaemia (Migoya et al., 2010), and to extend our observations to larger and more diverse groups of type 2 patients, over a longer duration.
Table 1. Postprandial glycaemic peak, ‘J’-index, and amplitude of glycaemic excursion (AGE), and incremental area under the curve (iAUC) for blood glucose, serum insulin, and plasma intact glucagon-like peptide 1 (GLP-1) in response to a carbohydrate meal after a preload of either D-xylose, or sucralose (control), with or without 100 mg sitagliptin (n = 12) \(^a\).

<table>
<thead>
<tr>
<th></th>
<th>PC</th>
<th>PX</th>
<th>SC</th>
<th>SX</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycaemic peak (mmol/L)</strong></td>
<td>14.8 ± 0.6</td>
<td>13.3 ± 0.6 (^b)</td>
<td>13.2 ± 0.6 (^c)</td>
<td>12.3 ± 0.6 (^{d e f})</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>‘J’-index (mmol(^2)/L(^2))</strong></td>
<td>54.7 ± 4.5</td>
<td>46.7 ± 3.8 (^b)</td>
<td>44.1 ± 3.9 (^c)</td>
<td>39.2 ± 3.4 (^{d e f})</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>AGE (mmol/L)</strong></td>
<td>8.7 ± 0.5</td>
<td>6.5 ± 0.5 (^b)</td>
<td>7.3 ± 0.5 (^c)</td>
<td>5.5 ± 0.5 (^{d e f})</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Glucose iAUC(_{40-0}) (mmol/L × min)</strong></td>
<td>3.8 ± 1.4</td>
<td>15.3 ± 4.5 (^b)</td>
<td>3.1 ± 2.0</td>
<td>12.2 ± 3.8 (^{d f})</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>Glucose iAUC(_{40-240}) (mmol/L × min)</strong></td>
<td>872.6 ± 101.4</td>
<td>797.1 ± 68.1</td>
<td>717.5 ± 103.8</td>
<td>630.7 ± 86.8 (^d)</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Insulin iAUC(_{40-0}) (mU/L × min)</strong></td>
<td>11.3 ± 5.0</td>
<td>54.7 ± 10.8 (^b)</td>
<td>17.3 ± 6.2</td>
<td>53.7 ± 17.9 (^{d f})</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Insulin iAUC(_{40-240}) (mU/L × min)</strong></td>
<td>6715.2 ± 1701.8</td>
<td>5343.4 ± 1203.0</td>
<td>5754.1 ± 1230.0</td>
<td>3972.9 ± 563.2 (^d)</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Intact GLP-1 iAUC(_{40-0}) (pmol/L × min)</strong></td>
<td>14.3 ± 6.7</td>
<td>52.2 ± 20.5</td>
<td>8.3 ± 3.3</td>
<td>86.8 ± 28.9 (^{d f})</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Intact GLP-1 iAUC(_{40-240}) (pmol/L × min)</strong></td>
<td>135.8 ± 36.7</td>
<td>125.0 ± 39.1</td>
<td>130.5 ± 38.5</td>
<td>492.1 ± 161.4 (^{d e f})</td>
<td>0.003</td>
</tr>
</tbody>
</table>

\(^a\) The four treatments were sitagliptin + D-xylose (SX), sitagliptin + control (SC), placebo + D-xylose (PX), and placebo + control (PC). One-factor repeated-measures ANOVA was used to determine statistical difference. Post hoc comparisons were adjusted by Bonferroni-Holm’s correction; data are means ± SEM. \(^b\) \(P < 0.05\), PX vs. PC; \(^c\) \(P < 0.05\), SC vs. PC; \(^d\) \(P < 0.05\), SX vs. PC; \(^e\) \(P < 0.05\), SX vs. PX; \(^f\) \(P < 0.05\), SX vs. SC.
Figure 1. Effects of D-xylose or sucralose (control), with or without sitagliptin, on blood glucose (A), serum insulin (B), insulin/glucose ratio (C), and plasma intact glucagon-like peptide-1 (GLP-1) (D) in response to a carbohydrate meal (n = 12). The four treatments were sitagliptin + D-xylose (SX), sitagliptin + control (SC), placebo + D-xylose (PX), and placebo + control (PC), respectively. Repeated-measures ANOVA was used to determine statistical difference. Post hoc comparisons were adjusted by Bonferroni-Holm’s correction. *P = 0.000 for each treatment × time interaction; **P < 0.05, PX vs. PC; ***P < 0.05, SC vs. PC; α P < 0.05, SX vs. PC; δ P < 0.05, SX vs. PX; ε P < 0.05, SX vs. SC. Data are means ± SEM.
Figure 2. Effects of D-xylose or sucralose (control), with or without sitagliptin, on breath hydrogen production in response to a carbohydrate meal (n = 12). The four treatments were sitagliptin + D-xylose (SX), sitagliptin + control (SC), placebo + D-xylose (PX), and placebo + control (PC), respectively. Repeated-measures ANOVA was used to determine statistical difference. * $P = 0.000$, PX vs. PC and SC; # $P = 0.000$, SX vs. PC and SC. Data are means ± SEM.
Figure 3. Effects of D-xylose or sucralose (control), with or without sitagliptin, on gastric emptying (half-emptying time, T50) (n = 12). The four treatments were sitagliptin + D-xylose (SX), sitagliptin + control (SC), placebo + D-xylose (PX), and placebo + control (PC), respectively. One-factor repeated-measures ANOVA was used to determine statistical difference. Post hoc comparisons were adjusted by Bonferroni-Holm’s correction. $P = 0.000$ for a treatment effect; $^* P < 0.01$, PX vs. PC and SC; $^# P < 0.001$, SX vs. PC and SC. Data are means ± SEM.
CHAPTER 6. EFFECTS OF ENTERIC-COATED LAURIC ACID PELLETS ON POSTPRANDIAL GLYCAEMIA IN LESS WELL-CONTROLLED PATIENTS WITH TYPE 2 DIABETES, WHEN GIVEN CONCURRENTLY WITH SITAGLIPTIN

6.1 Summary

Stimulation of endogenous glucagon-like peptide-1 (GLP-1) secretion by dietary strategies, such as giving a ‘preload’ in advance of the meal, has the capacity to slow gastric emptying and reduce postprandial glycaemic excursions (Chapter 4 and 5). The glucose-lowering effect can be further enhanced by concurrent inhibition of dipeptidyl peptidase-4 (DPP-4) (Chapter 5). We recently reported that a small dose of lauric acid delivered to a long segment of distal gut by enteric-coated pellets can stimulate a relatively large amount of GLP-1 and attenuate postprandial glycaemia in well-controlled type 2 patients. The study reported in Chapter 6 evaluated the effect of these pellets on glycaemia in less well-controlled type 2 patients, when given concurrently with the DPP-4 inhibitor, sitagliptin. 10 type 2 patients (HbA1c 8.2 ± 0.4%) were given a 100 mg morning dose of sitagliptin for 7 days, and each was studied on day 4 and day 7, when they consumed either 5 g active pellets (47% lauric acid by weight) or placebo with breakfast (at t = 0 min) and lunch (at t = 240 min), after taking their morning dose of 100 mg sitagliptin at t = -60 min, in double-blind, randomised fashion. Venous blood was sampled at intervals over 480 min.
mg $^{13}$C-octanoic acid was added to the lunch, and gastric emptying measured by breath test. Appetite sensations were evaluated by 100 mm visual analogue scales. After breakfast, there was no significant difference in blood glucose concentrations or fullness between active and placebo pellets. After lunch, however, postprandial blood glucose concentrations tended to be lower (treatment × time interaction, $P = 0.058$), gastric emptying was slower (half-emptying time: 197.4 ± 13.8 min for active vs. 168.4 ± 12.3 min for placebo pellets, $P = 0.041$), and fullness tended to be greater (treatment effect, $P = 0.088$), after active than placebo pellets. These observations suggest that the glycaemic status is an essential determinant of the glucose-lowering effect of active pellets, and should be considered when applying this novel approach for the therapy of type 2 diabetes.

6.2 Introduction

It is well established that optimisation of glycaemic control reduces the occurrence and progression of microvascular and probably macrovascular complications of type 1 and type 2 diabetes (DCCT, 1993, Stratton et al., 2000, UKPDS and Group, 1998). For most patients with relatively good glycaemic control, as measured by haemoglobin A1c (HbA1c), postprandial blood glucose is a better predictor of HbA1c than fasting blood glucose (El-Kebbi et al., 2004, Monnier et al., 2003). Accordingly, reducing postprandial glycaemic excursion is of fundamental importance in the management of these patients (Inzucchi et al., 2012).
Major determinants of postprandial glycaemia include the rate of gastric emptying and actions of the ‘incretin’ hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Chaikomin et al., 2006). Gastric emptying accounts for about 35% of the variance of in the initial rise and the peak of the blood glucose after oral glucose ingestion in both health (Horowitz et al., 1993) and type 2 diabetes (Jones et al., 1996). Acceleration of gastric emptying by erythromycin increases, whilst slowing gastric emptying by morphine reduces, postprandial glycaemia in type 2 patients (Gonlachanvit et al., 2003). In health, postprandial GLP-1 and GIP, together, mediate at least 50% of postprandial insulin secretion (the ‘incretin’ effect) after oral glucose (Holst and Gromada, 2004). Although the insulinotropic effect of GIP is diminished in type 2 diabetes, that of GLP-1 remains relatively intact (Nauck et al., 1993). In addition, GLP-1 slows gastric emptying (Deane et al., 2010) and suppresses glucagon secretion (Hare et al., 2010) and energy intake (Flint et al., 1998). Exogenous GLP-1 agonists have, accordingly, been developed as treatments for type 2 diabetes. However, an alternative strategy is to stimulate endogenous GLP-1 secretion.

GLP-1 is released from enteroendocrine L-cells that are predominantly distributed in the distal small intestine, colon and rectum (Eiselle et al., 1992), such that nutrient-induced GLP-1 secretion is highly dependent on the length (Lin et al., 1989, Lin et al., 1990) and the region (Little et al., 2006) of the gut
exposed. For example, GLP-1 secretion occurred when glucose was allowed to access to the entire small intestine, but not when glucose was restricted to the proximal 60 cm of the small intestine in healthy subjects (Little et al., 2006). Delivery of fatty acids directly into the ileum potently releases GLP-1 and peptide YY to suppress upper gastrointestinal motor activity, a phenomenon known as the ‘ileal brake’ (Lin et al., 1996, Lin et al., 1992).

To achieve selective interaction of nutrients with enteroendocrine L-cells in the ileum and colon in order to stimulate GLP-1, Meyer Nutriceuticals (Santa Monica, California, USA) has designed enteric-coated nutrient pellets (by weight: 47% lauric acid, a potent L-cell secretagogue in humans (Little et al., 2005, Feltrin et al., 2004, Feltrin et al., 2007); diameter: 0.5-1.7 mm). When active pellets are given with a ‘carrier meal’, their delivery into the ileum occurs within one hour of meal ingestion and continues over another 2-3 h. The subsequent dissolution and discharge of lauric acid from pellets allows the ileum to be exposed continuously for > 3 h and the colon for several more hours (Ma et al., 2013). Recently, our group has evaluated the effects of the active pellets in relatively well-controlled groups of type 2 patients managed by diet alone or metformin. Patients were studied on two days each, receiving active pellets on one day and matching placebo pellets on the other, in two dosage regimens: (A) 10 patients (mean HbA1c 5.9%) consumed 10 g pellets with breakfast (71 g carbohydrate, 4.3 g protein, 12 g fat, 415 kcal), while a second meal (lunch; 89 g carbohydrate, 29 g protein, 26 g fat, 708 kcal) was consumed at t = 240 min,
without any pellets (Rayner CK, 2010); (B) 8 patients (mean HbA1c 6.8%) consumed 5 g pellets with the same breakfast, followed by an identical meal with another 5 g pellets at t = 240 min (Ma et al., 2013). The blood glucose and plasma total GLP-1 concentrations demonstrate that these well-controlled type 2 patients were able to achieve significant improvement in postprandial glycaemia (mean peak reduction 0.5 to 1 mmol/L) for the 10 g and 5 g + 5 g dosage regimens, associated with stimulation of GLP-1, providing proof for the concept that exposure of the distal gut to a small quantity of nutrient can have substantial effects on GLP-1 release and hence glycaemic control.

As was demonstrated in the study reported in Chapter 5, the glucose-lowering efficacy of strategies stimulating endogenous GLP-1 can be further enhanced by concurrent inhibition of dipeptidyl peptidase 4 (DPP-4) (Wu et al., 2013d), the enzyme that inactivates intact GLP-1 (Drucker and Nauck, 2006). Patients who have type 2 diabetes that is sub-optimally controlled would be expected to have a greater margin for improved postprandial glycaemia than the well-controlled patients we have studied previously. Therefore, we hypothesised that the glucose-lowering effect of 5 g pellets, given with breakfast and lunch, in a less well-controlled group of type 2 patients exposed to the DPP-4 inhibitor, sitagliptin, would be more prominent than that was observed in the relatively well-controlled type 2 patients we had studied previously.
6.3 Subjects and methods

6.3.1 Subjects

10 patients with type 2 diabetes (8 males and 2 females) were studied, after they had provided written, informed consent. Two patients were managed with 850 mg metformin bd, one by a combination of 1000 mg metformin and 50 mg sitagliptin (janumet®, Merck, Sharp and Dohme) daily, and all others by diet alone. The mean age was 61.4 ± 3.1 years, body mass index (BMI) 30.5 ± 1.1 kg/m², HbA1c 8.2 ± 0.4%, and mean duration of known diabetes 6.6 ± 1.2 years. None had significant comorbidities, was a smoker, or was taking any medication known to affect gastrointestinal function. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

6.3.2 Protocol

Subjects were instructed to cease their usual oral hypoglycaemic agent (if they had one) and commence 100 mg sitagliptin (Januvia®; Merck, Sharp and Dohme) each morning for 7 days, during which each subject underwent two study visits on day 4 and day 7.

On the evening before each study visit (~1900), subjects consumed a standardised beef lasagne meal (2170 kJ; McCain Foods Proprietary Ltd, Australia), with bread, a non-alcoholic beverage and 1 piece of fruit, and
thereafter fasted until the following morning, when they attended the Discipline of Medicine, Royal Adelaide Hospital at 0800 h. On their arrival (t = -60 min), subjects took their morning dose of 100 mg sitagliptin with 30 mL water, and remained seated comfortably seated throughout the study. An intravenous cannula was then inserted into a forearm vein for repeated blood sampling. Between t = 0 and 5 min, they ate a standardised test meal (‘breakfast’) containing 5 g of either active or placebo pellets. The meal comprised 3 pikelets (Golden, North Ryde, NSW, Australia), coated with 9 g butter (Western Star, Rowville, VIC, Australia), 15 g golden syrup (Smith’s, Richlands, Australia) and 140 g pureed apple (Goulburn Valley, Shepparton, VIC, Australia), consumed with 100 mL water (Ma et al., 2013). Between t = 240 and 245 min, a second identical test meal (‘lunch’) was consumed, which again contained 5 g of either active or placebo pellets, and also 100 mg $^{13}$C-octanoic acid, as a marker of gastric emptying. Breath samples were collected every 5 minutes for the first hour after lunch, and every 15 minutes until t = 480 min.

Venous blood was sampled at t = 0, 15, 30, 45, 60, 90, 105, 120, 150, 180, 210, 240, 255, 270, 285, 300, 330, 360, 390, 420, and 480 min for the measurement of blood glucose, and plasma insulin, glucagon and GLP-1 (total and active). At the same intervals used for blood sampling, gastrointestinal sensations, including hunger, fullness, were assessed by 100 mm visual analogue scales (Parker et al., 2004). For the measurement of active GLP-1, venous blood was collected into ice-chilled EDTA tubes containing DPP-4 inhibitor (DPP4-010;
Linco Research Inc., St. Charles, MO, USA) (10 μL/mL blood). Samples were mixed six times by gentle inversion, and stored on ice before centrifugation at 3200 rpm for 15 min at 4 °C within 15 min of collection. Plasma was separated and stored at –70 °C for subsequent assays.

6.3.3 Measurements

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA).

$^{13}$CO$_2$ concentrations in breath samples were measured by an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas chromatographic purification system. The half-emptying time (T50) was calculated using the formula described by Ghoos et al (Ghoos et al., 1993). This method has been validated against scintigraphy for the measurement of gastric emptying (Chew et al., 2003).

6.3.4 Statistical analysis

Blood glucose, fullness and hunger were assessed using repeated measures ANOVA in the period of 0-240 min and 240-480 min, with treatment and time as factors. Blood glucose concentrations before breakfast (t = 0 min) and lunch (t = 240 min), and T50 of the lunch were compared by paired Student’s t-tests. All analyses were performed using SPSS software 19.0 (IBM Corporation, Armonk, NY, USA). The sample size of ten subjects was estimated to have 80%
power at the $P = 0.05$ significance level to detect a significant treatment × time effect on postprandial blood glucose concentrations after both breakfast and lunch (Ma et al., 2013, Rayner CK, 2010). Data are presented as mean values ± standard error; $P < 0.05$ was considered statistically significant.

6.4 Results

All patients tolerated the study well, and no adverse effects were reported.

6.4.1 Blood glucose

Blood glucose concentrations before breakfast ($t = 0$ min) and lunch ($t = 240$ min) did not differ between the two study days. There was a rise in blood glucose, which peaked 1-2 h after breakfast and lunch on both days. However, blood glucose concentrations did not differ after breakfast between active and placebo pellets, but tended to be lower after lunch ($P = 0.058$, treatment × time interaction for 240-480 min) after active than placebo pellets (Figure 1).

6.4.2 Gastric emptying

The half-emptying time of the lunch was greater after active than placebo pellets (T50: $197.4 \pm 13.8$ min vs. $168.4 \pm 12.3$ min, $P = 0.041$) (Figure 2).

6.4.3 Appetite sensations

Fullness increased, and hunger decreased, after both the breakfast and lunch on both days. Hunger did not differ after both breakfast and lunch between active
and placebo pellets, whereas fullness did not differ after breakfast, but tended to be greater after lunch after the active than placebo pellets (treatment effect, \( P = 0.088 \)) (Figure 3).

### 6.5 Discussion

The present study showed that the addition of active pellets to sitagliptin tended to lower postprandial blood glucose after lunch (when active pellets were releasing lauric acid both in the ileum and colon) but not after breakfast (when release of lauric acid was confined mainly to the ileum) in patients with less well-controlled type 2 diabetes, in a pattern similar to our previous observations in relatively well-controlled type 2 patients (Ma et al., 2013). This probably is due to the continuous release of lauric acid into the colon from the morning dose of pellets, which added to the effect of lunch dose. However, the reduction in blood glucose, as well as changes in hunger and fullness, failed to reach statistical significance in the current study.

The design and execution of the present study were the same as for our previous 5 g + 5 g study regimen (Ma et al., 2013), except that 100 mg sitagliptin was given before breakfast on each study day. As was demonstrated by the study reported in Chapter 5, administration of sitagliptin alone is associated with increased postprandial intact GLP-1 concentrations and decreased postprandial glycaemia (Wu et al., 2013d), and dietary strategies that stimulate endogenous GLP-1 secretion to improve postprandial glycaemia (e.g. ‘preload’ strategies)
can be further optimised by DPP-4 inhibition (Wu et al., 2013d). Therefore, the lack of substantial glucose-lowering by active pellets in these less well-controlled type 2 patients suggests that stimulation of GLP-1 secretion may have been relatively modest, although at the time of writing, neither total nor intact GLP-1 have been assayed.

In the present study, patients with more advanced type 2 diabetes were selected with an expectation that the margin for postprandial glycaemic improvement would be greater than that in the relatively well-controlled type 2 patients who had been studied previously (mean HbA1c 8.2% vs. 6.8%) (Ma et al., 2013). Indeed, both the fasting and postprandial blood glucose concentrations were much greater in the current group (i.e. fasting blood glucose concentrations: ~10.5 vs. ~7.5 mmol/L; postprandial glycaemic peaks: ~14 vs. ~10-11 mmol/L). However, hyperglycaemia, even within physiological range (i.e. 8 mmol/L), slows gastric emptying (Schvarcz et al., 1997), while more marked hyperglycaemia (i.e. 13-15 mmol/L) is reported to reduce small intestinal motility and transit (Byrne et al., 1998). The marked postprandial hyperglycaemia observed in the present study might have suppressed gastrointestinal motor activity, thereby impairing the delivery of lauric acid to the targeted gut regions.

The T50 was prolonged after active than placebo pellets. Although this prolongation was statistically significant, it appears not to have been sufficient
to have had a major effect on postprandial blood glucose concentrations. Rather, the delay of gastric emptying might slow the emptying of pellets, and hence, somewhat attenuate lauric acid-stimulated GLP-1 secretion. However, the mechanism by which active pellets slowed gastric emptying remains unclear. Although lauric acid can potently stimulate cholecystokinin secretion from enteroendocrine I-cells in the proximal small intestine (Little et al., 2005, Feltrin et al., 2004, Feltrin et al., 2007), lauric acid was mainly released in the ileum and colon in the current study. PYY is known to mediate the ileal brake, when the ileum is exposed to unabsorbed fatty acids (Lin et al., 1996). PYY also acts to induce satiation and suppress food intake (Batterham et al., 2002). Although PYY is also released from enteroendocrine L-cells, its pattern of secretion does not necessarily resemble GLP-1. For example, the magnitude of PYY secretion in response to rectally administered taurocholic acid is much greater than that of GLP-1 (Wu et al., 2013b). We observed a tendency to increase fullness after active than placebo pellets after lunch. Whether this was related to PYY stimulation remains to be determined, but the delayed gastric emptying after active pellets would be expected to increase gastric distension, which might, at least partly, account for the increase in fullness.

It should be noted that the number of subjects studied in the present study was small. Therefore, the outcomes should be interpreted with caution due to the possibility of type 2 errors. However, there is no doubt that in these sitagliptin-treated patients with less well-controlled type 2 diabetes, the glucose-lowering
Chapter 6. Enteric-coated nutrient pellets and glycaemia

effect of active pellets is rather blunted, despite the capacity of the active pellets
to slow gastric emptying and increase fullness. These observations suggest that
hyperglycaemia per se may confound intestinal transit of pellets and subsequent
release of lauric acid for GLP-1 stimulation, and that glycaemic status should be
taken into account when applying this novel approach for the therapy of type 2
diabetes, ie. it may be better suited to patients with relatively good glycaemic
control. Pellets that allow more rapid release of lauric acid should also be
evaluated.
Figure 1. Effect of enteric-coated lauric acid (‘active’) and placebo pellets on blood glucose concentrations in response to standardised breakfast and lunch in less well-controlled type 2 patients treated with sitagliptin (n = 10). Repeated-measures ANOVA was used to determine the statistical difference. $P = 0.058$, treatment × time interaction for 24-480 min. Data are means ± SEM.
Figure 2. Effect of enteric-coated lauric acid (‘active’) and placebo pellets on gastric emptying in response to standardised lunch in less well-controlled type 2 patients treated with sitagliptin (n = 10). Paired Student t-test was used to determine the statistical difference. * $P = 0.041$. Data are means ± SEM.
Figure 3. Effect of enteric-coated lauric acid (‘active’) and placebo pellets on fullness (A) and hunger (B) in response to standardised breakfast and lunch in less well-controlled type 2 patients treated with sitagliptin (n = 10). Repeated-measures ANOVA was used to determine the statistical difference. A. \( P = 0.088 \), treatment effect for 240-480 min. Data are means ± SEM.
CHAPTER 7. EFFECTS OF SITAGLIPTIN ON GLYCAEMIA, INCRETIN HORMONES, AND ANTROPYLORODUODENAL MOTILITY IN RESPONSE TO INTRADUODENAL GLUCOSE INFUSION IN HEALTHY LEAN AND OBESE HUMANS, AND PATIENTS WITH TYPE 2 DIABETES TREATED WITH OR WITHOUT METFORMIN

7.1 Summary
Inhibition of dipeptidyl peptidase 4 (DPP-4) lowers glycaemia by increasing intact glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino‑tropic polypeptide (GIP) concentrations, and the glucose-lowering in type 2 patients is potentiated by metformin, which augments plasma GLP-1 concentrations. Here we evaluated the effects of sitagliptin on glycaemic, incretin and antropyloroduodenal (APD) motor responses to intraduodenal (ID) glucose infusion at a controlled rate within the physiological range of gastric emptying (2 kcal/min), in healthy lean and obese subjects, and type 2 patients treated with and without metformin. 12 healthy lean and 12 non-diabetic obese males were each studied twice, and 12 male type 2 patients were studied on 4 occasions each: twice during therapy with metformin 850 mg bd for 7 days (days 5 and 8), and twice during treatment with a matching placebo bd for 7 days, with a 2-week ‘washout’. On each day, 100 mg sitagliptin or control (with 850 mg metformin or placebo in type 2 patients) was ingested (at t = -30 min), followed
by an ID glucose infusion (60 g over 120 min) and then an *ad libitum* buffet meal. Blood glucose and plasma hormones were evaluated frequently, APD motility recorded and energy intake determined. In response to 2 kcal/min ID glucose, sitagliptin increased plasma intact GIP substantially, and intact GLP-1 to a much lesser degree, enhanced insulin secretion, and attenuated glycaemic excursions in healthy lean and obese, but not type 2 subjects. Sitagliptin had minimal effect on the motor mechanisms that influence gastric emptying and no effect on plasma glucagon or energy intake. In contrast, metformin reduced fasting and glucose-stimulated glycaemia, associated with suppression of energy intake and modest augmentation of total and intact GLP-1, total GIP and glucagon in type 2 subjects, without any effect on insulin or APD motility. There was no evidence of synergy between metformin and sitagliptin. These observations indicate that the effect of DPP-4 inhibition on glycaemia in type 2 diabetes is likely to be dependent on the release of GLP-1, which may require a threshold of gastric emptying rate above 2 kcal/min, rather than on the secretion of GIP.

### 7.2 Introduction

In health, orally ingested glucose elicits a much greater insulin response than an ‘isoglycaemic’ intravenous glucose load, a phenomenon termed the ‘incretin effect’ (Nauck et al., 1986), which is mediated by the gut peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), released by the presence of intraluminal nutrients (Wu et al., 2010). Bioactive
GLP-1 and GIP are rapidly inactivated by dipeptidyl peptidase 4 (DPP-4) (Drucker and Nauck, 2006), and DPP-4 inhibitors have the potential to lower glycaemia by increasing postprandial intact GIP and GLP-1 concentrations (Drucker, 2007). In addition to its insulinotropic effect, GLP-1 also suppresses glucagon secretion (Schirra and Goke, 2005), appetite and food intake (Gutzwiller et al., 1999), and slows gastric emptying (Schirra et al., 2006, Deane et al., 2010). In type 2 diabetes, the insulinotropic effect of GIP is diminished, while that of GLP-1 remains intact (Nauck et al., 1993). As a result, the glucose-lowering effect of DPP-4 inhibitors in type 2 diabetes is likely to be dependent primarily on the bioavailability of endogenous GLP-1 rather than GIP, although this hypothesis has not been closely evaluated.

In both obesity (Seimon et al., 2013, Muscelli et al., 2008) and type 2 diabetes (Vollmer et al., 2008, Theodorakis et al., 2006), postprandial GLP-1 responses have inconsistently been reported to be reduced after ingestion of oral glucose or meals, and the secretion of GIP to be intact. However, these studies have not controlled for the rate of gastric emptying, which is known to be a major determinant of GLP-1 and GIP secretion (Wu et al., 2010). Even in health, there is a substantial inter-individual variation in the rate of gastric emptying (ie. 1-4 kcal/min) (Brener et al., 1983), and patients with long-standing type 2 diabetes frequently have accelerated or delayed gastric emptying (Horowitz et al., 2002). In both health and type 2 diabetes, the GIP response to intraduodenal glucose infusion increases in approximately linear fashion with increasing small
Chapter 7. Actions of sitagliptin and metformin

intestinal glucose exposure, whereas the GLP-1 response is not linear, being modest at 1-2 kcal/min and substantially greater at 4 kcal/min (Pilichiewicz et al., 2007a, Ma et al., 2012). Plasma DPP-4 activity is also known to vary between individuals, so that there is the potential for differences in the sensitivity to the effects of DPP-4 inhibition. For example, plasma DPP-4 activity increases with deterioration of glycaemic control in type 2 diabetes and decreases with aging (Ryskjaer et al., 2006). Non-diabetic individuals who are morbidly obese have been reported to have higher DPP-4 activity than lean controls (Lugari et al., 2004). The DPP-4 inhibitors have been administered to non-diabetic obese individuals and are well tolerated (Herman et al., 2006a), but whether their effects in this group are greater than in either lean or type 2 diabetic subjects has not been evaluated. The effect of DPP-4 inhibitors on gastric emptying has been inconsistently reported (Woerle et al., 2007, Vella et al., 2007, Wu et al., 2013d, Stevens et al., 2012); one study reported a small effect to slow gastric emptying (Woerle et al., 2007), while most have not observed any significant effect (Vella et al., 2007, Wu et al., 2013d, Stevens et al., 2012). However, the effect of DPP-4 inhibitors on the motor mechanisms that regulate gastric emptying, including antropyloroduododenal (APD) motility, has not been assessed, although endogenous GLP-1 was reported to suppress antral motility and stimulate pyloric contractions (Schirira et al., 2006). Clinical trial data indicate that sitagliptin is weight-neutral (Ahren, 2007), but reports on the effect of DPP-4 inhibition on appetite are also limited.
Recently, it has been shown that the combination of metformin and a DPP-4 inhibitor is more beneficial than either alone for the management of type 2 diabetes (Wu et al., 2013a), but the mechanism of this effect, and whether it is additive or synergistic, is unclear. In both rodents and healthy humans, metformin augments GLP-1, but not GIP, concentrations (Maida et al., 2011, Migoya et al., 2010), by means that remain to be established. Administration of metformin in mice for 12 weeks was also associated with increased expression of GLP-1 and GIP receptors in pancreatic islets (Maida et al., 2011). Although DPP-4 activity was reportedly reduced in type 2 patients treated with metformin (Lenhard et al., 2004), in vitro studies failed to show any direct effect (Lenhard et al., 2004, Hinke et al., 2002). Regardless, co-administration of metformin and sitagliptin resulted in an additive increase in plasma intact GLP-1 concentrations and improvement in postprandial glycaemia after mixed meals in both health and type 2 diabetes (Migoya et al., 2010, Solis-Herrera et al., 2013).

In the present study, our aim was to characterise the effects of DPP-4 inhibition when GIP is the major incretin hormone in the circulation. We employed a fixed rate of ID glucose infusion at 2 kcal/min, and evaluated the acute effects of sitagliptin on glycaemia, incretin hormones, APD motility, and appetite in response to ID glucose infusion in healthy lean and obese subjects, and in patients with type 2 diabetes treated with metformin or placebo.
7.3 Subjects and methods

7.3.1 Subjects

12 healthy lean subjects, 12 obese subjects without diabetes, and 12 patients with type 2 diabetes, were studied after they had provided written, informed consent. All were Caucasian males, and none had significant comorbidities, was a smoker, or was taking any medication known to affect gastrointestinal function. The healthy lean and obese subjects had been weight-stable and were unrestrained eaters. Type 2 subjects were all managed by diet alone, had glycated haemoglobin (HbA1c) < 7.5% (58 mmol/mol), and had no micro- or macro-vascular complications. Demographic and biochemical variables in the three groups are provided in Table 1. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

7.3.2 Protocol

Healthy lean and obese subjects each underwent two studies (ie. sitagliptin and control), in double-blind, randomised fashion, separated by 3 - 14 days. Subjects with type 2 diabetes were studied on 4 occasions each: twice during therapy with metformin 850 mg bd for 7 days (days 5 and 8, sitagliptin or control), and twice during treatment with a matching placebo bd for 7 days, with a ‘washout’ period of 14 days between the two treatment periods. In type 2 subjects, the order of both metformin and placebo, and sitagliptin and control, were
randomised, with the exception that during the second metformin/placebo treatment period, the order of sitagliptin and control was identical to the first treatment period (Figure 1).

On the evening before each study (~1900 h), subjects consumed a standardized beef lasagne meal (2170 kJ; McCain Foods Proprietary Ltd, Victoria, Australia) with bread, a non alcoholic beverage, and one piece of fruit, and then fasted until the following morning, when they attended the laboratory at ~0800 h. A silicone manometric assembly (Dentsleeve International, Mui Scientific, Ontario, Canada) was inserted through an anaesthetised nostril and allowed to pass into the duodenum by peristalsis (Feltrin et al., 2004). The assembly incorporated 16 manometric channels, spaced at 1.5 cm intervals, of which 7 were positioned in the antrum, 6 in the duodenum, and 3 on the side opposite a 4.5 cm sleeve sensor in the pylorus. An additional infusion channel opened ~12 cm beyond the pylorus. The correct positioning of the catheter was maintained by continuous measurement of the antral and duodenal transmucosal potential difference (TMPD), as described previously (Feltrin et al., 2004). All manometric channels were perfused with degassed 0.9 % saline (Feltrin et al., 2004).

An intravenous cannula was inserted for repeated blood sampling. A tablet of 100 mg sitagliptin or matching control (with a tablet of 850 mg metformin or matching placebo in type 2 subjects) was administered orally with 30 mL water (at t = -30 min). Thirty minutes later, an ID glucose infusion commenced (60 g
Chapter 7. Actions of sitagliptin and metformin

glucose dissolved in water to a total volume of 240 mL) and continued over 120 min (from t = 0 to 120 min; ie. 2 kcal/min). At the end of the infusion (t = 120 min), the catheter was removed. Subjects were then offered a standardised, cold buffet-style meal, to consume as much as they wished until they felt comfortably full (from t = 120 to 150 min), as described previously (Feltrin et al., 2004). Manometric data were recorded to a computer hard disk for subsequent analysis.

Venous blood was sampled at t = -30, 0, 15, 30, 45, 60, 75, 90, and 120 min for measurements of blood glucose and plasma hormones (total and intact GLP-1 and GIP, insulin and glucagon).

Blood samples were collected into ice-chilled EDTA tubes. For the measurement of intact GLP-1 and GIP, DPP-4 inhibitor (DPP4-010; Linco Research Inc., St. Charles, MO, USA) (10 μL/mL blood) was added to the tubes. Samples were mixed six times by gentle inversion, and stored on ice before centrifugation at 3200 rpm for 15 min at 4 °C within 15 min of collection. Plasma was separated and stored at –70 °C for subsequent analysis.

7.3.3 Measurements

Plasma glucose concentrations were measured by the glucose oxidase technique (YSI 2300 STAT Plus, Yellow Springs Instruments Corp., Yellow Springs, OH, USA). Plasma samples for GLP-1, GIP and glucagon analyses were extracted with ethanol (70% final concentration) and analysed as previously described (Vilsboll et al., 2003, Lindgren et al., 2011). Intact GLP-1 was measured using a
two-site (sandwich) ELISA, using two monoclonal antibodies: GLP-1F5 as a catching antibody (C-terminally directed) and Mab26.1 as a detecting antibody (N-terminally directed) (Vilsboll et al., 2003), while total GLP-1 was assayed using antiserum 89390, which has an absolute requirement for the intact amidated C-terminus of the molecule, and reacts equally with intact GLP-1 and the primary (N-terminally truncated) metabolite (Vilsboll et al., 2003). Intact and total GIP were analysed with the N-terminally and C-terminally directed antisera, 98171 (Vilsboll et al., 2003) and 80867 (Lindgren et al., 2011), respectively. Glucagon immunoreactivity was determined using the C-terminally directed antiserum 4305, which measures glucagon of pancreatic origin (Vilsboll et al., 2003). Insulin was measured by ELISA immunoassay (10-1113, Mercodia, Uppsala, Sweden) with sensitivity 1.0 mU/L, and intra- and inter-assay CVs of 2.7% and 10.9%, respectively.

Manometric pressures were recorded on a computer-based system (Flexisoft, Oakfield Instruments of Oxford, UK), and analysed using custom-designed software (Prof AJ Smout, Academic Medical Center, Amsterdam, The Netherlands) using accepted definitions (Feltrin et al., 2004) to determine the number of isolated pyloric pressure waves (IPPWs), and antral and duodenal pressure waves. The frequency of pressure waves was analysed over successive 15 min periods.
Energy intake at the buffet meal was analysed using commercially available software (Foodworks 3.01; Xyris Software, Highgate Hill, Queensland, Australia) (Feltrin et al., 2004).

### 7.3.4 Statistical analysis

Area under the curve (AUC) was calculated using the trapezoidal rule for plasma glucose, GLP-1 and GIP (total and intact), and glucagon, and serum insulin. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin sensitivity, which was calculated by the formula: fasting glucose (mmol/L) × fasting insulin (pmol/L)/22.5 (Herzberg-Schafer et al., 2010). AUC_{insulin}/AUC_{glucose} was calculated in order to compare insulin secretion whilst correcting for differences in blood glucose (Herzberg-Schafer et al., 2010). These variables, together with total numbers of pressure waves and energy intake, were analysed using paired Student’s t-test in the healthy lean and obese groups and two-factor repeated measures ANOVA, with sitagliptin and metformin as factors, in the type 2 patients, respectively. Repeated measures ANOVA, with treatment and time as factors, was also employed for intra-group comparisons. Inter-group comparisons were performed using one-factor ANOVA. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni’s correction, were performed, if ANOVAs revealed significant effects. Relationships between variables were assessed using Pearson correlation analysis. All analyses were performed using SPSS (version 19, IBM Corporation, Armonk, NY, USA). A sample size of 12 subjects was calculated to have 80%
power (at $\alpha = 0.05$) to detect 5–10% difference in AUC for blood glucose after sitagliptin than placebo in healthy lean and obese subjects (Stevens et al., 2012), and to detect an additive glucose-lowering effect between metformin and sitagliptin in type 2 subjects (Solis-Herrera et al., 2013). Data are presented as means ± standard error; $P < 0.05$ was considered statistically significant.

### 7.4 Results

Metformin was well tolerated by all type 2 patients. All subjects tolerated the study protocol well, and no adverse effects were reported.

#### 7.4.1 Blood glucose

In healthy lean and obese subjects, fasting blood glucose concentrations ($t = -30$ min) did not differ between the control and sitagliptin study days (lean: $4.7 \pm 0.1$ vs. $4.7 \pm 0.1$mmol/L; obese: $5.3 \pm 0.2$ vs. $5.4 \pm 0.2$mmol/L). Immediately before ID glucose infusion ($t = 0$ min), blood glucose concentrations remained unchanged on both days. During ID glucose infusion ($t = 0$-120 min), blood glucose concentrations increased, and were lower after sitagliptin than control in both groups (lean: $P = 0.001$ for AUC; $P < 0.001$ for treatment × time interaction with significant differences from $t = 120$ min ($P < 0.05$ for each); obese: $P = 0.004$ for AUC; $P < 0.001$ for treatment × time interaction with significant differences from $t = 30$ to 120 min ($P < 0.05$ for each)) (Figure 2A and 3A; Table 2).
In type 2 subjects, fasting blood glucose concentrations (t = -30 min) did not differ between the control and sitagliptin days during either placebo (6.6 ± 0.3 vs. 6.8 ± 0.4 mmol/L) or metformin (6.0 ± 0.3 vs. 6.2 ± 0.3 mmol/L) treatment, but were reduced by administration of metformin (treatment effect: \( P < 0.001 \)). Before ID glucose infusion (t = 0 min), blood glucose concentrations remained unchanged on each day. During ID glucose infusion (t = 0-120 min), blood glucose concentrations increased, and there was a significant treatment effect of metformin to reduce the AUC for blood glucose (\( P < 0.001 \)), without any effect of sitagliptin (\( P = 0.961 \)) or interaction between metformin and sitagliptin (\( P = 0.253 \)) (Figure 4A; Table 3).

Both fasting blood glucose concentrations (Table 1) and the AUC for blood glucose after ID glucose on the sitagliptin or control study days (Table 2) were greater in type 2 patients during the placebo treatment than healthy lean and obese subjects (\( P < 0.001 \) for each), without any difference between the latter two groups.

### 7.4.2 Plasma total and intact GLP-1

In healthy lean and obese subjects, fasting total GLP-1 concentrations (t = -30 min) did not differ between the control and sitagliptin study days (lean: 18.1 ± 1.5 vs. 17.2 ± 1.1 pmol/L; obese: 17.7 ± 2.1 vs. 16.5 ± 2.0 pmol/L). Before ID glucose infusion (t = 0 min), plasma total GLP-1 concentrations remained unchanged, and plasma intact GLP-1 concentrations also did not differ (lean: 0.4
Chapter 7. Actions of sitagliptin and metformin

± 0.3 vs. 0.6 ± 0.2 pmol/L; obese: 0.4 ± 0.1 vs. 0.6 ± 0.2 pmol/L). During ID glucose infusion (t = 0-120 min), plasma GLP-1 responses were minimal, but plasma total GLP-1 concentrations were lower after sitagliptin than control in healthy lean subjects (P = 0.067 for AUC; P < 0.05 for treatment effect, with significant differences at t = 15 and 30 min (P < 0.05 for each)), without any difference in obese subjects (Figure 2C and 3C; Table 2). In contrast, plasma intact GLP-1 concentrations were higher after sitagliptin than control in both healthy lean and obese subjects (lean: P = 0.016 for AUC; P = 0.011 for treatment effect, with significant differences at t = 45 and 60 min (P < 0.05 for each); obese: P < 0.001 for AUC; P = 0.016 for treatment × time interaction, with significant differences at t = 15, 45, 60, 90 and 120 min (P < 0.05 for each)) (Figure 2E and 3E; Table 2).

In type 2 subjects, fasting total GLP-1 concentrations did not differ between the control and sitagliptin days during either placebo (17.7 ± 1.5 vs. 17.3 ± 1.5 pmol/L) or metformin (21.9 ± 2.2 vs. 19.8 ±2.1 pmol/L) treatment, but were increased by administration of metformin (treatment effect: P = 0.016). Before ID glucose infusion (t = 0 min), plasma total GLP-1 concentrations remained unchanged on each day. Similarly, plasma intact GLP-1 concentrations did not differ between the control and sitagliptin days during either placebo (1.0 ± 0.3 vs. 0.9 ± 0.3 pmol/L) or metformin (2.0 ± 0.3 vs. 1.7 ± 0.4 pmol/L) treatment, but were increased by administration of metformin (treatment effect: P < 0.001). During ID glucose infusion (t = 0-120 min), plasma GLP-1 responses were
minimal, but there was a significant treatment effect of metformin to increase the AUC for plasma total GLP-1 \((P = 0.001)\), without any effect of sitagliptin \((P = 0.566)\) or interaction between metformin and sitagliptin \((P = 0.216)\). In contrast, there were significant treatment effects of both metformin \((P = 0.001)\) and sitagliptin \((P = 0.007)\) to increase the AUC for plasma intact GLP-1, without any interaction between metformin and sitagliptin \((P = 0.904)\) (Figure 4C and 4E; Table 3).

During fasting, neither total nor intact GLP-1 differed between the three groups (Table 1). There were significant differences in the AUCs for both total and intact GLP-1 after ID glucose between the three groups on the control, but not sitagliptin, days \((P = 0.039\) and 0.011, respectively), such that total GLP-1 AUC was less in obese than healthy lean subjects \((P < 0.05)\), and intact GLP-1 AUC was less in obese than type 2 subjects \((P < 0.05)\) (Table 2). On the control days, the AUC for plasma total (but not intact) GLP-1 after ID glucose in all groups combined was inversely related to BMI \(r = -0.41, P = 0.04\). After adjusting for BMI, the AUC for plasma total GLP-1 did not differ between the groups. Neither total nor intact GLP-1 AUC was related to age. However, after adjusting for BMI, the AUC for intact GLP-1 tended to be positively related to age on the control days \(r = 0.31, P = 0.06\).
7.4.3 Plasma total and intact GIP

In healthy lean and obese subjects, neither fasting (t = -30 min) total nor intact GIP concentrations differed between the control and sitagliptin study days (lean: 6.3 ± 1.6 vs. 7.2 ± 1.6 pmol/L for total GIP and 7.8 ± 0.4 vs. 8.0 ± 0.4 pmol/L for intact GIP; obese: 7.4 ± 2.2 vs 7.6 ± 1.2 pmol/L for total GIP and 8.3 ± 0.4 vs. 8.9 ± 0.4 pmol/L). Before ID glucose infusion (t = 0 min), plasma total and intact GIP concentrations remained unchanged on both days. During ID glucose infusion (t = 0-120 min), plasma GIP increased substantially to a plateau within 30 min, and plasma total GIP concentrations were lower after sitagliptin than control in healthy lean subjects (P = 0.019 for treatment × time interaction, with significant differences at t = 45 and 60 min (P < 0.05 for each)), without any differences in obese subjects (Figure 2D and 3D; Table 2). In contrast, plasma intact GIP concentrations were higher after sitagliptin than control in both healthy lean and obese subjects (both lean and obese: P < 0.001 for AUC; P < 0.001 for treatment × time interaction, with significant differences from t = 15 to 120 min (P < 0.05 for each)) (Figure 2F and 3F; Table 2).

In type 2 subjects, fasting GIP concentrations (t = -30 min) did not differ between the control and sitagliptin study days during either placebo (6.8 ± 1.6 vs. 7.8 ± 1.5 pmol/L for total GIP and 9.6 ± 0.5 vs. 8.8 ± 0.7 pmol/L for intact GIP) or metformin (8.5 ± 2.2 vs. 7.1 ± 2.0 pmol/L for total GIP and 9.8 ± 0.9 vs. 9.6 ± 0.9 for intact GIP) treatment, and were not altered by administration of metformin (P = 0.634 for total GIP and P = 0.384 for intact GIP, respectively).
Chapter 7. Actions of sitagliptin and metformin

Before ID glucose infusion (t = 0 min), plasma total and intact GIP concentrations remained unchanged on each day. During ID glucose infusion (t = 0-120 min), plasma GIP increased substantially to a plateau within 30 min, and there was a significant treatment effect of metformin to increase the AUC for plasma total GIP ($P = 0.014$), without any effect of sitagliptin ($P = 0.694$) or interaction between metformin and sitagliptin ($P = 0.477$). In contrast, there was a significant treatment effect of sitagliptin to increase the AUC for plasma intact GIP ($P = 0.008$), without any effect of metformin ($P = 0.107$), or interaction between metformin and sitagliptin ($P = 0.777$) (Figure 4D and 4F; Table 3).

During fasting, neither total nor intact GIP concentrations differed between the three groups (Table 1). The AUC for plasma total GIP after ID glucose on either control or sitagliptin study days (Table 2) also did not differ between the three groups. There was a significant difference in the AUC for plasma intact GIP on the control, but not sitagliptin, days ($P < 0.001$), such that intact GIP was greatest in type 2 subjects ($P < 0.01$ for each), without any difference between healthy lean and obese subjects. The AUC for plasma total or intact GIP was not related to BMI. However, the AUC for plasma intact GIP was positively related to age on the placebo, but not sitagliptin, days ($r = 0.65$, $P < 0.001$). This relationship remained significant when adjusting for the presence of type 2 diabetes ($r = 0.43$, $P = 0.01$).
7.4.4 Plasma glucagon

In healthy lean and obese subjects, fasting glucagon concentrations \((t = -30 \text{ min})\) did not differ between the control and sitagliptin study days (lean: \(6.3 \pm 0.8 \text{ vs. } 7.2 \pm 0.9 \text{ pmol/L}\); obese: \(10.3 \pm 1.4 \text{ vs. } 9.6 \pm 1.2 \text{ pmol/L}\)). Before ID glucose infusion \((t = 0 \text{ min})\), plasma glucagon concentrations remained unchanged on both days. After ID glucose infusion \((t = 0-120 \text{ min})\), plasma glucagon declined to comparably low levels \((\sim 2 \text{ pmol/L})\) in both groups, without any differences between sitagliptin and control (Figure 2G and 3G; Table 2).

In type 2 patients, fasting glucagon concentrations \((t = -30 \text{ min})\) did not differ between the control and sitagliptin study days during either placebo \((12.3 \pm 2.4 \text{ vs. } 12.5 \pm 2.8 \text{ pmol/L})\) or metformin \((15.5 \pm 2.9 \text{ vs. } 16.3 \pm 3.6 \text{ pmol/L})\) treatment, but were elevated during administration of metformin (treatment effect: \(P = 0.025\)). Before ID glucose infusion \((t = 0 \text{ min})\), there was a slight decline in plasma glucagon concentrations on each day. During ID glucose infusion \((t = 0-120 \text{ min})\), plasma glucagon was relatively stable during the first 30 min, followed by a decline to the levels of \(\sim 6 \text{ pmol/L}\) at \(t = 120 \text{ min}\). There was a treatment effect of metformin to increase the AUC for glucagon \((P = 0.003)\), without any effect of sitagliptin \((P = 0.938)\) or interaction between metformin and sitagliptin \((P = 0.681)\) (Figure 4G; Table 3).

Fasting plasma glucagon \((P = 0.034, \text{ Table 1})\) and the AUC for glucagon after ID glucose on both the control \((P = 0.023)\) and sitagliptin \((P = 0.019)\) study days.
differed between the three groups (Table 2), such that glucagon was greater in type 2 patients than healthy lean subjects ($P < 0.05$ for each), without significant differences between healthy lean and obese subjects, or between obese and type 2 subjects. Fasting glucagon was positively related to plasma intact GIP ($r = 0.529, P = 0.001$), even after adjusting the presence of type 2 diabetes ($r = 0.47, P = 0.005$).

7.4.5 Serum insulin, HOMA-IR, and $\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$

In healthy lean and obese subjects, neither fasting insulin concentrations ($t = -30$ min) nor HOMA-IR differed between the control and sitagliptin study days (lean: $3.2 \pm 0.6$ vs. $3.4 \pm 0.4$ mU/L for insulin, and $4.6 \pm 0.8$ vs. $4.9 \pm 0.7$ pmol.mmol.L$^{-2}$ for HOMA-IR; obese: $10.1 \pm 1.9$ vs. $11.2 \pm 2.2$ mU/L for insulin, and $16.3 \pm 3.3$ vs. $19.1 \pm 4.4$ pmol.mmol.L$^{-2}$ for HOMA-IR). Before ID glucose infusion ($t = 0$ min), serum insulin concentrations remained unchanged on both days. After ID glucose infusion ($t = 0$-120 min), serum insulin concentrations increased, and were higher after sitagliptin than control in both groups (lean: $P = 0.002$ for AUC; $P = 0.009$ for treatment $\times$ time interaction with significant differences from $t = 30$ to 90 min ($P < 0.05$ for each); obese: $P = 0.088$ for AUC; $P = 0.021$ for treatment $\times$ time interaction with significant difference at $t = 45$ min ($P < 0.05$)). In addition, the $\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$ was greater after sitagliptin than control in both groups (lean: $P < 0.001$, obese: $P = 0.032$) (Figure 2B and 3B; Table 2).
In type 2 patients, neither fasting insulin concentrations (t = -30 min) nor HOMA-IR differed between the control and sitagliptin study days during either placebo (8.6 ± 1.9 vs. 8.2 ± 1.7 mU/L for insulin, and 18.4 ± 4.6 vs. 18.6 ± 4.8 pmol.mmol.L^{-2} for HOMA-IR) or metformin (7.9 ± 1.5 vs. 7.6 ± 1.3 mU/L for insulin, and 15.5 ± 3.4 vs. 15.2 ± 3.1 pmol.mmol.L^{-2} for HOMA-IR) treatment, and neither was altered by administration of metformin (treatment effect: \( P = 0.522 \) and 0.217, respectively). Before ID glucose infusion (t = 0 min), serum insulin concentrations decreased slightly on each day. During ID glucose infusion (t = 0-120 min), serum insulin concentrations continuously increased. There was a treatment effect of sitagliptin to increase the AUC for serum insulin (\( P = 0.049 \)), without any effect of metformin (\( P = 0.142 \)) or interaction between metformin and sitagliptin (\( P = 0.827 \)). However, the AUC_{\text{insulin}}/AUC_{\text{glucose}} was increased by metformin (\( P = 0.019 \)), and tended to increase with sitagliptin (\( P = 0.065 \)), without any interaction between metformin and sitagliptin (\( P = 0.718 \)) (Figure 4B; Table 3).

Fasting serum insulin differed between the three groups (\( P = 0.005 \)), such that fasting insulin was greater in obese than lean subjects (\( P = 0.005 \)), and tended to be greater for type 2 than lean subjects (\( P = 0.07 \)), without any difference between obese and type 2 subjects (Table 1). HOMA-IR differed between the three groups (\( P = 0.009 \)), and was greater in obese and type 2 than lean subjects (\( P < 0.05 \) for each), without any difference between obese and type 2 subjects (Table 1). The AUC for insulin after ID glucose differed between the three
groups on the control ($P = 0.015$), but not sitagliptin ($P = 0.057$) day, such that insulin was greater for obese than type 2 subjects ($P = 0.020$), and tended to be greater for obese than lean subjects ($P = 0.073$), without any difference between lean and type 2 subjects (Table 2). Both fasting serum insulin and the AUC for serum insulin on the control days were positively related to BMI ($r = 0.67$ and $0.62$, $P < 0.001$ for each). The increase of $\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$ was positively related to the rise in plasma intact GIP ($r = 0.33$, $P < 0.05$), but not GLP-1.

7.4.6 APD pressure waves

The number of antral waves (AWs) in response to ID glucose ($t = 0-120$ min) did not differ between the groups on either the control or sitagliptin study days (Table 2). However, the number of AWs was less in healthy lean subjects ($P = 0.032$), and tended to be less in obese subjects ($P = 0.052$), after sitagliptin than control (Figure 5; Table 2). There was no effect of metformin ($P = 0.083$), or sitagliptin ($P = 0.863$), or interaction between metformin and sitagliptin ($P = 0.676$), on the number of AWs in type 2 subjects (Figure 6; Table 3).

The number of duodenal waves (DWs) in response to ID glucose ($t = 0-120$ min) did not differ between the groups on either the control or sitagliptin study days (Table 2). The number of DWs was less after sitagliptin than control in healthy lean subjects ($P = 0.018$), without any difference in obese subjects (Figure 5; Table 2). There was no effect of metformin ($P = 0.083$), or sitagliptin ($P =
0.863), or interaction between metformin and sitagliptin \((P = 0.676)\) on the number of DWs, in type 2 subjects (Figure 6; Table 3).

The number of isolated pyloric pressure waves (IPPWs) did not differ between the groups on either the control or sitagliptin study days (Table 2). The number of IPPWs did not differ between control and sitagliptin in healthy lean or obese subjects (Figure 5; Table 2). There was no effect of metformin \((P = 0.418)\), or sitagliptin \((P = 0.090)\), or interaction between metformin and sitagliptin \((P = 0.123)\), on the number of IPPWs in type 2 subjects (Figure 6; Table 3).

The numbers of AWs, DWs, or IPPWs were not related to the AUC for plasma glucose, GLP-1 or GIP (total and intact), glucagon, or serum insulin on the placebo day.

### 7.4.7 Energy intake

There was no difference in energy intake between control and sitagliptin in healthy lean or obese subjects (Table 2). There was a treatment effect of metformin to suppress energy intake in type 2 subjects \((P = 0.040)\), without any effect of sitagliptin \((P = 0.913)\), or interaction between metformin and sitagliptin \((P = 0.307)\) (Table 3). On both the control and sitagliptin study days, energy intake was greater in obese than type 2 subjects \((P < 0.05\) for each), without a difference between healthy lean and obese subjects, or healthy lean and type 2 subjects (Table 2).
7.5 Discussion

The amplitude of glucose-induced GLP-1 and GIP secretion is regulated by the rate of gastric emptying in both health and type 2 diabetes (Wu et al., 2010). GIP secretion is proportional to the glucose load, while there appears to be a threshold of the delivery rate of glucose into the duodenum above 2 kcal/min to induce substantial GLP-1 secretion (Pilichiewicz et al., 2007a, Ma et al., 2012), probably due to the different distribution of enteroendocrine K- and L-cells (Wu et al., 2010). It is unclear whether the effects of DPP-4 inhibition are dependent on the rate of carbohydrate entry into the small intestine and the resultant postprandial incretin profiles (ie. bioavailability of GLP-1 and/or GIP) in health, obesity and type 2 diabetes, and whether DPP-4 inhibition potentiates metformin-mediated glucose-lowering via the incretin-axis in type 2 diabetes. In the present study, we employed an ID glucose infusion at 2 kcal/min (ie. the low- to mid-point of the physiological range of gastric emptying(Brener et al., 1983)). Consistent with our previous findings with this infusion rate (Pilichiewicz et al., 2007a, Ma et al., 2012), GIP was stimulated to a much greater extent than was GLP-1 in each group. In this setting, a single dose of sitagliptin increased plasma intact GIP substantially, and intact GLP-1 to a much lesser degree, and enhanced insulin secretion. Glycaemic excursions were attenuated with sitagliptin in healthy lean and obese, but not type 2 subjects, without any effect on plasma glucagon or energy intake. In contrast, in type 2 patients, metformin reduced fasting and glucose-stimulated glycaemia,
associated with suppression of energy intake and modest augmentation of total and intact GLP-1, total GIP and glucagon concentrations, without any effect on insulin concentrations. There was no evidence of synergy between metformin and sitagliptin in their effects on glycaemia or intact incretin concentrations under the current experimental conditions. These observations indicate, in type 2 diabetes, that (i) there is a deterioration in the capacity of GIP to lower blood glucose, (ii) the glucose-lowering efficacy of DPP-4 inhibitors is likely to be dependent on the rate of nutrient delivery into the small intestine and the resultant magnitude of GLP-1 stimulation, and (iii) the action of metformin to lower blood glucose is not predominantly via the incretin-axis.

In both obesity and type 2 diabetes, postprandial GIP release is relatively intact, while GLP-1 secretion has been inconsistently reported to be either reduced or intact after ingestion of glucose or mixed meals (Seimon et al., 2013, Muscelli et al., 2008, Vollmer et al., 2008, Theodorakis et al., 2006). In the present study, variations in gut hormone responses that might result from differences in the rate of gastric emptying were circumvented by infusing glucose at a fixed rate directly into the duodenum. Basal GLP-1 and GIP concentrations (both total and intact) did not differ between the groups. After ID glucose, GIP secretion was intact in both obesity and type 2 diabetes when compared to lean healthy subjects, whereas the GLP-1 response was less in obesity, but was not diminished in type 2 diabetes per se. The latter finding is consistent with our previous report that concentrations of both incretins during different rates of ID
glucose infusion were comparable in BMI-matched healthy and type 2 subjects (Ma et al., 2012). Interestingly, plasma intact GLP-1 was less in obese than type 2 subjects, and tended to be less than in healthy lean subjects, while plasma intact GIP was greatest in type 2 subjects without any difference between the healthy lean and obese. This discrepancy is likely to be related to differences in DPP-4 activity, which is reported to increase in obesity (Lugari et al., 2004) and decrease with aging (Ryskjaer et al., 2006). In support of this, we observed positive relationships of plasma intact GLP-1 and GIP levels with age on the ‘control’ study days, after correcting for BMI. This correlation was more marked for GIP, probably because its stimulation by ID glucose was greater. Although fasting concentrations of intact GIP were associated with those of glucagon, the clinical implications of any age-related increase in plasma intact GIP remain to be elucidated.

The mechanism by which GLP-1 secretion is attenuated in obesity is not clear. Leptin resistance may play a role; in mice made leptin resistant by a high-fat diet, both basal and oral glucose-stimulated GLP-1 concentrations were decreased (Anini and Brubaker, 2003). Elevated non-esterified fatty acid (NEFA) concentrations in obesity would tend to enhance, rather than diminish GLP-1 release (Nauck et al., 2011). It has been postulated that age and plasma glucagon may be associated with GLP-1 concentrations after oral glucose or mixed meals (Nauck et al., 2011). However, we have previously reported that both GLP-1 and GIP responses to different rates of ID glucose infusion were comparable
between healthy young and old subjects (Trahair et al., 2012a). Intravenous administration of glucagon did not affect plasma GLP-1 concentrations in overweight/obese non-diabetic subjects (Tan et al., 2013). In line with these findings, we did not show any relationships between GLP-1 secretion and either age or glucagon concentrations. Furthermore, it remains unclear whether the apparent volume of distribution of both incretins is greater in obesity, thereby resulting in greater dilution of these hormones in the systemic circulation.

Sitagliptin resulted in slight reduction in total GLP-1 and GIP responses to ID glucose in healthy lean subjects, consistent with the existence of negative feedback on incretin secretion by the intact peptides (Deacon et al., 2002, Herman et al., 2006b, El-Ouaghlidi et al., 2007). However, this feedback signaling appeared to be diminished in obesity and type 2 diabetes – an observation that might be clinically important, since GIP acts on adipocytes to enhance fat deposition and impair insulin sensitivity (Irwin and Flatt, 2009).

As GIP was the dominant incretin to be released under the conditions of the present study, it is not surprising that sitagliptin resulted in a marked increase in plasma intact GIP, but minimal elevation in intact GLP-1 in the three groups. In health, GIP stimulates insulin secretion in a glucose-dependent manner (Wu et al., 2010). Intravenous infusion of GIP has the capacity to stimulate insulin secretion and improves postprandial glycaemia in healthy subjects (Edholm et al., 2010). In type 2 diabetes, both the insulinotropic property (Nauck et al.,
1993) and the glucose-lowering capacity (Amland et al., 1985) of GIP are reported to be diminished. Consistent with these concepts, we observed less of an increase in the \( \text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}} \) ratio and no effect on glycaemia in response to ID glucose after sitagliptin than control, in type 2 patients compared to the healthy lean and obese. It is noteworthy that both the obese subjects and type 2 patients displayed comparable insulin resistance as evaluated by HOMA-IR. Unlike the obese subjects, however, the type 2 patients failed to achieve a compensatory insulin response during the ID glucose infusion, suggesting an impaired \( \beta \)-cell function. Therefore, the lack of glucose-lowering by augmented intact GIP in type 2 patients is likely to be a consequence of insulin resistance and impaired \( \beta \)-cell function, as well as diminished responsiveness of the \( \beta \)-cell to GIP specifically. It appears that preservation of physiological levels of GIP was not sufficient to have a significant effect on glycaemia in type 2 diabetes, and that the therapeutic efficacy of DPP-4 inhibitors in this disorder may be dependent on the availability of endogenous GLP-1. Indeed, we have shown that the effect of sitagliptin to reduce glycaemia in type 2 patients can be enhanced by stimulating endogenous GLP-1 secretion by dietary strategies, such as consuming a small load of D-xylose before the main meal (Wu et al., 2013d). To further establish this concept, it would be of interest to investigate the effects of DPP-4 inhibition with a higher rate of ID glucose infusion that would be expected to stimulate greater endogenous GLP-1 secretion. Alternatively, the relative contributions of endogenous GLP-1 and GIP to the glucose-lowering in type 2 diabetes could be elucidated by infusing the specific GLP-1 antagonist,
exendin (9-39). Sitagliptin appears unlikely to have lowered blood glucose by suppressing glucagon in our study, since we did not demonstrate any differences in plasma glucagon between the sitagliptin and control days in any group.

Consistent with previous reports (Maida et al., 2011, Migoya et al., 2010), we observed an increase in both plasma total and intact GLP-1 after metformin in type 2 patients. The mechanism of this effect remains unclear. It has been suggested that metformin increases preproglucagon gene expression in the intestine (Migoya et al., 2010), but this has not been a consistent observation (Maida et al., 2011). A recent study by Mulherin et al. failed to show any acute effect of metformin on L-cell secretion in vitro, but suggested an indirect stimulation of GLP-1 by metformin via peripheral M3- and gastrin releasing protein receptor-dependent pathways in vivo (Mulherin et al., 2011). Notably, metformin was associated with a modest increase in glucose-induced GIP secretion in our experiments, which has not been reported after either oral glucose or mixed meals, probably because previous experimental models were less controlled than ours (Maida et al., 2011, Migoya et al., 2010). The mechanism and clinical implications of enhanced GIP stimulation by metformin remain to be established. As expected, metformin lowered both fasting blood glucose and the glycaemic response to ID glucose in type 2 patients. However, enhancement of incretin secretion does not appear to be the dominant mechanism for this glucose-lowering effect, since the magnitude of the increase in both incretins was modest and addition of sitagliptin did not further potentiate
the reduction in glycaemia. Our observations are in line with the findings by Maida et al. that the glucose-lowering effect of metformin persists in GLP-1 and/or GIP receptor knockout mice (Maida et al., 2011). Again, the failure to detect any synergy between metformin and sitagliptin on glycaemia in this experimental setting probably reflects the fact that stimulation of endogenous GLP-1 was minimal. It is well established that metformin has a favourable effect on insulin-mediated peripheral glucose disposal and potently suppresses hepatic glucose production (Bailey and Turner, 1996). The latter effect counteracts the gluconeogenic and glycogenolytic actions of glucagon on the liver. Recently, Miller et al. demonstrated that metformin suppresses hepatic glucagon signalling by decreasing production of cyclic AMP (Miller et al., 2013). Hence, the observed increase in plasma glucagon after metformin in the present study could reflect a reactive response of pancreatic α-cells to antagonism of glucagon signaling (Gu et al., 2009). The increase in $\text{AUC}_{\text{insulin}}/\text{AUC}_{\text{glucose}}$ after metformin is likely due to improved β-cell responsiveness, as a result of attenuation in glycaemia (Ferner et al., 1988).

Sitagliptin was associated with fewer antral and duodenal pressure waves in the lean, and a tendency for reduction in antral waves in the obese, but had no effect on APD motility in patients with type 2 diabetes. Gastric emptying is driven by antral and duodenal contractions against pyloric resistance. Since pyloric motility, as evaluated by the frequency of IPPWs, was not altered in any group, the modest suppression of antral and duodenal motility in the lean and obese by
sitagliptin may not be sufficient to affect gastric emptying (Stevens et al., 2012). Sitagliptin might potentially have had a larger effect on IPPWs if endogenous GLP-1 had been stimulated to a greater extent. However, we recently demonstrated in type 2 patients that even when the effect of sitagliptin on postprandial glycaemia was optimised by boosting endogenous GLP-1 secretion, gastric emptying was not affected (Wu et al., 2013d). This might relate to the effects of peptide YY (PYY), which is co-secreted with GLP-1 from enteroendocrine L-cells(Savage et al., 1987). Degradation of PYY 1-36 to PYY 3-36 occurs via DPP-4, and the latter form is reportedly more potent at retarding emptying (Witte et al., 2009). Therefore, DPP-4 inhibition might blunt any PYY-mediated slowing of gastric emptying. Our motility data are in keeping with accumulating evidence that DPP-4 inhibition does not influence gastric emptying (Wu et al., 2013d, Stevens et al., 2012, Vella et al., 2007). Metformin was reported to slow gastric emptying in mice (Maida et al., 2011), but we did not observe any effect of metformin on APD motility in the current experimental setting.

Sitagliptin had no effect on energy intake in any group, consistent with the clinical observation that DPP-4 inhibitors are weight neutral (Ahren, 2007). This could be due either to the fact that GLP-1 stimulation was modest, and/or to the potential decrease in plasma PYY 3-36 which also has satiating effects (Batterham et al., 2002). In contrast, energy intake was less after administration of metformin in type 2 patients, consistent with its known anorectic effect and
its clinical impact to induce weight loss (Lee and Morley, 1998). This effect was not explained by induction of nausea, and is probably mediated primarily by GLP-1-independent mechanisms, since the increase in plasma GLP-1 after metformin was modest, and metformin is known to suppress food intake comparably in GLP-1 receptor knockout and wild type mice (Maida et al., 2011).

Our study has several limitations. Firstly, the number of subjects studied was relatively small; however, the effects examined were consistent between subjects, and increasing the sample size is unlikely to substantially change the study outcomes. Secondly, because the blood glucose concentrations had not returned to baseline by 120 min, we could not determine the effect on the overall glycaemic response. Thirdly, the obese subjects, who had evidence of metabolic dysfunction (insulin resistance and non-significant elevation of fasting glucagon), were not screened by a 75 g oral glucose tolerance test to exclude glucose intolerance. However, their blood glucose and glucagon responses to ID glucose resembled those of healthy lean subjects. Fourthly, age was not well matched between the three groups. Although age may not influence secretion of either incretin (Trahair et al., 2012a), it seems to have an impact on incretin metabolism (Ryskjaer et al., 2006). Finally, glucose was infused at a single rate (2 kcal/min), and it would be of great interest to determine whether the relative efficacy of sitagliptin in lowering glycaemia would increase with a higher rate of small intestinal glucose delivery.
In conclusion, when ID glucose is infused at a rate that approximates the low- to mid-point of the physiological range of gastric emptying, resulting in substantial release of GIP, but minimal stimulation of GLP-1, sitagliptin reduces glycaemic excursions in healthy lean and obese humans, but not in patients with type 2 diabetes. In contrast, metformin improves glycaemia, augments plasma total and intact GLP-1, total GIP, and glucagon, and suppresses energy intake in type 2 diabetes. There is no evidence of synergy between metformin and sitagliptin, probably because GIP is the dominant incretin secreted in our experimental setting. The effect of DPP-4 inhibition on glycaemia in type 2 diabetes is likely to be critically dependent on the rate of carbohydrate entry into the small intestine.
Table 1. Demographic and biochemical variables in the three study groups.

<table>
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<tr>
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<th>Lean</th>
<th>Obese</th>
<th>T2DM</th>
<th>P value</th>
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<tbody>
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<td>12 males</td>
<td>12 males</td>
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</tr>
<tr>
<td>Ethnic characteristics</td>
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<td>Caucasian</td>
<td>Caucasian</td>
<td>-</td>
</tr>
<tr>
<td>Age (yr)</td>
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<td>38.8 ± 4.5</td>
<td>63.7 ± 1.9</td>
<td>*,# &lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5 ± 0.6</td>
<td>34.5 ± 1.2</td>
<td>29.9 ± 1.2</td>
<td>*,# &lt; 0.001</td>
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<tr>
<td>Duration of known diabetes (yr)</td>
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<td>-</td>
<td>3.5 ± 0.9</td>
<td>-</td>
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<td>HbA1c (%)</td>
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<td>-</td>
<td>6.5 ± 0.1</td>
<td>-</td>
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<tr>
<td>(mmol/mol)</td>
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<td></td>
<td>47.6 ± 1.4</td>
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<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>4.7 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>6.7 ± 0.3</td>
<td>*,# &lt; 0.001</td>
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<td>Fasting plasma glucagon (pmol/L)</td>
<td>6.3 ± 0.8</td>
<td>10.0 ± 1.2</td>
<td>12.4 ± 2.5</td>
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<td>Fasting serum insulin (mU/L)</td>
<td>3.3 ± 0.5</td>
<td>10.6 ± 2.0</td>
<td>8.4 ± 1.8</td>
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<td>HOMA-IR (pmol.mmol.L⁻²)</td>
<td>4.8 ± 0.7</td>
<td>17.7 ± 3.8</td>
<td>18.5 ± 4.6</td>
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<tr>
<td>Fasting total GLP-1 (pmol/L)</td>
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<td>Fasting total GIP (pmol/L)</td>
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<td>Fasting intact GLP-1 (pmol/L)</td>
<td>0.5 ± 0.2</td>
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<td>Fasting intact GIP (pmol/L)</td>
<td>7.9 ± 0.3</td>
<td>8.6 ± 0.4</td>
<td>9.2 ± 0.6</td>
<td>0.109</td>
</tr>
</tbody>
</table>

One factor ANOVA was used to determine the statistical significance. Post hoc comparisons were adjusted by Bonferroni’s correction. * P < 0.001 and δ P < 0.05 for comparisons of lean vs. obese, or T2DM; # P < 0.01 for comparisons of obese vs. T2DM. Data are means ± SEM.
### Table 2. Areas under the curve (AUC) for plasma glucose, GLP-1 and GIP (total and intact), and glucagon, serum insulin, and the total number of antral waves (AWs), duodenal waves (DWs), and isolated pyloric pressure waves (IPPWs) in response to intraduodenal glucose infusion (2 kcal/min, during t = 0-120 min) after control (C) or sitagliptin (S) (at t = -30 min) in healthy lean and obese subjects, and type 2 diabetic subjects treated with placebo (P) (n = 12 for each group)\(^1,2\).

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th></th>
<th>Obese</th>
<th></th>
<th>T2DM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
<td>C</td>
<td>S</td>
<td>P + C</td>
<td>P + S</td>
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<tr>
<td>Glucose AUC  (mmolL(^{-1})min)</td>
<td>967.8 ± 29.7</td>
<td>887.7 ± 35.9 *</td>
<td>1033.4 ± 42.0</td>
<td>955.4 ± 53.9 *</td>
<td>1353.2 ± 45.4 (^{*,\varepsilon})</td>
<td>1334.8 ± 51.1 (^{*,\varepsilon})</td>
</tr>
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<td>Total GLP-1 AUC (pmolL(^{-1})min)</td>
<td>2924.2 ± 238.2</td>
<td>2532.7 ± 148.9</td>
<td>2290.2 ± 140.1 *</td>
<td>2249.6 ± 230.4</td>
<td>2556.5 ± 204.8</td>
<td>2599.2 ± 193.0</td>
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<tr>
<td>Total GIP AUC (pmolL(^{-1})min)</td>
<td>3207.9 ± 319.1</td>
<td>2900.6 ± 411.5</td>
<td>3247.1 ± 392.2</td>
<td>3144.8 ± 411.7</td>
<td>2865.4 ± 247.1</td>
<td>2919.6 ± 351.2</td>
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<td>Intact GLP-1 AUC (pmolL(^{-1})min)</td>
<td>79.1 ± 21.1</td>
<td>156.4 ± 31.1 *</td>
<td>16.9 ± 4.5</td>
<td>123.1 ± 21.9 *</td>
<td>108.0 ± 30.2 (^{\varepsilon})</td>
<td>169.9 ± 32.4 (^{\varepsilon})</td>
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<tr>
<td>Intact GIP AUC (pmolL(^{-1})min)</td>
<td>1611.9 ± 67.1</td>
<td>2435.6 ± 113.7 *</td>
<td>1608.8 ± 68.8</td>
<td>2558.1 ± 155.8 *</td>
<td>2010.0 ± 93.2 (^{\varepsilon})</td>
<td>2640.6 ± 219.4 *</td>
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<tr>
<td>Glucagon AUC (pmolL(^{-1})min)</td>
<td>547.3 ± 67.0</td>
<td>544.6 ± 84.3</td>
<td>921.6 ± 128.7</td>
<td>812.5 ± 88.4</td>
<td>1282.1 ± 287.3 (^{\delta})</td>
<td>1309.8 ± 309.6 (^{\delta})</td>
</tr>
<tr>
<td>Insulin AUC (mUL(^{-1})min)</td>
<td>3040.7 ± 401.6</td>
<td>3713.3 ± 475.0 *</td>
<td>4944.4 ± 824.8</td>
<td>6603.1 ± 1498.5</td>
<td>2607.0 ± 472.6 (^{\varepsilon})</td>
<td>3500.4 ± 816.8 (^{\varepsilon})</td>
</tr>
<tr>
<td>AUC(<em>{insulin})/AUC(</em>{glucose}) (mU/mmol)</td>
<td>3.1 ± 0.4</td>
<td>4.2 ± 0.5 *</td>
<td>4.9 ± 0.9</td>
<td>6.7 ± 1.4 *</td>
<td>1.9 ± 0.4 (^{\varepsilon})</td>
<td>2.7 ± 0.7 (^{\varepsilon})</td>
</tr>
<tr>
<td>Total AWs (number)</td>
<td>86.4 ± 22.1</td>
<td>31.8 ± 6.3 *</td>
<td>164.1 ± 36.9</td>
<td>98.6 ± 21.9</td>
<td>102.3 ± 18.0</td>
<td>93.3 ± 34.1</td>
</tr>
<tr>
<td>Total DWs (number)</td>
<td>1179.6 ± 168.8</td>
<td>779.9 ± 137.5 *</td>
<td>1193.2 ± 208.2</td>
<td>1077.3 ± 241.3</td>
<td>1163.1 ± 115.5</td>
<td>956.5 ± 102.9</td>
</tr>
<tr>
<td>Total IPPWs (number)</td>
<td>84.4 ± 15.6</td>
<td>73.2 ± 20.6</td>
<td>87.0 ± 30.1</td>
<td>76.1 ± 24.3</td>
<td>65.2 ± 7.4</td>
<td>59.7 ± 8.7</td>
</tr>
<tr>
<td>Energy intake (kJ)</td>
<td>5005.6 ± 615.3</td>
<td>4446.3 ± 521.6</td>
<td>5809.3 ± 619.7</td>
<td>6022.1 ± 497.4</td>
<td>4184.6 ± 465.9 (^{\varepsilon})</td>
<td>3900 ± 488.6 (^{\varepsilon})</td>
</tr>
</tbody>
</table>

1. Data are means ± SEM; paired Student’s t-test for intra-group comparisons: * \(P < 0.05\), C vs. S;
2. One-factor ANOVA for inter-group comparisons, adjusted by Bonferroni’s correction for post hoc comparisons: * \(P < 0.05\) for lean vs obese; \(^{\delta}\) \(P < 0.05\) for lean vs. T2DM; \(^{\varepsilon}\) \(P < 0.05\) for obese vs. T2DM.
### Table 3. Areas under the curves (AUCs) for blood glucose, plasma GLP-1 and GIP (total and intact), plasma glucagon, serum insulin, and antropyloroduodenal (APD) pressure waves in response to intraduodenal glucose infusion (2 kcal/min, during t = 0-120 min) after placebo (P) + control (C), P + sitagliptin (S), metformin (M) + C, or M + S in patients with type 2 diabetes (n = 12) \(^1\).

<table>
<thead>
<tr>
<th></th>
<th>P + C</th>
<th>P + S</th>
<th>M + C</th>
<th>M + S</th>
<th>S effect</th>
<th>M effect</th>
<th>MS interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose AUC (mmolL(^{-1})min)</td>
<td>1353.2 ± 45.4</td>
<td>1334.8 ± 51.1</td>
<td>1227.2 ± 44.4</td>
<td>1247.4 ± 40.4</td>
<td>0.961</td>
<td>0.000</td>
<td>0.253</td>
</tr>
<tr>
<td>Total GLP-1 AUC (pmolL(^{-1})min)</td>
<td>2556.5 ± 204.8</td>
<td>2599.2 ± 193.0</td>
<td>3062.1 ± 240.1</td>
<td>2906.5 ± 240.1</td>
<td>0.566</td>
<td>0.001</td>
<td>0.216</td>
</tr>
<tr>
<td>Total GIP AUC (pmolL(^{-1})min)</td>
<td>2865.4 ± 247.1</td>
<td>2919.6 ± 351.2</td>
<td>3551.3 ± 407.9</td>
<td>3351.5 ± 284.1</td>
<td>0.694</td>
<td>0.014</td>
<td>0.477</td>
</tr>
<tr>
<td>Intact GLP-1 AUC (pmolL(^{-1})min)</td>
<td>108.0 ± 30.2</td>
<td>169.9 ± 32.4</td>
<td>191.3 ± 33.2</td>
<td>256.8 ± 41.0</td>
<td>0.007</td>
<td>0.001</td>
<td>0.904</td>
</tr>
<tr>
<td>Intact GIP AUC (pmolL(^{-1})min)</td>
<td>2865.4 ± 247.1</td>
<td>2919.6 ± 351.2</td>
<td>3551.3 ± 407.9</td>
<td>3351.5 ± 284.1</td>
<td>0.008</td>
<td>0.107</td>
<td>0.777</td>
</tr>
<tr>
<td>Glucagon AUC (pmolL(^{-1})min)</td>
<td>1282.1 ± 287.3</td>
<td>1309.8 ± 309.6</td>
<td>1677.1 ± 351.8</td>
<td>1661.0 ± 406.6</td>
<td>0.938</td>
<td>0.003</td>
<td>0.681</td>
</tr>
<tr>
<td>Insulin AUC (mUL(^{-1})min)</td>
<td>2607.0 ± 472.6</td>
<td>3500.4 ± 816.8</td>
<td>2779.4 ± 473.8</td>
<td>3697.0 ± 832.7</td>
<td>0.049</td>
<td>0.142</td>
<td>0.827</td>
</tr>
<tr>
<td>AUC(<em>{insulin/AUC</em>{glucose}}) (mU/mmol)</td>
<td>1.9 ± 0.4</td>
<td>2.7 ± 0.7</td>
<td>2.3 ± 0.5</td>
<td>3.1 ± 0.8</td>
<td>0.065</td>
<td>0.019</td>
<td>0.718</td>
</tr>
<tr>
<td>Total AWs (number)</td>
<td>102.3 ± 18.0</td>
<td>93.3 ± 34.1</td>
<td>142.7 ± 21.8</td>
<td>118.0 ± 27.9</td>
<td>0.863</td>
<td>0.083</td>
<td>0.676</td>
</tr>
<tr>
<td>Total DWs (number)</td>
<td>1163.1 ± 115.5</td>
<td>956.5 ± 102.9</td>
<td>1204.5 ± 111.1</td>
<td>959.8 ± 129.8</td>
<td>0.100</td>
<td>0.594</td>
<td>0.673</td>
</tr>
<tr>
<td>Total IPPWs (number)</td>
<td>65.2 ± 7.4</td>
<td>59.7 ± 8.7</td>
<td>66.3 ± 11.5</td>
<td>38.8 ± 10.1</td>
<td>0.090</td>
<td>0.418</td>
<td>0.123</td>
</tr>
<tr>
<td>Energy intake (kJ)</td>
<td>4184.6 ± 465.9</td>
<td>3900 ± 488.6</td>
<td>3497.0 ± 472.3</td>
<td>3735.0 ± 392.8</td>
<td>0.913</td>
<td>0.040</td>
<td>0.307</td>
</tr>
</tbody>
</table>

\(^1\) Data are means ± SEM; two-factor repeated ANOVA was used to determine statistical significance, with S and M as factors.
Figure 1. Schematic representation of the study protocol.
Chapter 7.

Actions of sitagliptin and metformin

A. **Plasma glucose**

B. **Serum insulin**

C. **Plasma total GLP-1**

D. **Plasma total GIP**

E. **Plasma intact GLP-1**

F. **Plasma intact GIP**

G. **Plasma glucagon**

*ID glucose (2 kcal/min)*

Plots showing changes in various plasma levels over time post-glucose administration.
Figure 2. Blood glucose, plasma glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (total and intact), plasma glucagon, and serum insulin in response to intraduodenal glucose infusion (2 kcal/min, during t = 0 to 120 min) after control (C) and sitagliptin (S) in healthy lean subjects (n = 12). Repeated measures ANOVA was used determine the statistical significance, with treatment and time as factors. Post hoc comparisons, adjusted by Bonferroni’s correction, were performed, if ANOVAs were significant. A: $P < 0.001$, treatment × time interaction; B: $P = 0.009$, treatment × time interaction; C: $P < 0.05$, treatment effect; D: $P = 0.019$, treatment × time interaction; E: $P = 0.011$, treatment × time interaction; F: $P < 0.001$ treatment × time interaction. * $P < 0.05$ for each. Data are means ± SEM.
Chapter 7. Actions of sitagliptin and metformin

**A. Plasma glucose**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma glucose over time (min)

**B. Serum insulin**

- ID glucose (2 kcal/min)
- Graph showing changes in serum insulin over time (min)

**C. Plasma total GLP-1**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma total GLP-1 over time (min)

**D. Plasma total GIP**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma total GIP over time (min)

**E. Plasma intact GLP-1**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma intact GLP-1 over time (min)

**F. Plasma intact GIP**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma intact GIP over time (min)

**G. Plasma glucagon**

- ID glucose (2 kcal/min)
- Graph showing changes in plasma glucagon over time (min)
Figure 3. Blood glucose, plasma glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (total and intact), plasma glucagon, and serum insulin in response to intraduodenal glucose infusion (2 kcal/min, during $t = 0$ to 120 min) after control (C) and sitagliptin (S) in healthy obese subjects ($n = 12$). Repeated measures ANOVA was used determine the statistical significance, with treatment and time as factors. Post hoc comparisons, adjusted by Bonferroni’s correction, were performed, if ANOVAs were significant. A: $P < 0.001$, treatment $\times$ time interaction; B: $P = 0.021$, treatment $\times$ time interaction; E: $P = 0.016$ treatment $\times$ time interaction; F: $P < 0.001$ treatment $\times$ time interaction. * $P < 0.05$ for each. Data are means $\pm$ SEM.
Chapter 7. Actions of sitagliptin and metformin
Figure 4. Blood glucose, plasma glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (total and intact), plasma glucagon, and serum insulin in response to intraduodenal glucose infusion after placebo (P) + control (C), P + sitagliptin (S), metformin (M) + C, or M + S in patients with type 2 diabetes (n = 12). Repeated measures ANOVA was used determine the statistical significance, with treatment and time as factors. Post hoc comparisons, adjusted by Bonferroni’s correction, were performed, if ANOVAs were significant. A: $P = 0.003$, treatment $\times$ time interaction; B: $P < 0.001$, treatment $\times$ time interaction; C: $P < 0.001$, treatment effect; D: $P = 0.03$, treatment effect; E: $P = 0.007$, treatment effect; F: $P < 0.001$, treatment $\times$ time interaction; G: $P = 0.003$, treatment $\times$ time interaction. $^\circ P < 0.05$, P + C vs. P + S; $^* P < 0.05$, P + C vs. M + C; $^\# P < 0.05$, P + C vs. M + S; $^\delta P < 0.05$, P + S vs. M + S; $^\epsilon P < 0.5$, M + C vs. M + S. Data are means ± SEM.
Figure 5. The frequency of antral waves (AWs), duodenal waves (DWs), and isolated pyloric pressure waves (IPPWs) in response to intraduodenal glucose infusion (2 kcal/min, during t = 0-120 min) after sitagliptin (S) or control (C) in healthy lean and obese subjects (n = 12 for each group). Repeated measures ANOVA was used determine the statistical significance, with treatment and time as factors. Post hoc comparisons, adjusted by Bonferroni’s correction, were performed, if ANOVAs were significant. A and D: $P < 0.05$, treatment effect; * $P < 0.05$ for post hoc comparisons. Data are means ± SEM.
Figure 6. The frequency of antral waves (AWs) (A), duodenal waves (DWs) (B), and isolated pyloric pressure waves (IPPWs) (C) in response to intraduodenal glucose infusion (2 kcal/min, during t = 0-120 min) after placebo (P) + control (C), P + sitagliptin (S), metformin (M) + C, or M + S in patients with type 2 diabetes (n = 12). Repeated measures ANOVA was used to determine the statistical significance, with treatment and time as factors.
CHAPTER 8. EFFECTS OF RECTAL ADMINISTRATION OF TAUROCHOLIC ACID ON THE SECRETION OF GLUCAGON-LIKE PEPTIDE-1 AND PEPTIDE YY IN HEALTHY HUMANS.


8.1 Summary
Glucagon like peptide-1 (GLP-1) and peptide YY (PYY) are secreted by enteroendocrine L-cells located most densely in the colon and rectum, and are of fundamental importance in blood glucose and appetite regulation. In vitro and animal studies suggest that bile salts can stimulate GLP-1 and PYY secretion by TGR5 receptor activation. In this chapter, we evaluated the effects of taurocholic acid (TCA), administered in either an enema or a suppository formulation, on the release of GLP-1 and PYY in healthy humans. All subjects were studied after an overnight fast, in double-blind, randomised order. In Study 1, 10 healthy male subjects were evaluated on 3 occasions each, following rectal administration of a 20 mL enema containing 1500 mg or 3500 mg of TCA, or vehicle only. In Study 2, 12 healthy male subjects were evaluated twice, following administration of a 3 mL suppository containing 1500 mg TCA or vehicle only. Plasma total GLP-1 and PYY, and gastrointestinal sensations (100 mm visual analogue scales) were measured at intervals for 180 min. TCA in an enema formulation stimulated secretion of both GLP-1 (P = 0.019) and PYY (P
= 0.0005), and the incremental area under the curve for each demonstrated a
dose-dependent relationship (r = 0.48, P = 0.004 for GLP-1, and r = 0.56, P =
0.001 for PYY). Fullness was greater after 3500mg TCA than placebo (P < 0.05),
without a significant difference between 1500mg TCA and placebo. In contrast,
1500 TCA in a suppository formulation had no effects on GLP-1 or PYY
secretion, or fullness, compared to placebo. TCA either in an enema or a
suppository increased desire to defecate and frequency of defecation (P < 0.01
for each), when compared to placebo. In conclusion, rectal TCA formulated as
an enema stimulates GLP-1and PYY, and increases fullness, in a dose-
dependent manner in healthy humans, suggesting that topical application of bile
acids to the distal gut has therapeutic potential for the management of diabetes
and obesity.

8.2 Introduction
The interaction of ingested nutrients with the small intestine gives rise to
secretion of gastrointestinal hormones that are fundamental in determining
subsequent gastric emptying and transit of luminal contents, appetite, and blood
glucose homeostasis (Cummings and Overduin, 2007, Ma et al., 2009b). Of
particular importance are the peptides released from the enteroendocrine L cells,
located predominantly in the distal small intestine, colon and rectum (Eissele et
al., 1992). These include glucagon-like peptide 1 (GLP-1), which induces
insulin secretion in a glucose dependent manner, suppresses glucagon, slows
gastric emptying, and suppresses appetite (Baggio and Drucker, 2007), and
peptide YY (PYY), which also slows gastric emptying and potently suppresses food intake (Batterham et al., 2002). Both GLP-1 and PYY contribute to the phenomenon known as the “ileal brake”, whereby exposure of the ileum to nutrient retards further gastric emptying and small intestinal transit of chyme (Wen et al., 1995). Administration of exogenous gut peptides and their analogues have been proposed as treatments for obesity and diabetes, and GLP-1 agonists such as exenatide and liraglutide are now approved and marketed for the treatment of type 2 diabetes. Stimulating endogenous hormone secretion could be cheaper and better tolerated than injecting exogenous GLP-1 and PYY agonists for treating diabetes or obesity. The potential for such a strategy is supported by the observation that the release of both GLP-1 and PYY is increased following gastric bypass surgery for obesity, an effect that has been implicated in the weight loss and improvement in glucose tolerance that results from this procedure (le Roux et al., 2007, Morinigo et al., 2006).

Bile salts are high affinity ligands for the G-protein coupled TGR5 receptor (Kawamata et al., 2003). In vitro studies have revealed the presence of TGR5 receptors on cultured enteroendocrine L-cells, and have demonstrated that bile salts potently release PYY and GLP-1 from these cells (Katsuma et al., 2005). Bile acids in the isolated perfused rat colon also promote GLP-1 release (Plaisancie et al., 1995), while sigmoid perfusion with the bile salt deoxycholate in humans was reported to be a potent stimulus for PYY and enteroglucagon (Adrian et al., 1993). In the colon and rectum, intraluminal bile salt
concentrations are low under physiological conditions (Adrian et al., 1993), and it is logical that excessive bile salts in this region constitute a signal that chyme flow exceeds the absorptive capacity of the small intestine. Secretion of PYY and GLP-1 from the L-cells in response to luminal bile salts can therefore be seen to mediate a physiologically appropriate response, i.e. reduction of ingestion and slowing of gastrointestinal transit.

Taurocholic acid (TCA), a bile acid found physiologically in the lumen of the human gut, is a potent TGR5 ligand (Sato et al., 2008). TCA induces PYY secretion when infused into the colon in dogs (Izukura et al., 1991). A recent report indicated that TCA enemas could stimulate GLP-1 and PYY secretion in obese patients with type 2 diabetes receiving the dipeptidyl peptidase-4 (DPP-4) inhibitor, sitagliptin (Adrian et al., 2012). The current study was designed to evaluate the effects of different doses and formulations of TCA on the release of total GLP-1 and PYY, and on appetite, when applied to the rectum without DPP-4 inhibition in healthy humans.

8.3 Subjects and methods

8.3.1 Subjects

Twenty two healthy male subjects were studied, after they provided written informed consent. Females were excluded, since the secretion of gut hormones, including GLP-1, is known to vary with the phases of the menstrual cycle (Brennan et al., 2009). Subjects taking any medication known to influence
gastrointestinal function, and those who consumed > 20 g alcohol on a daily basis, or were smokers, were excluded. Ten subjects (mean age: 36.3 ± 4.6 years; body mass index (BMI): 26.5 ± 0.9 kg/m²) underwent Study 1; twelve subjects (mean age: 27.3 ± 3.8 years; BMI: 22.7 ± 0.7 kg/m²) underwent Study 2. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

8.3.2 Protocols

On the evening before each study day (~1900), subjects consumed a standardised evening beef lasagne meal (McCain Foods Proprietary Ltd, Victoria, Australia) with bread, a nonalcoholic beverage, and one piece of fruit. Subjects then fasted from solids, and refrained from consuming liquids after 2200. On each study day, subjects attended the laboratory at ~0830, and were asked to defecate. An intravenous cannula was inserted into a forearm vein for repeated blood sampling, and subjects were then positioned in the left lateral decubitus position for the remainder of the study.

8.3.2.1 Study 1: Administration of TCA in an enema formulation

10 subjects were studied on 3 occasions each, separated by ≥ 3 days, in double-blind, randomised order. They received a 20 mL aqueous gel (1% carboxymethyl cellulose) containing 1500 mg or 3500 mg TCA (NZ Pharmaceuticals Ltd. Palmerston North, New Zealand), or vehicle only, infused
into the rectum using a syringe and 10 cm soft cannula over 2 minutes (T = -2 to 0 min).

8.3.2.2 Study 2: Administration of TCA in a suppository formulation

12 subjects were studied on 2 occasions each, separated by \( \geq 3 \) days, in double-blind, randomised order. They received a 3 mL suppository (carboxymethyl cellulose, xanthan gum, fumed silica, calcium polycarbophil, and fatty base material) containing 1500 mg TCA (NZ Pharmaceuticals Ltd. Palmerston North, New Zealand), or vehicle only, applied at T = 0 min.

In each Study, blood was sampled at T= -5, 10, 30, 60, 90, 120, and 180 min for immediate measurement of blood glucose and subsequent measurements of total GLP-1 and PYY. At the same intervals, subjects were asked to rate gastrointestinal sensations. All subjects were telephoned the day after each visit to determine whether they had experienced any late adverse effects.

8.3.3 Measurements

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA).

For subsequent assays for GLP-1 and PYY, 10 mL venous blood samples were collected in ice-chilled EDTA-treated tubes. Plasma was obtained by centrifugation of blood samples at 3200 rpm for 15 min at 4 °C. The plasma
samples were frozen at -70 °C. Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK; Linco Research, St. Charles, MO, USA) with sensitivity 3 pmol/L, and intra- and inter-assay coefficients of variation (CVs) 6.8% and 8.5%. Plasma total PYY was measured by radioimmunoassay using an adaptation of a previously described method (Pilichiewicz et al., 2006). An antiserum (kindly donated by Dr. B Otto, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Munich, Germany), raised in rabbits against human PYY (1-36) (Sigma-Aldrich), was employed; i.e. the assay does not distinguish between PYY (1-36) and PYY (3-36). The antiserum showed < 0.001% cross-reactivity with human pancreatic polypeptide and sulfated cholecystokinin-8 and 0.0025% cross-reactivity with human neuropeptide Y. The sensitivity was 1.5 pmol/L, and intra- and inter-assay CVs 12.3% and 16.6%.

Gastrointestinal sensations, including desire to eat, projective consumption, hunger, fullness, and desire to defecate, were assessed using validated 100 mm visual analogue scales (Parker et al., 2004).

8.3.4 Statistical analysis

Incremental area under the curve (iAUC) was calculated using the trapezoidal rule (Wolever, 2004) for blood glucose, total GLP-1 and PYY concentrations, and analyzed by repeated one-factor ANOVA for Study 1 and by paired Student t-test for Study 2. Gastrointestinal sensations were assessed using repeated-
measures ANOVA, with treatment and time as factors for both studies. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni’s correction, were performed if ANOVAs revealed significant effects. 10 subjects were calculated to have 80% power ($\alpha = 0.05$) to detect a linear relationship between log dose and log GLP-1 and PYY responses with a slope of at least one standard deviation for Study 1, and 12 subjects to have 80% power ($\alpha = 0.05$) to detect differences in iAUC for total GLP-1 and PYY (Adrian et al., 2012). The dose-dependent effect for Study 1 was determined by correlation analysis. Frequency of defecation was analysed by Chi-square test. All analyses were performed using SPSS software (version 17.0). Data are presented as mean values ± standard error; $P < 0.05$ was considered statistically significant.

8.4 RESULTS

The protocols were tolerated well by all subjects, and no adverse effects were reported on the follow up telephone call.

8.4.1 Study 1: Administration of TCA in an enema formulation

8.4.1.1 Blood glucose concentrations

Baseline blood glucose concentrations did not differ between the three days, and blood glucose concentrations were unchanged after placebo or any dose of TCA (Figure 1A).
8.4.1.2 Plasma total GLP-1

Baseline plasma GLP-1 did not differ between the three study days. There was a rise in plasma GLP-1 after 1500 mg and 3500 mg TCA, with a significant treatment effect on the iAUC for plasma GLP-1 (1500 mg TCA: 233.4 ± 116.3 pmol/L × min, 3500 mg TCA: 390.2 ± 84.7 pmol/L × min vs. placebo: 61.4 ± 23.4 pmol/L × min, \( P = 0.019 \)), and the iAUC for GLP-1 concentrations demonstrated a dose-dependent relationship \( (r = 0.48, P = 0.004) \) (Figure 1B).

8.4.1.3 Plasma PYY

Baseline plasma PYY did not differ between the three study days. There was a rise in plasma PYY after 1500 mg and 3500 mg TCA, with a significant treatment effect on the iAUC for plasma PYY (1500 mg TCA: 452.8 ± 192.2 pmol/L × min, 3500 mg TCA: 827.2 ± 199.2 pmol/L × min vs placebo: 4.8 ± 3.2 pmol/L × min, \( P = 0.0005 \)), and the iAUC for PYY demonstrated a dose-dependent relationship \( (r = 0.56, P = 0.001 \text{ for PYY}) \) (Figure 1C).

8.4.1.4 Desire to defecate and frequency of defecation

Desire to defecate (Figure 2) and frequency of defecation within the study period (0 – 180 min) were greater after 1500 mg and 3500 mg TCA enemas than placebo \( (P < 0.01 \text{ for each}) \), with no difference between 1500 mg and 3500 mg TCA. No subject had a bowel action after placebo, while 9 subjects defecated after both 1500 mg TCA enema (median 35 min; range: 10 – 79 min), and 3500 mg TCA enema (median 30 min; range: 8 – 80 min).
8.4.1.5 Appetite sensations

There were no differences in hunger or desire to eat between the three study days (data not shown). However, fullness was greater after 3500mg TCA enema than placebo \((P < 0.05)\) (Figure 3A), and prospective consumption tended to be less after 3500 mg TCA enema than placebo \((P = 0.08)\) (Figure 3B), without a significant difference between 1500mg TCA and placebo.

8.4.2 Study 2: Administration of TCA in a suppository formulation

8.4.2.1 Blood glucose, plasma GLP-1 and PYY

Baseline blood glucose, plasma total GLP-1 and PYY did not differ between the two study days. Blood glucose concentrations were unchanged after either 1500 mg TCA suppository or placebo (Figure 4A). There was a slight decrease in plasma GLP-1 and PYY over time on both study days, with no difference between 1500 mg TCA suppository and placebo (Figure 4B and 4C).

8.4.2.2 Desire to defecate and frequency of defecation

Desire to defecate (Figure 5) and frequency of defecation within the study period \((0 – 180 \text{ min})\) were greater after 1500 mg TCA than placebo \((P < 0.01 \text{ for each})\). No subject had a bowel action after placebo, while 9 subjects defecated after 1500 mg TCA (median 180 min; range: 10 – 180 min).
8.4.2.3 Appetite sensations

There was no difference in hunger, prospective consumption, desire to eat, or fullness, between TCA and placebo (data not shown).

8.5 Discussion

We have shown that rectal administration of the bile acid, TCA, stimulates the secretion of GLP-1 and PYY, and increases the sensation of fullness, in a dose-dependent manner in healthy humans. Our study is one of the first to demonstrate the potential for topical application of bile acids to stimulate enteroendocrine L-cells, which are most densely located in the colon and rectum (Eissele et al., 1992). We have also demonstrated the importance of the formulation used to deliver TCA, with efficacy observed when TCA was given in an enema, but not in a suppository formulation.

GLP-1 contributes to the regulation of glucose homeostasis in a number of ways, including pancreatic and extrapancreatic actions on insulin secretion, improvement of insulin sensitivity and peripheral glucose disposal, inhibition of gastric emptying, and suppression of glucagon secretion and food intake (Wu et al., 2010), while PYY also mediates slowing of gastric emptying and potently suppresses energy intake (Batterham et al., 2002). Therefore, strategies that stimulate endogenous GLP-1 and PYY may be of therapeutic potential in the management of diabetes and obesity. We have previously shown that administration of either fat (Gentilcore et al., 2006), protein (Ma et al., 2009c),
or poorly absorbed carbohydrates (Wu et al., 2012) as a ‘preload’ reduces postprandial glycaemic excursions by stimulating endogenous GLP-1 and slowing of gastric emptying in both health and type 2 diabetes. Rectal application of bile acids, such as TCA, may represent a promising alternative to stimulate endogenous GLP-1 and PYY.

Both GLP-1 and PYY were released dose-dependently by TCA given in an enema, consistent with the previous report that colonic perfusion with deoxycholate induces PYY and enteroglucagon (i.e. GLP-1) release in healthy subjects (Adrian et al., 1993), and with the recent study of Adrian et al that rectal application of TCA with concurrent systemic administration of sitagliptin induced dose-dependent release of active GLP-1 and total PYY in obese patients with type 2 diabetes (Adrian et al., 2012). In line with the finding that L-cells in the human colon store more PYY than enteroglucagon (El-Salhy et al., 2012), we observed that TCA stimulated PYY to a greater magnitude than GLP-1. However, the elevation of plasma GLP-1 and PYY was transient – both increased within 15 min, but returned to baseline concentrations within an hour of rectal TCA perfusion. This was probably due to the limited duration of exposure of TCA to the rectal mucosa, given that few subjects were able to retain the formulation for more than 40 minutes. The stimulation of defecation after rectal TCA administration would be expected, given the capacity of unabsorbed bile acids to accelerate colonic transit (Wong et al., 2011, Sadik et al., 2004), an effect that is probably TGR5-dependent (Camilleri et al., 2011).
In parallel with GLP-1 and PYY stimulation, we observed that the sensation of fullness was increased, and ratings of prospective consumption tended to decrease, after rectal TCA administration. This is in keeping with the report of Adrian et al. that rectally administered TCA inhibited food intake (Adrian et al., 2012), although this might be partly attributable to the side effects. We did not evaluate energy intake, since our priority was to study the profile of gut hormone release over time, in order to guide optimal timing of TCA administration in relation to meals in subsequent studies evaluating the capacity for TCA to lower postprandial glycaemia and inhibit energy intake. The lack of any change in blood glucose, despite the stimulation of GLP-1, was anticipated, given that healthy subjects were studied and remained fasted during the study. Adrian et al. reported a lowering of blood glucose concentrations by rectal TCA, but they studied patients with type 2 diabetes who had fasting hyperglycaemia, and who also received sitagliptin (Adrian et al., 2012). We did not measure insulin and glucagon concentrations in the current study, given that the insulinotropic and glucagonostatic effects of GLP-1 are glucose-dependent (Nauck et al., 2002, Andersen et al., 1978).

A suppository formulation of TCA would represent a more practical dosage form than an enema, but the TCA suppository failed to stimulate GLP-1 or PYY in our study. The volume of the suppository (3 mL) was less than enema (20 mL), which would presumably result in a smaller surface area of mucosa being
exposed to TCA. Furthermore, release of TCA from the solid suppository formulation would be delayed by the time required for melting after application. Finally, the dose of TCA that could be incorporated into a suppository could not feasibly be increased above 1500 mg.

In conclusion, our results have confirmed the concept that topical application of bile acids to the distal gut mucosa has the capacity to stimulate the secretion of endogenous GLP-1 and PYY, and provide a potential new management approach for diabetes and obesity. Efforts to refine the tolerability of the formulation should now be a priority.
Figure 1. Effects of taurocholic acid (TCA) in an enema formulation on blood glucose (A), plasma glucagon-like peptide 1 (GLP-1) (B), and plasma peptide YY (PYY) (C) in healthy humans. Incremental areas under the curves (iAUC) were analyzed by one-factor ANOVA, with post hoc comparisons adjusted for Bonferroni’s correction. (B) There was a significant treatment effect of TCA enemas on GLP-1 ($P = 0.019$), such that GLP-1 stimulation was dose-dependent ($r = 0.48$, $P = 0.004$); (C) there was a significant treatment effect of TCA enemas on PYY ($P = 0.005$), such that PYY stimulation demonstrated a dose-dependent relationship ($r = 0.56$, $P = 0.001$). Data are means ± SEM.
Figure 2. Effects of taurocholic acid (TCA) in an enema on desire to defecate in healthy humans. Repeated-measures ANOVA was used to determine statistical significance. Desire to defecate was greater after either 1500 mg or 3500 mg TCA ($P < 0.01$ for each), compared to placebo, without significant any difference between 1500 mg TCA and 3500 mg TCA.
Figure 3. Effects of taurocholic acid (TCA) in an enema on fullness (A) and prospective consumption (B) in healthy humans. Repeated-measures ANOVA was used to determine statistical significance. (A) Fullness was greater after 3500 mg TCA compared to placebo ($P < 0.05$), without any significant difference between 1500 mg TCA and placebo. (B) Prospective consumption tended to be less after 3500 mg TCA than placebo ($P = 0.08$), with no difference between 1500 mg TCA and placebo.
Figure 4. Effects of taurocholic acid (TCA) in a suppository on blood glucose (A), plasma glucagon-like peptide 1 (GLP-1) (B), and plasma peptide YY (PYY) (C) in healthy humans. Incremental areas under the curves (iAUC) were analyzed by two-tailed paired Student’s t-test. There was no treatment effect on either blood glucose, plasma GLP-1, or plasma PYY.
Figure 5. Effects of taurocholic acid (TCA) in a suppository on desire to defecate in healthy humans. Repeated-measures ANOVA was used to determine statistical significance. Desire to defecate was greater after 1500 mg TCA suppository than placebo ($P < 0.001$).
CHAPTER 9. EFFECTS OF TAuroCHOLic ACID ON GLYCAEMIC, GlUCAGoN-LIKE PEPTIDE-1, AND INsULIN RESPONSES TO SMALL INTESTINAL GLUCOSE INFUSION IN HEALTHY HUMANS.


9.1 Summary

In vitro and animal studies suggest that bile acids have the capacity to reduce blood glucose by stimulating glucagon-like peptide-1 (GLP-1) and, thereby, insulin. The study in this Chapter evaluated the effects of intrajejunal taurocholic acid (TCA) on blood glucose, GLP-1 and insulin responses to jejunal glucose infusion in healthy humans. Ten healthy males were each studied on 2 days, in double-blind, randomised order. After an overnight fast, a jejunal catheter was positioned and a balloon inflated 30cm beyond the pylorus with aspiration of endogenous bile. 2 g TCA in saline, or saline control, was infused beyond the balloon over 30 min, followed by 2 g TCA or control, together with 60 g glucose, over the next 120 min. Blood was sampled frequently for the measurements of blood glucose, total GLP-1, insulin, C-peptide and glucagon. Intrajejunal infusion of TCA alone (t = -30 to 0 min) had no effect on blood glucose, GLP-1, insulin, C-peptide, or glucagon concentrations. During intrajejunal glucose infusion (t = 0 to 120 min), blood glucose concentrations were lower (P < 0.001), and plasma GLP-1 (P < 0.001) and the C-
peptide/glucose ratio ($P = 0.008$) were both greater, whereas plasma insulin, C-peptide, and glucagon levels were not significantly different, after TCA than control. In conclusion, in healthy humans, small intestinal infusion of TCA potently reduces the glycaemic response to small intestinal glucose, associated with an increase in GLP-1 and C-peptide/glucose ratio. These observations indicate the potential for bile acid-based therapy in type 2 diabetes.

9.2 Introduction

While bile acids in the small intestine are known to inhibit nutrient-stimulated cholecystokinin (CCK) release, gall bladder contraction, and small intestinal transit (Koop et al., 1996, Penagini et al., 1988), it is now emerging that bile acids function as important signalling molecules, and are essential in blood glucose regulation (Nguyen and Bouscarel, 2008).

Bile acids are dual ligands of the nuclear receptor, farnesoid X receptor (FXR), and the membrane G-protein coupled receptor, TGR5, which are both expressed abundantly in the intestine, liver, skeletal muscles and pancreas (Nguyen and Bouscarel, 2008). In vitro and in vivo animal studies have demonstrated that FXR activation increases glycogen synthesis and insulin sensitivity, and inhibits gluconeogenesis, so as to improve glucose tolerance (Ma et al., 2006). TGR5 is expressed on enteroendocrine L-cells in the small and large intestine (Reimann et al., 2008), which secrete glucagon-like peptide-1 (GLP-1). This hormone, together with glucose-dependent insulinotropic polypeptide (GIP), accounts for
the much greater insulin response to enteral compared to intravenous glucose – the so-called ‘incretin’ effect (Holst et al., 2009). In type 2 diabetes, the insulinotropic effect of GIP is largely diminished, while that of GLP-1 is preserved (Holst et al., 2009), and GLP-1 has, accordingly, been the focus of incretin-based therapies for diabetes.

GLP-1 is released primarily from L-cells in the distal small intestine, colon, and rectum (Eissele et al., 1992). Bile acids can stimulate GLP-1 release from L-cells in vitro in a TGR5-dependent manner (Thomas et al., 2009), while in mice, co-administration of taurocholic acid (TCA) with glucose by gavage reduces postprandial glycaemia, associated with enhanced GLP-1 secretion, when compared to glucose alone (Rafferty et al., 2011). We, and others, recently reported that rectal administration of TCA dose-dependently stimulates GLP-1 secretion in healthy humans (Wu et al., 2013b) and patients with type 2 diabetes (Adrian et al., 2012). Whether bile acids delivered into the upper small intestine also stimulate GLP-1 secretion, and thereby improve blood glucose in humans has not been established.

The current study evaluated the effects of TCA on blood glucose, GLP-1 and insulin responses to intrajejunal glucose infusion.
9.3 Materials and methods

9.3.1 Subjects

10 healthy males (mean age: 33.4 ± 6.0 years; body mass index: 24.5 ± 1.1 kg/m²) were studied, after giving written, informed consent. Females were excluded, since the secretion of some gut hormones, including GLP-1, is known to be affected by the phase of the menstrual cycle (Brennan et al., 2009). Subjects taking any medication known to influence gastrointestinal function, and those who consumed > 20 g alcohol on a daily basis, or were smokers, were excluded. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

9.3.2 Materials

Bovine TCA (4 g) (NZ Pharmaceuticals, Palmerston North, New Zealand) was made up to 240 mL in 0.9% saline, and 240 mL saline control was matched for osmolarity.

9.3.3 Protocol

Each subject was studied twice, separated by ≥ 1 week, in double-blind fashion, randomized by the hospital pharmacy. On the evening before each study (~1900h), subjects consumed a standardized lasagna meal (McCain Foods), then fasted from solids, and refrained from liquids after 2200h. Subjects attended the laboratory at ~0800h the following day, when a multilumen silicone rubber
catheter (Dentsleeve International, Ontario, Canada) was inserted through transnasally and allowed to pass into the small intestine by peristalsis (Little et al., 2006). The catheter was positioned with an infusion port located 50 cm beyond the pylorus (proximal jejunum), and incorporated a balloon (5 cm long) 30 cm beyond, and an aspiration channel 25 cm beyond, the pylorus. Catheter position was monitored continuously by measuring the transmucosal potential difference in the stomach (~ -40 mV) and duodenum (~ 0 mV) (Little et al., 2006). The balloon was inflated with air (~ 30 – 40 mL) until the subject reported a sensation of pressure, without discomfort. Intraballoon pressure was monitored continuously to ensure sustained inflation (von Richter et al., 2001, Little et al., 2006).

A cannula was inserted into a forearm vein for blood sampling. Intrajejunal infusion of TCA or control then commenced, with 120 mL (2 g TCA) infused from t = -30 to 0 min, and then 120 mL (2 g TCA) infused over the next 120 min (t = 0 to 120 min). During the latter period, glucose (25%) was infused at 0.5 g/min (2 kcal/min) for 120 min. Blood was sampled every 15 min into ice-chilled EDTA tubes, and immediately centrifuged at 3200 rpm for 15 min at 4 °C. Plasma was separated and stored at −70 °C for subsequent analysis.

9.3.4 Measurements

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). Plasma
total GLP-1 was measured by radioimmunoassay (GLPIT-36HK; Linco Research, St. Charles, MO, USA) with sensitivity 3 pmol/L, and intra- and inter-assay coefficients of variation (CV) of 6.8% and 8.5%. Plasma insulin was measured by ELISA (10-1113, Mercodia, Uppsala, Sweden) with sensitivity 1.0 mU/L, and intra- and inter-assay CVs of 2.7% and 7.8%. Plasma C-peptide was measured by ELISA immunoassay (10-1136-01, Mercodia, Uppsala, Sweden) with sensitivity 15 pmol/L, and intra- and inter-assay CVs of 11.4% and 1.5%. Glucagon was measured by radioimmunoassay (GL-32K, Millipore, Billerica, MA) with sensitivity 20 pg/ml, and intra- and inter-assay CVs of 15 % and 10.5 %.

9.3.5 Statistical analysis

Incremental area under the curve (iAUC) was calculated using the trapezoidal rule for blood glucose, and plasma GLP-1, insulin, C-peptide and glucagon concentrations, and these were compared using paired t-tests. These variables, and the C-peptide/glucose ratio, were also analyzed using repeated measures ANOVA, with treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni’s correction, were performed if ANOVAs revealed significant effects. All analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA). 10 subjects were calculated to have 80% power ($\alpha = 0.05$) to detect a five-fold difference in GLP-1 secretion, compared to control (Wu et al., 2013b). Data are presented as means ± standard error; $P < 0.05$ was considered statistically significant.
9.4 Results

All subjects tolerated the study well, and no adverse effects were reported.

9.4.1 Blood glucose

Fasting blood glucose concentrations did not differ between study days. Blood glucose concentrations were unchanged when either TCA or control was infused alone (t = -30 to 0 min). During intrajejunal glucose infusion (t = 0 to 120 min), blood glucose increased on both days, but was lower during TCA than control infusion ($P = 0.02$ for overall iAUC; $P < 0.001$ for treatment × time interaction, with significant differences at $t = 45$ and 105 min ($P < 0.05$ for each)) (Figure 1A; Table 1).

9.4.2 Plasma total GLP-1

Fasting GLP-1 concentrations did not differ between study days. When TCA or control was infused alone (t = -30 to 0 min), GLP-1 concentrations remained unchanged. During intrajejunal glucose infusion (t = 0 to 120 min), a sustained rise in GLP-1 was evident with TCA infusion, whereas with control, GLP-1 increased and then plateaued from $\sim t = 30$ min, such that GLP-1 concentrations were higher with TCA ($P = 0.04$ for overall iAUC; $P < 0.001$ for treatment × time interaction, with significant differences at $t = 90$, 105, and 120 min ($P < 0.05$ for each)) (Figure 1B; Table 1).
9.4.3 Plasma insulin, C-peptide and glucagon

Fasting insulin, C-peptide, and glucagon concentrations did not differ between study days, and were unchanged during TCA or control infusion alone (t = -30 to 0 min). During intrajejunal glucose infusion (t = 0 to 120 min), insulin and C-peptide concentrations increased on both days, without any difference between TCA and control (Figure 1C, 1D and 1F; Table 1). However, the C-peptide/glucose ratio was higher with TCA ($P = 0.008$ for treatment × time interaction, with significant differences at t = 45, 60, and 105 min ($P < 0.05$ for each)) (Figure 1E).

9.5 Discussion

This study establishes that in healthy humans, the presence of TCA in the jejunum has no effect on fasting blood glucose, GLP-1, insulin, or C-peptide, but potently reduces the glycaemic excursion in response to a small intestinal glucose infusion, associated with an increase in GLP-1 and C-peptide/glucose ratio.

Determinants of postprandial blood glucose include the rate of gastric emptying and small intestinal absorption, postprandial secretion of incretin hormones and insulin, and hepatic glucose metabolism. Studies in healthy humans have established that bile acids in the small intestine can trigger inhibitory feedback to slow small intestinal transit (Penagini et al., 1988), and we have reported previously that inhibition of flow of luminal contents can delay small intestinal...
glucose absorption (Chaikomin et al., 2007). Intraluminal bile acids can also inhibit intestinal absorption of glucose in humans (Brown and Ammon, 1981). These mechanisms may have contributed to the reduction in glycaemia observed with TCA, but ought to result in lowering, rather than the observed relative stimulation, of insulin secretion.

The strengths of our study are that we excluded endogenous bile acids by occluding the distal duodenal lumen and aspirating all proximal secretions, and we infused glucose at a controlled rate that approximated physiological gastric emptying of a glucose drink (Brener et al., 1983). Although we measured blood glucose with a glucometer, the differences between study days were consistent, and unlikely to be substantially altered by using a different assay. When given alone, TCA failed to stimulate GLP-1 release, in contrast to previous reports of rectal administration of supraphysiological doses (Wu et al., 2013b, Adrian et al., 2012). A 30 minute infusion period is sufficient to observe GLP-1 secretion in response to nutrient stimuli such as fatty acids (Little et al., 2005). We administered 4 g of bile acid that approximates the physiological postprandial bile acid output (Koop et al., 1996), but a higher dose might possibly have had a greater stimulatory effect. We infused TCA alone, rather than a more physiological mixture of bile acids, given its relative potency for the TGR5 receptor in vitro (Sato et al., 2008). The proximal site of infusion might account for diminished effect when compared to rectal administration, given that L-cells are most densely distributed in the distal gut (Eissele et al., 1992). The
stimulation of GLP-1 by TCA observed during intrajejunal glucose infusion occurred relatively late, which might reflect the delay in TCA reaching this region. Alternatively, if TCA inhibited small intestinal glucose absorption (Brown and Ammon, 1981), there would be greater exposure of glucose to the more distal gut, with enhancement of late GLP-1 secretion – a similar phenomenon is evident when sucrose is given with the α-glucosidase inhibitor, acarbose (Qualmann et al., 1995).

Although insulin and C-peptide concentrations per se did not differ between study days, these must be interpreted in the context of the blood glucose concentration, since stimulation of insulin secretion by GLP-1 is glucose-dependent (Holst et al., 2009). The increase in C-peptide/glucose ratio during TCA exposure therefore supports a tendency for enhanced insulin secretion, which is likely driven by GLP-1 rather than GIP, since TCA is reported not to induce GIP secretion in healthy humans (Bjornsson et al., 1982). We did not demonstrate any suppression of glucagon that would contribute to the glucose-lowering effect of TCA.

In addition to stimulation of GLP-1 and insulin secretion, activation of the FXR pathway might be important in the lowering of blood glucose; in rodents, FXR activation gives rise to increased glycogenesis and decreased gluconeogenesis (Ma et al., 2006). Co-administration of TCA with glucose reduces postprandial glycaemia even in GLP-1 receptor knockout mice (Rafferty et al., 2011). In line
with these observations, we observed that the glucose-lowering effect of TCA occurred earlier than stimulation of GLP-1, supporting the existence of GLP-1-independent mechanisms.

The present findings warrant further clarification of the mechanisms involved, both to elucidate the physiology of these responses – which may be relevant, for example, to Roux-en-Y gastric bypass surgery – and to develop a therapeutic approach for type 2 diabetes.
Table 1. Fasting concentrations and incremental area under the curves (iAUC) for blood glucose, plasma total glucagon-like peptide-1 (GLP-1), insulin, C-peptide, and glucagon in response to taurocholic acid (TCA) or control, both before (t = -30 to 0 min) and during (t = 0 to 120 min) intrajejunal glucose infusion (at the rate of 0.5 g/min) in healthy males (n = 10) a.

<table>
<thead>
<tr>
<th></th>
<th>TCA (4 g)</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>0.33</td>
</tr>
<tr>
<td>Fasting plasma total GLP-1 (pmol/L)</td>
<td>16.9 ± 1.9</td>
<td>18.0 ± 1.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Fasting plasma insulin (mU/L)</td>
<td>3.4 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>0.70</td>
</tr>
<tr>
<td>Fasting plasma C-peptide (pmol/L)</td>
<td>319.4 ± 36.7</td>
<td>298.5 ± 34.5</td>
<td>0.35</td>
</tr>
<tr>
<td>Fasting plasma glucagon (pg/mL)</td>
<td>52.8 ± 5.8</td>
<td>58.6 ± 9.5</td>
<td>0.38</td>
</tr>
<tr>
<td>Glucose iAUC-30-0 (mmol/L × min)</td>
<td>0.2 ± 0.2</td>
<td>2.2 ± 1.0</td>
<td>0.07</td>
</tr>
<tr>
<td>Glucose iAUC0-120 (mmol/L × min)</td>
<td>169.4 ± 32.9</td>
<td>262.1 ± 25.5</td>
<td>0.02</td>
</tr>
<tr>
<td>GLP-1 iAUC-30-0 (pmol/L × min)</td>
<td>15.3 ± 5.7</td>
<td>14.5 ± 10.7</td>
<td>0.94</td>
</tr>
<tr>
<td>GLP-1 iAUC0-120 (pmol/L × min)</td>
<td>1513.9 ± 264.7</td>
<td>1076.5 ± 248.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Insulin iAUC-30-0 (mU/L × min)</td>
<td>4.5 ± 2.9</td>
<td>7.6 ± 5.7</td>
<td>0.64</td>
</tr>
<tr>
<td>Insulin iAUC0-120 (mU/L × min)</td>
<td>4692.4 ± 1036.6</td>
<td>4573.1 ± 1225.9</td>
<td>0.90</td>
</tr>
<tr>
<td>C-peptide iAUC-30-0 (pmol/L × min)</td>
<td>263.3 ± 119.0</td>
<td>157.5 ± 263.3</td>
<td>0.59</td>
</tr>
<tr>
<td>C-peptide iAUC0-120 (pmol/L × min)</td>
<td>132448.5 ± 22590.3</td>
<td>148032.0 ± 19380.1</td>
<td>0.41</td>
</tr>
<tr>
<td>Glucagon iAUC-30-0 (pg/mL × min)</td>
<td>69.3 ± 30.2</td>
<td>92.3 ± 69.3</td>
<td>0.73</td>
</tr>
<tr>
<td>Glucagon iAUC0-120 (pg/mL × min)</td>
<td>293.8 ± 109.4</td>
<td>654.0 ± 277.8</td>
<td>0.27</td>
</tr>
</tbody>
</table>

a Paired Student’s t-tests were used to determine statistical significance; data are means ± SEM.
Chapter 9.  Bile salts and glycaemia

A  **Blood glucose**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)

B  **Plasma GLP-1**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)

C  **Plasma insulin**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)

D  **Plasma C-peptide**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)

E  **C-peptide/glucose ratio**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)

F  **Plasma glucagon**

- 2g TCA/control
- 2g TCA/control+glucose (0.5g/min)
Figure 1. Effects of intrajejunal taurocholic acid (TCA) or control on blood glucose (A), plasma total glucagon-like peptide-1 (GLP-1) (B), plasma insulin (C), plasma C-peptide (D), C-peptide/glucose concentration ratio (E), and plasma glucagon (F), before (t = -30 to 0 min; 2 g TCA or control) and during (t = 0 to 120 min; 2 g TCA or control) intrajejunal glucose infusion at the rate of 0.5 g/min (i.e. 2 kcal/min) in healthy males (n = 10). Repeated-measures ANOVA was used to determine statistical significance. Post hoc comparisons were adjusted by Bonferroni’s correction. $P < 0.001$ for A and B, and $P = 0.008$ for E, treatment × time interaction; $* P < 0.05$, TCA vs. control. Data are means ± SEM.
CHAPTER 10. ARTIFICIAL SWEETENERS HAVE NO EFFECT ON GASTRIC EMPTYING OF, OR THE GLUCAGON-LIKE PEPTIDE-1 AND POSTPRANDIAL GLYCAEMIC RESPONSES TO, ORAL GLUCOSE IN HEALTHY HUMANS

Adapted from Wu T. et al. Diabetes Care. 2013 (In press).

10.1 Summary

Intestinal exposure to glucose stimulates the release of glucagon-like peptide-1 (GLP-1), slows subsequent gastric emptying, and reduces appetite. The presence of glucose is likely to be signaled, in part, by intestinal ‘sweet taste receptors’ (STRs). It is unclear whether stimulation of intestinal STRs by artificial sweeteners could augment these responses to glucose and thereby modulate postprandial glycaemia. The study described in this Chapter was to evaluate the acute effects of two artificial sweeteners, alone or in combination, on gastric emptying, GLP-1 and glycaemia in response to oral glucose in healthy humans. 10 healthy males were each studied on 4 occasions, on which they consumed 240 ml water alone, or sweetened with either 52 mg sucralose, 200 mg acesulfame potassium (AceK), or 46 mg sucralose + 26 mg AceK (equivalent sweetness), followed, after 10 min, by 75 g glucose with 150 mg $^{13}$C acetate in a 300 ml aqueous solution. Gastric emptying was evaluated by breath test, and blood glucose, plasma insulin and total GLP-1 were measured at frequent intervals. The rate of gastric emptying did not differ between any of the study
days. Blood glucose, plasma insulin and GLP-1 concentrations increased after oral glucose ($P < 0.001$ for each), but there was no difference in the response between the four days. In conclusion, acute administration of sucralose, AceK, or a combination of both, had no effect on gastric emptying or the blood glucose, insulin and GLP-1 responses to a subsequent glucose load in healthy humans.

**10.2 Introduction**

Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), the so-called ‘incretin’ hormones, are released from the intestine in response to nutrient exposure (Wu et al., 2010), and account for at least 50% of insulin secretion in healthy humans after oral glucose (Baggio and Drucker, 2007). In patients with type 2 diabetes, the insulinotropic effect of GIP is diminished, while that of GLP-1 remains relatively intact (Nauck et al., 1993). GLP-1 also slows gastric emptying (Schirra et al., 2006, Deane et al., 2010), and suppresses both glucagon secretion (Baggio and Drucker, 2007) and appetite (Flint et al., 1998). Accordingly, GLP-1, rather than GIP, has hitherto been the focus of incretin-based approaches to treat type 2 diabetes.

The mechanisms underlying nutrient detection in the small intestine and consequent stimulation of incretin release are poorly understood. Intestinal ‘sweet taste receptors’ (STRs), including T1R2, T1R3, and their cellular signaling partners $\alpha$-gustducin and TRPM5, are important molecules for sensing glucose (Jang et al., 2007, Young et al., 2009), and stimulation of STRs has been
linked to glucose-induced incretin release in both animals and humans (Gerspach et al., 2011, Kokrashvili et al., 2009, Steinert et al., 2011b). For example, α-gustducin knockout mice exhibit a defective GLP-1 response and impaired glucose tolerance after oral glucose administration (Jang et al., 2007, Kokrashvili et al., 2009). In humans, blockade of STRs by lactisole, a T1R2/T1R3 antagonist, decreases GLP-1 secretion and increases glycaemic excursions in response to either intragastric or intraduodenal administration of glucose (Gerspach et al., 2011, Steinert et al., 2011b). Artificial sweeteners, such as sucralose, also stimulate GLP-1 release from L-cells in vitro (Jang et al., 2007); however, in vivo studies from our group (Ma et al., 2009a) and others (Steinert et al., 2011a, Fujita et al., 2009) failed to demonstrate any GLP-1 or GIP release in both rodents and healthy humans after acute exposure to various non-caloric sweeteners. These observations suggest that the intestinal sweet taste system is necessary, but not sufficient, to induce GLP-1 secretion.

There are inconsistent observations relating to the potential for synergy between artificial sweeteners and glucose in stimulating GLP-1 secretion. It is important to clarify this issue, since beverages sweetened by artificial sweeteners are consumed in large volumes on a daily basis in the modern society. We have shown that sucralose, when given intraduodenally at a relatively high concentration, had no effect on the GLP-1 response to a subsequent glucose infusion in healthy humans (Ma et al., 2010). In contrast, a recent study by Brown et al in healthy and type 1 diabetic human subjects reported that oral
ingestion of ‘diet soda’, which contained both sucralose and acesulfame potassium (AceK), augmented GLP-1 release by more than one third after an oral glucose load given 10 minutes later, when compared to carbonated water, although blood glucose, insulin, GIP, and peptide YY concentrations were unaffected (Brown et al., 2012, Brown et al., 2009). Sucralose and AceK bind STRs in different ways, with the former binding preferentially to the N-terminal domain of T1R2 and the latter non-preferentially to T1R2 and T1R3 (Nie et al., 2005, Xu et al., 2004). The design of the latter study was, however, suboptimal, as the diet soda contained a number of substances (including caramel colour, gum acacia, natural flavors, citric acid, potassium benzoate, phosphoric acid and potassium citrate) that were not controlled for and may have potentially influenced on GLP-1 secretion.

The current study was designed to determine whether oral administration of sucralose and AceK in comparable doses to those employed by Brown et al (Brown et al., 2012, Brown et al., 2009), would augment the GLP-1 response to oral glucose and modulate gastric emptying and/or glycaemia in healthy humans.

**10.3 Research design and methods**

**10.3.1 Subjects**

10 healthy males (mean age: 33.6 ± 5.9 years; body mass index (BMI): 25.5 ± 1.0 kg/m²) were studied, after they provided written, informed consent. Females were excluded, since the secretion of some gut hormones, including GLP-1, and
gastric emptying, are known to be affected by the phase of the menstrual cycle (Brennan et al., 2009). Subjects taking any medication known to influence gastrointestinal function, and those who consumed > 20 g alcohol on a daily basis, or were smokers, were excluded. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

10.3.2 Protocols

Each subject was studied on four occasions, separated by at least 3 days, in single-blinded, randomized fashion. On the evening preceding each study day (~1900), subjects ate a standardized beef lasagne meal (McCain Foods Proprietary Ltd, Wendouree, Victoria, Australia), consumed with bread, a non-alcoholic beverage and one piece of fruit, and were instructed to then fast until the following morning. Subjects attended the laboratory at approximately 0830 h and were seated comfortably for the duration of the study. An intravenous cannula was inserted into a forearm vein for repeated blood sampling. Between t = -11 to -10 min, subjects consumed either 240 ml water (control), or 240 ml water equivalently sweetened with either (i) 52 mg sucralose, (ii) 200 mg AceK, or (iii) 46 mg sucralose + 26 mg AceK (Steinert et al., 2011a). Ten min later (t = 0 to 2 min), each drank 75 g glucose made up to 300 ml with water, and containing 150 mg $^{13}$C-acetate. Breath samples were collected immediately before, and every 5 min after, oral glucose ingestion during the first hour and
then every 15 min for a further 3 hours. Venous blood samples were taken immediately before administration of the water or artificial sweetener drinks (t = -11 min), and at t = 0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min, and were placed in ice-chilled-EDTA tubes on ice before centrifugation at 3200rpm for 15 min at 4 °C. Plasma was separated and stored at −70 °C for subsequent analysis.

10.3.3 Measurements

$^{13}$CO$_2$ concentrations in breath samples were measured by an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas chromatographic purification system. The half-emptying time (T50) was calculated using the formula described by Ghoos et al (Ghoos et al., 1993). This method has been validated against scintigraphy for the measurement of gastric emptying (Chew et al., 2003).

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). Plasma insulin was measured by ELISA (10-1113, Mercodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L and the coefficient of variation (CV) was 2.7% within assays and 7.8% between assays. Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK; Linco Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and intra- and inter-assay CVs were 6.8% and 8.5% respectively.
10.3.4 Statistical analysis

Areas under the curves (AUC) were calculated for blood glucose and plasma insulin and GLP-1, and these, together with the rate of gastric emptying (T50), were compared between study days using one-factor repeated measures ANOVA, corrected for multiple comparisons by Holm-Bonferroni’s correction. These variables were also assessed using repeated measures ANOVA, with treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Holm-Bonferroni’s correction, were performed if ANOVAs revealed significant effects. All analyses were performed using SPSS (version 19, IBM Corporation, Armonk, NY, USA). Ten subjects were calculated to have 80% power (at $\alpha = 0.05$) to detect at least 30% difference in GLP-1 secretion, when compared to water (Brown et al., 2012, Brown et al., 2009). Data are presented as mean values ± standard error; $P < 0.05$ was considered statistically significant.

10.4 Results

All subjects tolerated the study well and there were no adverse effects.

10.4.1 Blood glucose, plasma insulin and total GLP-1

Fasting blood glucose, plasma insulin and total GLP-1 concentrations did not differ between the four study days. After water and sweetened drinks, there were no changes in blood glucose, plasma insulin or total GLP-1 concentrations prior to oral glucose ingestion. After oral glucose, there were increases in blood
glucose, plasma insulin, and total GLP-1 concentrations ($P < 0.001$ for each), without any difference between the four days (Figure 1A, 1B and 1C, and Table 1).

### 10.4.2 Gastric emptying

There was a rapid rise in the $^{13}\text{CO}_2:^{12}\text{CO}_2$ ratio during the first 30 min after oral glucose, followed by a plateau for about 60 min and a slow decline, without any difference between the four days (Figure 2). There was no difference in the T50 between the four study days (121.1 ± 8.0 min for sucralose; 123.7 ± 5.4 min for AceK; 123.7 ± 5.1 min for sucralose + AceK vs. 122.2 ± 7.0 min for water).

### 10.5 Discussion

We demonstrated that acute activation of STRs by sucralose and AceK, either alone or in combination, at doses typically used in commercial beverages, has no effect on blood glucose, plasma insulin or GLP-1 concentrations before or after a subsequent oral glucose load in healthy humans, nor do these sweeteners delay gastric emptying of a glucose solution. These observations apparently contrast with those of Brown et al that diet soda (containing 46 mg sucralose + 26 mg AceK) increased plasma GLP-1 in response to a subsequent oral glucose load in healthy humans and patients with type 1 diabetes, when compared with carbonated water (Brown et al., 2012, Brown et al., 2009). The doses of sucralose and AceK, and the load and timing of the subsequent glucose drink were identical in our study, but it is unclear whether other components of diet
soda that were not controlled for by Brown et al may have had the capacity to stimulate GLP-1 secretion.

Our findings are, however, consistent with previous reports that artificial sweeteners, including sucralose and AceK, when given alone by oral (Ford et al., 2011, Wu et al., 2013d, Wu et al., 2012), intragastric (Steinert et al., 2011a, Ma et al., 2009a), or intraduodenal (Ma et al., 2010) routes, has no effect on GLP-1 secretion, insulin or blood glucose concentrations in health or type 2 diabetes, and are in keeping with our observations that sucralose had no effect on GLP-1 secretion or the glycaemic response to intraduodenal glucose (Ma et al., 2010). That the STR antagonist, lactisole, attenuates glucose-stimulated GLP-1 secretion (Gerspach et al., 2011, Steinert et al., 2011b), suggests that activation of STRs is necessary, but not sufficient, to stimulate L-cell secretion.

There might be a potential interaction between intestinal STRs and sodium glucose co-transporter-1 (SGLT1) in determining GLP-1 secretion. Sweet tastants that are also substrates of SGLT1 are potent stimuli for GLP-1 secretion, regardless of whether they are metabolized (Wu et al., 2012, Moriya et al., 2009). For example, 3-O-methylglucose, a non-metabolized SGLT1 substrate, stimulates GLP-1 secretion in rodents and humans (Wu et al., 2012, Moriya et al., 2009), while SGLT1 blockade with phoridzin inhibits the release of GLP-1 in response to monosaccharides in animal models (Sugiyama et al., 1994, Moriya et al., 2009). It has also been reported that levels of intestinal SGLT1 are
increased after 3 or more hours exposure to a broad array of sweet tastants, including artificial sweeteners such as sucralose, AceK, and saccharin in several animal species (Stearns et al., 2010, Moran et al., 2010). Conversely, STR knockout mice failed to show upregulation of SGLT1 after 2 weeks of dietary supplementation with glucose or sucralose, in contrast to wild type mice (Margolskee et al., 2007). However, the duration of STR activation in advance of oral glucose in the current study was probably insufficient to affect intestinal levels of SGLT1 (Stearns et al., 2010, Ma et al., 2010), and further studies are warranted to investigate the metabolic impacts of prolonged exposure to artificial sweeteners, in particular small intestinal absorption of glucose and secretion of incretin hormones.

The number of subjects studied, though relatively small, should have provided sufficient power to detect meaningful differences in GLP-1 secretion after oral glucose, so it is unlikely that increasing the sample size would result in a substantially different outcome.

In conclusion, sucralose and AceK, either alone or in combination, have no acute effect on blood glucose, GLP-1, or gastric emptying in response to oral glucose in healthy humans. These observations suggest that there is no synergy between artificial sweeteners and glucose in GLP-1 stimulation.
Table 1. Area under the curve (AUC) before (t = -10 to 0 min) and after (t = 0 to 240 min) 75 g oral glucose for blood glucose, plasma insulin and glucagon-like peptide 1 (GLP-1) after ingestion of either water, sucralose, acesulfame potassium (AceK), or sucralose + AceK in healthy humans (n = 10) *

<table>
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<tr>
<th></th>
<th>Water</th>
<th>Sucralose</th>
<th>AceK</th>
<th>Sucralose + AceK</th>
<th>P values</th>
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<tr>
<td>Blood glucose AUC-10-0min (mmol/L × min)</td>
<td>53.0 ± 1.7</td>
<td>54.9 ± 1.6</td>
<td>54.2 ± 1.8</td>
<td>54.8 ± 1.2</td>
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<tr>
<td>Blood glucose AUC0-240min (mmol/L × min)</td>
<td>1437.9 ± 78.6</td>
<td>1446.8 ± 56.4</td>
<td>1453.4 ± 56.8</td>
<td>1431.0 ± 63.4</td>
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<td>Plasma insulin AUC-10-0min (mU/L × min)</td>
<td>57.9 ± 7.9</td>
<td>72.5 ± 6.3</td>
<td>76.0 ± 11.6</td>
<td>77.5 ± 7.8</td>
<td>0.199</td>
</tr>
<tr>
<td>Plasma insulin AUC0-240min (mU/L × min)</td>
<td>7089.7 ± 1010.7</td>
<td>7976.6 ± 1451.2</td>
<td>7464.0 ± 1440.6</td>
<td>7143.5 ± 762.3</td>
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<td>Plasma GLP-1 AUC-10-0min (pmol/L × min)</td>
<td>224.5 ± 21.4</td>
<td>255.9 ± 40.9</td>
<td>237.5 ± 24.3</td>
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<tr>
<td>Plasma GLP-1 AUC0-240min (pmol/L × min)</td>
<td>5327.6 ± 418.9</td>
<td>5365.9 ± 543.2</td>
<td>5337.5 ± 446.2</td>
<td>5684.9 ± 826.4</td>
<td>0.769</td>
</tr>
</tbody>
</table>

* Values are means ± SEM. One-factor repeated measures ANOVA was used to determine the statistical significance.
Chapter 10. Gut responses to artificial sweeteners

Figure 1. Effects of ingestion of either water or artificial sweeteners (sucralose, acesulfame potassium (AceK), or sucralose + AceK) (at t = -10 min) on blood glucose (A), plasma insulin (B) and glucagon-like peptide 1 (GLP-1) (C) in response to 75 g oral glucose (at t = 0 min) in healthy humans (n = 10). Repeated-measures ANOVA was used to determine the statistical significance, with treatment and time as factors. (A) P = 0.947 for treatment effect, P < 0.001 for time effect, and P = 0.997 for treatment × time interaction; (B) P = 0.695 for treatment effect, P < 0.001 for time effect, and P = 0.810 for treatment × time interaction; (C) P = 0.923 for treatment effect, P < 0.001 for time effect, and P = 0.979 for treatment × time interaction. Data are means ± SEM.
Figure 2. Effects of ingestion of either water or artificial sweeteners (sucralose, acesulfame potassium (AceK), or sucralose + AceK) (at t = -10 min) on $^{13}\text{CO}_2$:$^{12}\text{CO}_2$ ratio in response to 75 g oral glucose (at t = 0 min) in healthy humans (n = 10). Repeated-measures ANOVA was used to determine the statistical significance, with treatment and time as factors. $P = 0.969$ for treatment effect, $P < 0.001$ for time effect, and $P = 0.788$ for treatment × time interaction. Data are means ± SEM.
CHAPTER 11. DISORDERED CONTROL OF INTESTINAL SWEET TASTE RECEPTOR EXPRESSION AND GLUCOSE ABSORPTION IN TYPE 2 DIABETES


11.1 Summary

We previously established that the intestinal sweet taste receptors (STRs), T1R2 and T1R3, were expressed in distinct epithelial cells in the human proximal intestine, and that their transcript levels varied with glycaemic status in patients with type 2 diabetes. Here we determined whether STR expression was (i) acutely regulated by changes in luminal and systemic glucose levels, (ii) disordered in type 2 diabetes, and (iii) linked to glucose absorption. Fourteen healthy subjects and 13 patients with type 2 diabetes were studied twice, at euglycaemia (5.2 ± 0.2 mmol/L) or hyperglycaemia (12.3 ± 0.2 mmol/L). Endoscopic biopsies were collected from the duodenum at baseline and after a 30 min intraduodenal glucose infusion (30 g/150 ml water plus 3 g 3-O-methylglucose, 3-OMG). STR transcripts were quantified by RT-PCR and plasma assayed for 3-OMG concentration. Intestinal STR transcript levels at baseline were unaffected by acute variations in glycaemia in healthy subjects and type 2 patients. T1R2 transcript levels increased after luminal glucose infusion in both groups during euglycaemia (+5.8 × 10^4 and +5.8 × 10^4 copies, respectively), but decreased in healthy subjects during hyperglycaemia (-1.4 ×
10^4 copies). T1R2 levels increased significantly in type 2 patients under the same conditions \(+6.9 \times 10^5\) copies. Plasma 3-OMG concentrations were significantly higher in type 2 patients than healthy controls during acute hyperglycaemia. Intestinal T1R2 expression is reciprocally regulated by luminal glucose in health according to glycaemic status, but is disordered in type 2 diabetes during acute hyperglycaemia. This defect may enhance glucose absorption in type 2 patients and exacerbate postprandial hyperglycaemia.

### 11.2 Introduction

Glucose in the small intestinal lumen induces feedback that regulates gastric emptying, absorptive function and energy intake (Raybould and Holzer, 1992, Horowitz et al., 1993, Pilichiewicz et al., 2007a), mediated both by vagal nerve pathways and secretion of gut peptides (Young, 2011), including glucose-dependent insulino tropic polypeptide (GIP) from enteroendocrine K-cells, and glucagon-like peptide 1 (GLP-1) from L-cells. These ‘incretins’ substantially augment insulin secretion when glucose is given orally, when compared to an isoglycaemic intravenous infusion (Holst and Gromada, 2004). Both the rate of gastric emptying and the secretion and action of the incretin hormones are key determinants of postprandial glycaemia. However, the precise mechanism of glucose detection in the small intestine remains unclear.

Lingual sweet taste cells possess two G-protein coupled receptors, T1R2 and T1R3, which form a heterodimeric sweet taste receptor (STR) for sugars, D-
amino acids, sweet proteins and artificial sweeteners (Nelson et al., 2001, Li et al., 2002). T1R2/R3 activation liberates the alpha-subunit of the G-protein gustducin (α-gustducin), leading to intracellular Ca\(^{2+}\) release, gating of a taste-specific transient receptor potential ion channel TRPM5 (Perez et al., 2002) cellular depolarisation, and release of mediators that activate lingual afferent nerves.

We, and others, have shown that STRs, α-gustducin and TRPM5 are also expressed with cellular and regional specificity in the animal and human intestine where they may serve as glucose sensors (Hofer et al., 1996, Dyer et al., 2005, Bezencon et al., 2007, Sutherland et al., 2007, Young et al., 2009, Young, 2011). In addition to expression in intestinal sweet taste cells, some of these taste components are also expressed in separate intestinal cell populations that detect umami (T1R3, α-gustducin, TRPM5), bitter and fats (α-gustducin, TRPM5) (Young, 2011). STR activation may be linked to gut hormone secretion, since mice deficient in T1R3 or α-gustducin exhibit defective glucose-induced GLP-1 release (Jang et al., 2007) while the STR blocker, lactisole, decreases GLP-1 secretion and increases glycaemic excursions after intragastric or intraduodenal glucose infusion in humans (Steinert et al., 2011b, Gerspach et al., 2011). Animal studies also indicate that STR activation increases the availability and function of the primary intestinal glucose transporter, sodium-glucose cotransporter-1 (SGLT-1) (Margolskee et al., 2007, Stearns et al., 2010), although this link has not been assessed directly in humans.
Patients with type 2 diabetes frequently demonstrate disordered gastrointestinal responses to nutrients, with delayed gastric emptying in up to 30-50%, and abnormally rapid emptying in a few (Horowitz et al., 2002) and a high prevalence of gastrointestinal symptoms (Bytzer et al., 2001). GLP-1 and GIP secretion has been inconsistently reported to be diminished in type 2 patients (Toft-Nielsen et al., 2001, Vilsboll et al., 2001), while intestinal levels of SGLT-1, and the capacity for glucose absorption, may be increased (Dyer et al., 2002). Any of these abnormalities could potentially relate to disordered intestinal sensing of glucose. We previously reported that duodenal expression of STRs during fasting was comparable in unselected patients with type 2 diabetes and non-diabetic controls, but was inversely related to the blood glucose concentration at the time of biopsy in type 2 patients (Young et al., 2009). In rodents, we and others have also shown that intestinal STR transcript and protein levels are rapidly downregulated upon acute luminal exposure to glucose or artificial sweeteners (Mace et al., 2007, Young et al., 2009). Our current aims were, therefore, to evaluate the modulation of duodenal STR expression in response to acute changes in luminal and systemic glucose exposure in healthy humans, and to determine whether STR regulation is disordered in type 2 diabetes, and related to changes in glucose absorption and/or gut hormone secretion.
11.3 Methods

11.3.1 Subjects

Fourteen healthy subjects and 13 patients with type 2 diabetes were studied in randomised, cross-over fashion. The mean duration of known diabetes in the latter group was 5 ± 1 y, glycated haemoglobin (HbA1c) was 6.3 ± 0.2% (45 ± 2 mmol/mol) and all were free of significant comorbidities and managed by diet alone. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and conducted in accordance with the Declaration of Helsinki as revised in 2000. Each subject provided written informed consent.

11.3.2 Screening visit

Each subject attended the laboratory at 0830 h after an overnight fast (12 h for solids, 10 h for liquids). An intravenous cannula was inserted for blood sampling, and subjects consumed a glucose drink (75 g glucose dissolved in water to 300 mL, labelled with 150 mg $^{13}$C acetate) within 5 min ($T = -5$ to 0 min). Blood was sampled at $T = -5, 30, 60, 120, 180$ min to measure blood glucose by glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). Breath samples were collected before, and every 5 min after, oral glucose during the first hour, and every 15 min for a further 2 h to measure $^{13}$CO$_2$ concentrations by isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK). The gastric half-emptying time was calculated using the formula of Ghoos et al (Ghoos et al., 1993). Gastrointestinal symptoms were
assessed by a standard questionnaire (maximum score 27), as previously (Horowitz et al., 1991). Autonomic nerve function was assessed in the type 2 patients using standardised cardiovascular reflex tests, with a score $\geq 3$ (out of a maximum of 6) indicating autonomic dysfunction (Ewing and Clarke, 1982).

### 11.3.3 Endoscopy Protocol

After the screening visit each subject was studied twice, separated by at least a week, with female subjects studied exclusively during the follicular phrase of the menstrual cycle to limit variations in gut hormone concentrations (Brennan et al., 2009). Subjects attended the laboratory at 0830 h following an overnight fast, and an insulin/glucose clamp was established to achieve euglycaemia (~5 mmol/L) or hyperglycaemia (~12 mmol/L) (Rayner et al., 2000c). A 50 mL iv bolus of 25% glucose (Baxter Healthcare, Old Toongabbie, NSW, Australia) was administered on the hyperglycaemic day, and 0.9% saline (Baxter Healthcare) on the euglycaemic day, over 1 min each, followed by continuous infusion of the same solution starting at 150 mL/h and adjusted according to blood glucose measurements every 5 minutes on the hyperglycaemic day, or remaining at 150 mL/h on the euglycaemic day. On the euglycaemic day, 25% dextrose was infused iv if the blood glucose concentration fell below 5 mmol/L. In addition, 100 IU of insulin (Actrapid; Novo Nordisk, Baulkham Hills, NSW, Australia), in 500 mL 4% succinylated gelatin solution (Gelofusine; B. Braun Australia, Bella Vista, NSW, Australia), was infused iv at a variable rate to maintain euglycaemia. Once blood glucose concentrations were stable for 30
min (12.3 ± 0.2 mmol/L on the hyperglycaemic day, 5.2 ± 0.2 mmol/L on the
euglycaemic day) a small diameter video endoscope (GIF-XP160, Olympus,
Tokyo, Japan) was passed via an anaesthetised nostril into the second part of the
duodenum, from which mucosal biopsies were collected using standard biopsy
forceps, and placed into either RNAlater (Qiagen, Sydney, NSW, Australia) or 4%
paraformaldehyde for 2 h. At T = 0 an intraduodenal infusion containing 30 g
glucose and 3 g of the glucose absorption marker 3-O-methyglucose (3-OMG,
Sigma-Aldrich, St Louis, MO, USA) was commenced via the biopsy channel of
the endoscope, and continued for 30 min (1 g/min; 4 kcal/min). At T = 10 and
T = 30 min additional biopsies to be collected. Blood samples (20 mL) were
taken every 10 min over 1 h to determine concentrations of 3-OMG, C-peptide,
GLP-1 and GIP.

11.3.4 Assays

Plasma total GLP-1 concentrations were measured by radioimmunoassay
(GLPIT-36HK; Millipore, Billerica, MA) with sensitivity of 3 pmol/L and intra-
and inter-assay coefficients of variation (CV) of 4.2% and 10.5%. Total plasma
GIP was measured by RIA as previously, with sensitivity of 2 pmol/L and intra-
and inter-assay CV of 6.1% and 15.4%, respectively. Plasma C-peptide
concentrations were measured by ELISA (10-1136-01, Mercodia, Uppsala,
Sweden), with sensitivity of 15 pmol/L, and intra- and inter-assay CV of 3.6%
and 3.3%. Serum 3-OMG concentrations were measured by liquid
chromatography and mass spectrometry with sensitivity of 10 pmol/L (Deane et al., 2011).

### 11.3.5 Quantification of gene expression by real time RT-PCR

RNA was extracted from tissues using an RNeasy Mini kit (Qiagen) following manufacturer instructions, and RNA yield and quality determined using a NanoDrop (NanoDrop Technologies, Wilmington, DE). Quantitative real time reverse transcriptase PCR (RT-PCR) was then used to determine the absolute expression of sweet taste molecules. Validated human primers for T1R2, α-gustducin and TRPM5 were used as primer assays (QuantiTect, Qiagen), while T1R3 primers were designed using Primer 3.0 software (Applied Biosystems, Foster City, CA) based on target sequences obtained from the NCBI nucleotide database (Table 1). Absolute standard curves were generated by including known copy number standards in RT-PCR for each target (Table 2), as described (Young et al., 2009). RT-PCR was performed on a Chromo4 (MJ Research, Waltham, MA, USA) real time instrument attached to a PTC-200 Peltierthermal cycler (MJ Research) using a QuantiTect SYBR Green one-step RT-PCR kit (Qiagen) according to the manufacturer’s specifications, as previously (Young et al., 2009). Each assay was performed in triplicate and included internal no-template and no-RT controls. All replicates were averaged for final mRNA copy number, which was expressed as copies per 50 ng of total RNA.
11.3.6 Immunohistochemistry

Fixed tissues were cryoprotected (30% sucrose in phosphate-buffered saline), embedded in cryomolds, and frozen, before sectioning at 6-10 μm (Cryocut 1800, Leica Biosystems, Nussloch, Germany) and thaw-mounting onto gelatin-coated slides. Immunoreactivity was detected using rabbit T1R2 primary (H90, 1:400, SC-50305, Santa Cruz Biotechnology, CA, USA), goat GLP-1 primary (1:400, SC-7782, Santa Cruz), and monoclonal 5-HT (1:1000, M0758, Dako Australia, Victoria, Australia) and GIP primary antibodies (1:800, AB30679, Abcam). All were visualised using species-specific secondary antibodies conjugated to Alexa Fluor dyes (1:200 in PBST) as previously described (Sutherland et al., 2007, Young et al., 2009). Antigen retrieval (S1700, Dako) was performed for T1R2 according to manufacturer instructions. Nucleated epithelial cells immunopositive for individual targets were counted per square millimetre of high power field and averaged over at least 10 intact transverse sections per subject.

11.3.7 Data analysis

The incremental area under the curve (iAUC) for 3-OMG, GLP-1 and GIP concentrations was calculated using the trapezoidal rule (Wolever, 2004) and analysed by one-factor ANOVA using Prism software (version 6.0; Graphpad, La Jolla, CA, USA). These variables were also assessed using repeated-measures ANOVA, with treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Holm-Sidak’s correction, were performed.
if ANOVAs showed significant effects. One-way ANOVA, with Holm-Sidak’s post hoc test, was used to compare differences in duodenal levels of STR transcripts between healthy subjects and type 2 diabetic patients. Relationships between transcript expression and other factors were evaluated by Pearson correlation coefficient (r). Twelve subjects were calculated to have 80% power to detect a one third difference in duodenal T1R2 expression in paired studies (α = 0.05), compared to control (Young et al., 2009). P values ≤ 0.05 were considered statistically significant. Data are expressed as mean ± SEM.

11.4 Results
All subjects tolerated the study well. The patients with type 2 diabetes were older than the healthy subjects, but gastrointestinal symptom scores, BMI and gastric emptying of glucose did not differ (Table 3). Five type 2 patients had autonomic dysfunction, but none had evidence of peripheral neuropathy, nephropathy, retinopathy, or macro-vascular complications. As expected, blood glucose concentrations were higher in type 2 patients during fasting and after oral glucose (P < 0.05, Figure 1A).

11.4.1 Baseline STR expression
Transcripts for T1R2, T1R3, α-gustducin and TRPM5 were readily detected in duodenal biopsies by quantitative RT-PCR. TRPM5 was the most abundant STR transcript in all subjects, with lower levels of α-gustducin and much lower levels of T1R2 and T1R3; T1R2 was the least expressed transcript (Figure 2A).
TRPM5 transcript levels in healthy subjects during euglycaemia were $34 \pm 8$ fold higher than those of T1R2 ($P < 0.001$), while $\alpha$-gustducin levels were $22 \pm 7$ fold higher ($P < 0.05$) and T1R3 levels $12 \pm 5$ fold higher.

### 11.4.2 Effects of acute changes in glycaemia on STR expression

Fasting expression of STR transcripts was unaffected by glycaemic state in either health or type 2 diabetes, and did not differ between the groups (Figure 2B-E).

### 11.4.3 Effects of luminal glucose on duodenal STR expression

Due to inter-subject variability in STR expression, responses to luminal glucose were evaluated as changes from baseline. During euglycaemia, T1R2 transcript levels increased in response to duodenal glucose infusion in health and type 2 diabetes after 30 min ($+5.9 \times 10^4$ and $+5.8 \times 10^4$ copies, Figure 3A). During hyperglycaemia, T1R2 transcript levels decreased in healthy subjects after 30 min ($-1.4 \times 10^4$ copies), but increased in type 2 patients ($+6.9 \times 10^5$ copies), so that levels in health were lower at 30 min during hyperglycaemia than euglycaemia and lower than in type 2 patients during either glycaemic state (subject $\times$ time interactions; $P < 0.01$ for each). Levels of T1R3, $\alpha$-gustducin and TRPM5 transcript, in contrast, did not significantly change in response to luminal glucose under either glycaemic condition (Figure 3B-D).
11.4.4 Plasma hormone concentrations

Fasting plasma GLP-1 concentrations did not differ between health and type 2 diabetes, and were not acutely affected by the glycaemic state. Plasma GLP-1 increased in response to duodenal glucose infusion in all groups (Figure 1C; $P < 0.001$), with higher concentrations evident in type 2 patients at 40 min irrespective of glycaemic status (subject $\times$ time interactions; $P < 0.01$), and at 50 min during euglycaemia, compared to healthy subjects (subject $\times$ time interactions; $P < 0.05$). The iAUC for GLP-1 was higher in type 2 patients during euglycaemia and hyperglycaemia, when compared to healthy subjects (Table 4; $P < 0.05$, each).

Fasting plasma GIP concentrations did not differ between healthy subjects and type 2 patients, and were not acutely affected by the glycaemic state. Plasma GIP increased in response to duodenal glucose infusion in both groups (Figure 1D; $P < 0.001$), with higher GIP concentrations evident in type 2 diabetes patients at 40 min irrespective of glycaemic status, and higher concentrations during euglycaemia at 20 and 50 min, compared to healthy subjects (subject $\times$ time interaction; $P < 0.05$). The iAUC was higher in type 2 patients during euglycaemia in comparison to healthy subjects (Table 4; $P < 0.05$).

Fasting C-peptide concentrations were higher during hyperglycaemia than during euglycaemia in healthy subjects ($P < 0.001$, Figure 1E), but not in type 2 patients. C-peptide concentrations increased in response to duodenal glucose
infusion in both subject groups during hyperglycaemia (subject × time interaction; \( P < 0.05 \) and iAUC \( P < 0.001 \)), but not during euglycaemia (Figure 1E, Table 4). C-peptide concentrations during hyperglycaemia were higher in healthy subjects than in type 2 patients throughout the glucose infusion (subject × time interaction; \( P < 0.05 \) and iAUC \( P < 0.001 \)).

### 11.4.5 Serum 3-OMG concentrations

Serum 3-OMG concentrations increased over time in all groups, but were higher at 60 min in type 2 patients during hyperglycaemia than in any other group (subject × time interaction; \( P < 0.001 \), Figure 1F). The iAUC for 3-OMG was higher in both type 2 patients and healthy subjects during hyperglycaemia than euglycaemia (Table 4; \( P < 0.05 \)).

### 11.4.6 Phenotype of human intestinal sweet taste cells

Immunolabelling for T1R2 was evident in single cells dispersed throughout the mucosal epithelium in healthy subjects and type 2 patients (Figure 4). Immunopositive cells showed a homogenous distribution of label throughout the cytoplasm, were largely open or ‘flask’ shaped and found with equal frequency within villi or crypts. In dual labelling experiments in healthy subjects, 19 ± 11\% of T1R2 labelled duodenal cells co-expressed GLP-1, while 13 ± 8\% of L- cells co-expressed T1R2 (Figure 4A). In a similar manner, 15 ± 10\% of T1R2 labelled duodenal cells co-expressed GIP, while 12 ± 8\% of K-cells co-expressed T1R2 (Figure 4B). Separate populations of T1R2 labelled cells co-
expressed 5-HT (31 ± 6%), while 5 ± 1% of EC-cells co-expressed T1R2 in healthy subjects (Figure 4C). During fasting, an equivalent number of T1R2 immunopositive cells were evident in healthy subjects and type 2 patients, under euglycaemia or hyperglycaemia, and the number did not change during duodenal glucose infusion. Similarly, the proportion of cells immunopositive for GLP-1, GIP and 5-HT did not differ between healthy subjects and type 2 patients, or with glycaemic state or exposure to luminal glucose, although a trend for increased L-cells in fasting type 2 patients was evident (P = 0.07, data not shown).

11.4.7 Relationships between variables

Absolute copy numbers of STR transcripts during fasting, and after 30 min glucose infusion, did not correlate with age, gender, BMI, symptom score or gastric half-emptying time in either group, and in type 2 patients, they were not related to duration of diabetes, HbA1c, autonomic dysfunction, or symptom score. In contrast, the change in T1R2 transcript level after luminal glucose exposure correlated with the iAUC for 3-OMG in healthy subjects during euglycaemia (r = 0.73, P < 0.05), and the change in TRPM5 transcript level with plasma GLP-1 concentrations at 30 min (r = 0.62, P < 0.05) in the same group. Changes in T1R2 (r = 0.78, P < 0.01) and T1R3 transcript levels (r = 0.59, P < 0.05) in type 2 patients during hyperglycaemia also correlated with plasma GIP concentrations at 30 min, and the change in T1R2 correlated with the iAUC for GIP (r = 0.69, P = 0.03).
11.5 Discussion

This study is the first to define changes in expression of intestinal STR transcripts in healthy humans and patients with type 2 diabetes, in response to acute changes in systemic and luminal glucose. We have shown that absolute levels of STR transcripts are unaffected by acute variations in glycaemia during fasting in either group, but that T1R2 expression increases upon exposure to luminal glucose during euglycaemia. In contrast, T1R2 expression decreases markedly in response to luminal glucose during hyperglycaemia in health, but in type 2 diabetes increases under the same conditions. Type 2 patients also exhibit increased glucose absorption during acute hyperglycaemia compared to healthy subjects, suggesting that dysregulated expression of intestinal STRs can perpetuate postprandial hyperglycaemia in this group.

We confirmed our previous observation that fasting STR transcript levels are similar in health and type 2 diabetes irrespective of age, gender or BMI (Young et al., 2009). While we previously observed that levels of STR transcript were inversely related to fasting blood glucose concentrations in unselected type 2 patients presenting for endoscopy, we have now established unequivocally that acute changes in glycaemia do not influence fasting intestinal STR expression in either health or ‘well-controlled’ type 2 diabetes. The apparent discrepancy in these observations may reflect the effects of more longstanding hyperglycaemia or differences in the duration of fasting in the earlier cross-sectional study. We
have now shown that the intestinal STR system is, in contrast, highly responsive to the presence of luminal glucose, with rapid, and reciprocal, regulation of T1R2 transcripts in health, depending on the prevailing blood glucose concentration. Comparable changes were evident in T1R3 and TRPM5 transcript levels, although these were not statistically significant. Increased inter-subject variability seen for T1R3 and TRPM5 transcript levels may be due to their expression in additional populations of intestinal cells tuned to detect other taste modalities and, therefore, unresponsive to luminal and/or systemic glucose.

Healthy subjects that displayed the largest glucose-induced increase in duodenal T1R2 transcript levels during euglycaemia had the highest plasma concentrations of the glucose absorption marker 3-OMG. As SGLT-1 is responsible for the active transport of luminal 3-OMG, our findings support a role of intestinal T1R2 signals in the regulation of glucose absorption via SGLT-1. Indeed, intestinal STR activation has been shown to upregulate SGLT-1 transcript, apical protein and function in a number of species (Margolskee et al., 2007, Stearns et al., 2010). Accordingly, reciprocal regulation of T1R2 in human health may increase SGLT-1 function at euglycaemia to facilitate glucose absorption, and reduce SGLT-1 function during hyperglycaemia to limit postprandial glycaemic excursion. However, despite a reduction in T1R2 transcript after luminal glucose exposure during hyperglycaemia, our healthy subjects still displayed greater rates of glucose absorption than during
euglycaemia, which might be accounted for by changes in SGLT-1 lagging behind those in T1R2. Our finding that plasma 3-OMG concentrations were elevated in type 2 patients during hyperglycaemia is in keeping with the concept that SGLT-1 transporter capacity was maintained, or increased, in the presence of luminal glucose under these conditions. In fact, even small changes in SGLT-1 may increase this risk, as type 2 patients are reported to have up to 4-fold higher levels of transcript, protein and function of this transporter at baseline compared to healthy controls (Dyer et al., 2002). It should be noted that an increased level of facilitated glucose transport via the basolateral glucose transporter GLUT2 may contribute to plasma levels of 3-OMG in the current study, however the role of STR signals to direct the apical insertion of GLUT2 in enterocytes appears to be limited to rodents (Mace et al., 2007, Shirazi-Beechey et al., 2011a).

The link between STR stimulation and incretin hormone release in healthy humans is not clear. Most *in vivo* studies indicate that acute administration of non-nutritive sweeteners does not trigger incretin secretion in either humans or rodents (Ma et al., 2009a, Wu et al., 2012, Brown et al., 2009). Nonetheless, we observed that subsets of duodenal L-cells, K-cells and EC-cells were immunopositive for T1R2, in accord with previous reports (Jang et al., 2007, Sutherland et al., 2007, Young, 2011). Together with positive associations between luminal glucose-induced changes in some STR transcripts and measures of GLP-1 and GIP secretion in the current study, it remains possible
that STRs do have a regulatory role in gut hormone release. The inhibition of glucose-induced GLP-1 secretion in healthy humans by the STR blocker, lactisole (Steinert et al., 2011b), supports this concept. It must also be recognized that STR signals may serve autocrine and/or paracrine functions within the intestinal mucosa that are not reflected in circulating gut hormone concentrations; the latter appear to be a blunt marker for local concentrations of GLP-1 (D'Alessio et al., 2007). There is also a large body of evidence indicating that the intestinotrophic gut peptide, glucagon-like peptide 2 (GLP-2), co-released from L-cells with GLP-1, is a powerful local stimulus to increase intestinal glucose transport via SGLT-1 and GLUT2 in rodents, and in patients with short bowel syndrome (Cheeseman, 1997, Au et al., 2002, Jeppesen et al., 2001). Importantly, GLP-2 release has recently been revealed as STR-dependent in animals and a human enteroendocrine cell line (Daly et al., 2012, Sato et al., 2013), highlighting an important link between STRs and GLP-2 in the regulation of intestinal glucose transport.

Reports concerning postprandial incretin hormone release in patients with type 2 diabetes have been inconsistent, with plasma GLP-1 concentrations after a mixed meal being either reduced (Toft-Nielsen et al., 2001) or intact (Vollmer et al., 2008), although such studies are potentially confounded by failure to control for differences in the rate of gastric emptying, which is frequently delayed in longstanding diabetes or during acute hyperglycaemia (Horowitz et al., 2002). Our observation that GLP-1 and GIP responses to a standardised rate of
duodenal glucose infusion were maintained, and indeed increased, in type 2 patients, supports our previous findings (Ma et al., 2012), and is in keeping with the trend for increased L-cell density in these patients in the current study and a report of an increased density of L-cells, and mixed L/K-cells, in the duodenum of well-controlled type 2 patients (Theodorakis et al., 2006). There is now strong evidence that SGLT-1 transport is a key stimulus for release of GLP-1 and GIP, which occurs even after exposure to non-metabolised SGLT-1 substrates, and is inhibited by pharmacological blockade or genetic ablation of SGLT-1 in rodents (Parker et al., 2012, Gorboulev et al., 2012, Parker et al., 2010). Therefore, increased SGLT-1 capacity could explain enhanced glucose-induced GLP-1 and GIP responses in our type 2 patients. Any deficiency in the incretin effect in type 2 diabetes is likely to be explained by impaired ß-cell function, rather than deficient incretin hormone secretion (Woerle et al., 2012, Ma et al., 2012), and indeed, defective C-peptide responses in our type 2 patients during hyperglycaemia support this assertion. Acute hyperglycaemia had no effect on GLP-1 or GIP secretion, as noted previously (Vilsboll et al., 2003, Kuo et al., 2010). While SGLT-1 transport appears a major determinant of GLP-1 and GIP release, other transporters (Cani et al., 2007, Parker et al., 2012) or signalling pathways (Reimann et al., 2008) may also be involved, so increased glucose absorptive capacity during hyperglycaemia may not necessarily result in enhanced GLP-1 or GIP concentrations.
Our study had a number of limitations. While transcriptional regulation of intestinal T1R2 occurred rapidly in humans, we did not quantify changes in STR protein in parallel due to ethical considerations on the additional biopsies required. However, similarly rapid changes in these proteins following glucose or sucralose exposure are known to occur in apical membrane vesicles of rat jejunum (Mace et al., 2007). We have not assessed effects on SGLT-1 transcript or protein here, although measures of glucose absorption with 3-OMG reflect, in large part, SGLT-1 function as the primary intestinal glucose transporter in humans. There was considerable inter-individual variability in baseline expression of intestinal STR transcripts, so that our study was insufficiently powered to detect relationships between absolute transcript levels and concentrations of gut hormones and 3-OMG. Our 3-OMG measurements were limited to 60 min, and differences between groups or glycaemic states may have become more marked after this point. The duodenal glucose infusion was also relatively brief, being limited by the tolerability of unsedated endoscopy. Our type 2 patients had relatively good glycaemic control, and more marked differences from health might be observed in patients with a higher HbA1c. The type 2 patients were older than the healthy controls, although we have not previously shown any age-related differences in postprandial GLP-1 responses (Trahair et al., 2012b).

In conclusion, we have shown that the intestinal STR system is reciprocally regulated in the presence of luminal glucose according to glycaemic status in
health, but not in type 2 diabetes. In the latter, T1R2 dysregulation potentially increases the risk of postprandial hyperglycaemia, but the intestinal STR system appears unlikely to be a major determinant of circulating GLP-1 or GIP concentrations in humans.
### Table 1. Human primers used for absolute quantification of target genes in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Primer information</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R2 (TASR2)</td>
<td>NM_152232</td>
<td>QT01026508 Forward (5’ to 3’): CAAAACCCAGACGACATCG</td>
<td>94</td>
</tr>
<tr>
<td>T1R3 (TASR3)</td>
<td>NM_152228</td>
<td>Reverse (5’ to 3’): CATGCCAGGAACCGAGAC</td>
<td>101</td>
</tr>
<tr>
<td>Go$_{gust}$ (GNAT3)</td>
<td>XM_001129050</td>
<td>QT00049784</td>
<td>111</td>
</tr>
<tr>
<td>TRPM5</td>
<td>NM_014555</td>
<td>QT00034734</td>
<td>115</td>
</tr>
</tbody>
</table>

QT = QuantiTect primer assay (Qiagen)

### Table 2. Human primers used to generate RT-PCR product containing target amplicon to create absolute standard curves

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R2</td>
<td>TACCTGCCTGGGGATTAC</td>
<td>AAATAGGGAGAGGAAGTTGG</td>
<td>390</td>
</tr>
<tr>
<td>T1R3</td>
<td>AAGGGCTAAATCACCACCAGA</td>
<td>CCAGGTACCAGGTGCACAGT</td>
<td>953</td>
</tr>
<tr>
<td>Go$_{gust}$</td>
<td>GAGGACCAACGACAACTTTA</td>
<td>ACAATGGAGGTTGGTTGAAAA</td>
<td>491</td>
</tr>
<tr>
<td>TRPM5</td>
<td>CTTGCTGCCCTAGTGAAC</td>
<td>CTGCAGGAAGTCCCTTGAGTA</td>
<td>639</td>
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Table 3. Demographic, anthropometric, metabolic and gastrointestinal parameters of the study participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy</th>
<th>Type 2 Diabetic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>9M : 5F</td>
<td>4M : 9F</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>31 ± 3</td>
<td>66 ± 2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 ± 1</td>
<td>27 ± 1</td>
<td>Not significant</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td></td>
<td>6.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Duration of known diabetes (yr)</td>
<td></td>
<td>5.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose, at screening (mmol/L)</td>
<td>5.9 ± 0.2</td>
<td>7.4 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2h blood glucose after oral load (mmol/L)</td>
<td>6.3 ± 0.4</td>
<td>12.3 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gastrointestinal symptom score (maximum 27)</td>
<td>1.9 ± 0.6</td>
<td>1.2 ± 0.3</td>
<td>Not significant</td>
</tr>
<tr>
<td>Autonomic function score (maximum 6)</td>
<td>-</td>
<td>2.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Gastric half-emptying (min)</td>
<td>123 ± 8</td>
<td>130 ± 12</td>
<td>Not significant</td>
</tr>
</tbody>
</table>
Table 4. Incremental area under the curve (iAUC) for glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), C-peptide and 3-O-methyl glucose (3-OMG) in healthy subjects and type 2 patients.

<table>
<thead>
<tr>
<th>iAUC&lt;sub&gt;60&lt;/sub&gt; (pmol/L.min)</th>
<th>Healthy Subjects</th>
<th>Type 2 Patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Euglycaemia</td>
<td>Hyperglycaemia</td>
<td></td>
</tr>
<tr>
<td>GLP-1</td>
<td>1530 ± 152</td>
<td>1403 ± 122</td>
<td></td>
</tr>
<tr>
<td>GIP</td>
<td>1308 ± 126</td>
<td>1261 ± 160</td>
<td></td>
</tr>
<tr>
<td>C-Peptide</td>
<td>2028 ± 178</td>
<td>7796 ± 715&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>3-OMG</td>
<td>542 ± 45</td>
<td>747 ± 55&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Significantly different from A. healthy euglycaemia, B. healthy hyperglycaemia, C. type 2 diabetes euglycaemia. Data are mean ± SEM.
Figure 1. Effects of oral glucose or intraduodenal glucose infusion on blood glucose levels and plasma levels of hormones and the glucose absorption marker 3-OMG in healthy subjects and type 2 patients during euglycaemia or hyperglycaemia. (A) Blood glucose levels following a glucose drink in healthy control (HC) subjects and type 2 patients (T2D); *P < 0.05, #P < 0.01, P < 0.001, T2D compared to HC. (B) Blood glucose levels following intraduodenal (ID) glucose infusion during glycaemic clamp; P < 0.001, HC euglycaemic compared to hyperglycaemic groups, also, T2D euglycaemic compared to T2D hyperglycaemic; *P < 0.05, T2D euglycaemic compared to HC euglycaemic; **P < 0.05, T2D hyperglycaemic compared to HC hyperglycaemic. (C) Plasma GLP-1, *P < 0.05, T2D groups compared to HC euglycaemic; #P < 0.01, T2D groups compared to HC hyperglycaemic. (D) Plasma GIP, *P < 0.05, T2D groups compared to HC euglycaemic; **P < 0.05, T2D groups compared to HC hyperglycaemic; P < 0.05, T2D euglycaemic compared to other groups. (E) C-peptide, P < 0.001, HC hyperglycaemic compared to euglycaemic groups; *P < 0.05, T2D hyperglycaemic compared to other groups. (F) 3-OMG, P < 0.001, T2D hyperglycaemic compared to other groups. Data are mean ± SEM; significance represents treatment × time interactions. GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic polypeptide; 3-OMG, 3-O-methy glucose.
Chapter 11. Sweet taste sensing in the intestine

A

Healthy

B

Type 2 Diabetes

C

T1R2

D

T1R3

E

α-gustducin

F

TRPM5

RNA copy number: 50 µg total RNA

RNA copy number: 50 µg total RNA

RNA copy number: 50 µg total RNA

Euglycemia

Hyperglycemia

HC

T2D

HC

T2D

HC

T2D
Figure 2. Absolute transcript levels of STR in the duodenum of healthy subjects and type 2 patients at stable euglycaemia and hyperglycaemia. Absolute expression (copy number) of STR transcripts at baseline in the duodenum of healthy subjects (A) or patients with type 2 diabetes (B). (A) TRPM5 levels were 15-fold higher, α-gustducin 9-fold higher and T1R3 3-fold higher than T1R2 levels in healthy subjects. (B) TRPM5 levels were 29-fold higher, α-gustducin 11-fold higher and T1R3 5-fold higher than T1R2 levels in patients with type 2 diabetes. * P < 0.05, # P < 0.01 compared to T1R2. Duodenal levels of T1R2 (C), T1R3 (D), α-gustducin (E) and TRPM5 (F) transcript in healthy subjects and type 2 patients (T2D) at stable euglycaemia or hyperglycaemia. There were no significant differences in transcript levels detected at stable baseline. Data are mean ± SEM.
Figure 3. Effects of intraduodenal glucose infusion on sweet taste molecule transcript levels in healthy subjects and type 2 patients during euglycaemia or hyperglycaemia. (A) Change in absolute expression of T1R2 in human duodenum during ID glucose infusion under euglycaemic or hyperglycaemic clamp; #P < 0.01, HC hyperglycaemic compared to all other groups. Data are mean ± SEM. (B) T1R3, (C) α-gustducin, (D) TRPM5.
Figure 4. Subsets of L-cells, K-cells and EC-cells express STR in healthy human duodenum. (A) Immunolabelling for GLP-1 was present in 19 ± 11% of T1R2 labelled duodenal cells in HC subjects at euglycaemia, while 13 ± 8% of L-cells co-expressed T1R2. (B) GIP was present in 15 ± 10% of T1R2 labelled cells in HC subjects at euglycaemia, while 12 ± 8% of K-cells co-expressed T1R2. (C) In similar manner, separate populations of T1R2 labelled cells co-expressed 5-HT (31 ± 6%), while 5 ± 1% of EC-cells co-expressed T1R2. Scale Bar (A-C) = 20 µm.
CHAPTER 12: CONCLUSIONS

The studies reported in this thesis have yielded improved understanding of the role of the gastrointestinal tract in blood glucose regulation, and have evaluated the efficacy of potential dietary and/or pharmacological strategies for the management of postprandial glycaemia in type 2 diabetes.

Slowing the rate of gastric emptying and stimulation of the incretin hormones, GLP-1 and to some extent GIP, represent important mechanisms for improving postprandial glycaemic excursions (Chaikomin et al., 2006). Therefore, dietary and/or pharmacological strategies that are capable of inducing these functional changes in the gut appear promising for improving glycaemic control in type 2 diabetes. It has been established that macronutrient preloads, such as fat (Gentilcore et al., 2006) and protein (Ma et al., 2009c), when consumed in advance of the main meal, have the capacity to reduce postprandial glycaemic excursions in type 2 patients by these mechanisms. However, the long-term benefits of this approach might be limited by the provision of additional energy in the preload. In the study reported in Chapter 4, we have demonstrated in healthy humans that the non-metabolised SGLT1 substrate, 3OMG, stimulates GLP-1 and GIP release, and slows gastric emptying, and that a mixture of poorly absorbed tagatose and isomalt mixture (TIM), which probably stimulates a long length of the gut, results in later and sustained GLP-1 secretion and slowing of gastric emptying, without any effect on GIP, when compared to the
artificial sweetener, sucralose. These findings indicate that non-nutrient substrates of SGLT1 (e.g. 3OMG), or poorly absorbed carbohydrates (e.g. TIM), could be used as alternative preloads to improve postprandial glycaemia in type 2 diabetes, entailing no or minimal additional energy intake.

In the study reported in Chapter 5, we evaluated the effects of D-xylose (a poorly absorbed pentose) when given as a preload, with or without sitagliptin, on gastric emptying, GLP-1, and postprandial glycaemia in patients with relatively well-controlled type 2 diabetes. The main observations in this study were that (i) consumption of D-xylose in advance of a high-carbohydrate meal attenuates the postprandial glycaemic excursion, in association with stimulation of GLP-1 secretion before the meal and slowing of gastric emptying, (ii) a single dose of the DPP-4 inhibitor, sitagliptin, increases postprandial intact GLP-1 concentrations, and reduces postprandial glycaemia, without slowing gastric emptying or stimulating postprandial insulin secretion, and (iii) the combination of a D-xylose preload with sitagliptin reduces the postprandial glycaemic excursion more than either treatment alone. These findings indicate that D-xylose has the potential as a preload for reducing postprandial glycaemia in type 2 diabetes and provide proof for the concept that the glucose-lowering efficacy of a DPP-4 inhibitor can be influenced by dietary intake.

We have recently reported that a small dose of lauric acid delivered over a long segment of distal gut via enteric-coated pellets can stimulate a relatively large
amount of GLP-1 and improve postprandial glycaemia in patients with well-controlled type 2 diabetes. We hypothesised that this glucose-lowering effect would be more prominent in patients with less well-controlled type 2 diabetes when treated with sitagliptin. The study presented in Chapter 6 showed that the addition of active pellets to sitagliptin tended to lower postprandial blood glucose after lunch, but not breakfast, in patients with less well-controlled type 2 diabetes, in a pattern similar to our previous observations in relatively well-controlled type 2 patients (Ma et al., 2013). This probably is due to the continuous release of lauric acid into the colon from the morning dose of pellets, which added to the effect of lunch dose. However, the reduction in blood glucose, as well as changes in hunger and fullness, failed to reach statistical significance, although gastric emptying of the lunch was modestly slower after active than placebo pellets. We have not yet measured plasma total and active GLP-1, but would not expect GLP-1 to have been stimulated to a clinically significant degree by active pellets. Marked hyperglycaemia in this patient group may have impaired the delivery of lauric acid, and consequently, the effect on postprandial glycaemia.

The study of Chapter 7 has shown, in healthy lean and obese humans, and patients with type 2 diabetes treated with or without metformin, that when ID glucose is infused at the rate of 2 kcal/min (the low to mid point of the physiological range of gastric emptying), GIP is the dominant incretin, and its secretion remains intact in obesity and type 2 diabetes, while GLP-1 secretion is
minimal, and is blunted in obesity, but not otherwise in type 2 diabetes. Under these conditions, sitagliptin augments plasma intact GLP-1 and GIP concentrations, increases insulin secretion, and reduces glycaemic excursions in healthy lean and obese humans, but not patients with type 2 diabetes. Moreover, sitagliptin has minimal effect on the motor mechanisms that influence gastric emptying, without any effect on energy intake. In contrast, metformin improves glycaemia, augments fasting and post-glucose GLP-1 and glucagon concentrations, and glucose-stimulated GIP secretion, and suppresses energy intake in type 2 diabetes, without any effect on APD motility. There is no evidence of synergy between metformin and sitagliptin in this experimental setting, probably because GIP is the dominant incretin secreted. The effect of DPP-4 inhibition on glycaemia in type 2 diabetes is likely to be critically dependent on the rate of carbohydrate entry into the small intestine, ie. these agents are probably more effective when the rate of glucose entry into the small intestine is sufficient to induce substantial GLP-1 release.

Bile acids are now generating considerable interest for their role in glucose homeostasis (Nguyen and Bouscarel, 2008). Altered flow of bile has been proposed as one mechanism by which gastric bypass surgery ameliorates type 2 diabetes (Pournaras et al., 2012). Bile acids bind to the G-protein coupled receptor TGR5, expressed on enteroendocrine L-cells in the small and large intestine (Reimann et al., 2008). The studies reported in Chapter 8 showed that rectal administration of the bile acid, taurocholic acid (TCA), stimulates GLP-
1 and PYY, and increases fullness, in a dose-dependent manner in healthy humans, consistent with the concept that topical application of bile acids to the distal gut may have a potential role in the management of diabetes and obesity. We have also demonstrated the importance of the formulation used to deliver TCA, with efficacy observed when TCA was given in an enema, but not in a suppository formulation. However, intraluminal bile acids are extremely low in the large intestine, but are physiologically present in the upper small intestine. In the study reported in Chapter 9, we evaluated the effect of intrajejunal TCA on GLP-1, insulin and glycaemic responses to small intestinal glucose infusion. We found that in healthy humans, the presence of TCA in the jejunum has no effect on fasting blood glucose, GLP-1, insulin, or C-peptide, but potently reduces the glycaemic excursion in response to a small intestinal glucose infusion, associated with an increase in GLP-1 and C-peptide/glucose ratio. In addition, we observed that the glucose-lowering effect of TCA occurred earlier than stimulation of GLP-1, supporting the existence of GLP-1-independent mechanisms.

Stimulation of the intestinal sweet taste receptor (STR) system has been linked to L-cell secretion in humans. For example, the STR antagonist, lactisole, attenuates glucose-stimulated GLP-1 and PYY secretion (Gerspach et al., 2011, Steinert et al., 2011b). The study of Chapter 10 has shown that the activation of STRs by two artificial sweeteners, sucralose and acesulfame potassium, either alone or in combination, has no effect on blood glucose, plasma insulin or GLP-
Conclusions

1 concentrations before or after a subsequent oral glucose load in healthy humans, nor do they delay gastric emptying of the glucose solution. Therefore, activation of STRs appears to be necessary, but not sufficient, to stimulate L-cell secretion.

The expression of STRs (T1R2 and T1R3) in distinct epithelial cells in the human proximal intestine, and that their transcript levels, were shown to vary with glycaemic status in patients with type 2 diabetes (Young et al., 2009). In the study reported in Chapter 11, by clamping blood glucose at either euglycaemia (5.2 ± 0.2 mmol/L) or hyperglycaemia (12.3 ± 0.2 mmol/L), we have shown that absolute levels of STR transcripts are unaffected by acute variations in glycaemia during fasting in either group, but that T1R2 expression increases upon exposure to luminal glucose during euglycaemia. In contrast, T1R2 expression decreases markedly in response to luminal glucose during hyperglycaemia in health, but increases in type 2 diabetes under the same conditions. Type 2 patients also show increased glucose absorption during acute hyperglycaemia compared to healthy subjects, suggesting that dysregulated expression of intestinal STRs can perpetuate postprandial hyperglycaemia in type 2 diabetes. We also confirmed that subsets of duodenal L-, K- and EC-cells express T1R2. However, the intestinal STR system appears unlikely to be a major determinant of plasma concentrations of GLP-1 or GIP in humans.
In summary, gastrointestinal motor and sensory function, and hormone secretion are fundamental to blood glucose homeostasis. Gastrointestinal motility, in particular gastric emptying, determines the rate of nutrient entry into the small intestine, and hence, the size of the postprandial glycaemic excursion and stimulation of GLP-1 and GIP secretion. Accordingly, the glucose-lowering efficacy of DPP-4 inhibitors, which prolong the half-life of active forms of incretin hormones in the circulation, seems to be dependent on the rate of nutrient entry to the small intestine. Although GLP-1 secretion is attenuated in obesity, the incretin hormone responses are not otherwise affected in type 2 diabetes. Probably due to the diminished insulinotropic action of GIP and in the presence of insulin resistance, stimulation of endogenous GIP secretion does not seem to have a major influence on blood glucose in type 2 diabetes. In contrast, strategies that stimulate GLP-1 secretion are promising for the management of postprandial glycaemia. SGLT-1 substrates and bile salts can potently stimulate L-cell secretion. Poorly absorbed carbohydrates and enteric-coated nutrient pellets, which can be exposed to a long segment of the gut, result in later and sustained GLP-1 secretion. These stimuli, when given in advance of, or together with, either a meal or an intraluminal glucose infusion, have the capacity to attenuate glycaemic excursions in both health and type 2 diabetes, and therefore warrant long-term evaluation in the management of the latter. Although metformin augments both GLP-1 and GIP secretion, it lowers blood glucose predominantly via the mechanisms independent of the incretin-axis. Stimulation of the intestinal STRs on L-cells by artificial sweeteners does not yield
improved GLP-1 secretion in response to oral glucose, and the dysregulated intestinal STR system in type 2 diabetes does not alter the GLP-1 response to small intestinal glucose. However, a role of STRs in the regulation of glucose absorption is implicated. Further studies are required to determine the consequences of the dysregulated STR system in type 2 diabetes.


gastrointestinal hormones, appetite, and energy intake in healthy men.


more rapid small intestinal glucose delivery increases plasma insulin, GIP, and GLP-1 but does not improve overall glycemia in healthy subjects. *Am J Physiol Endocrinol Metab*, 289, E504-7.


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