Gastrointestinal motor function and glycaemic control in diabetes

A thesis submitted by

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THESIS ABSTRACT

This thesis focuses on the role of gastric emptying and gastrointestinal hormones in the regulation of glycaemia and appetite in health and patients with type 2 diabetes. The key issues relate to: 1) dietary strategies to improve postprandial glycaemia and appetite by modulation of gastric emptying and gut hormone release, 2) comparative patterns of incretin hormone secretion in response to known rates of small intestinal glucose delivery in healthy subjects and type 2 patients, and 3) whether non-nutrient substances impact on gastric emptying and hormone secretion.

The rate of gastric emptying and secretion of the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are major determinants of glucose homeostasis. Dietary strategies that aim to increase endogenous GLP-1 secretion and slow gastric emptying are therapeutically appealing in type 2 diabetes. The study reported in Chapter 5 evaluated the acute effects of whey protein, when given as a „preload‟, on gastric emptying, incretin hormones and postprandial glycaemia in patients with type 2 diabetes. Different doses of whey preload were evaluated for their effects on glycaemia, fullness and energy intake in Chapter 6. The study reported in Chapter 7 addressed the question of whether the acute effects of whey are sustained with „chronic‟ administration.

The study reported in Chapter 8 evaluated plasma GLP-1 and glycaemic responses to a small load of enterically coated lauric acid, designed to be released over a long length of terminal ileum, after both breakfast (with which the formulation was given), and a subsequent lunch, in patients with type 2 diabetes.

Patients with type 2 diabetes have been reported to have impaired postprandial secretion of GLP-1, but gastric emptying was not quantified in these studies. It has been established that the rate of gastric emptying is an important determinant of incretin hormone secretion, and gastric emptying is frequently abnormally slow in patients with diabetes. In Chapter 9, glycaemia, insulinaemia, and incretin hormone release were evaluated in response to duodenal glucose loads in patients with type 2 diabetes, and were compared to healthy controls.

Sucralose, a noncaloric sweetener, has been reported to stimulate GLP-1 release from L-cells in vitro. The study described in Chapter 10 evaluated whether sucralose stimulated GLP-1 release or affected gastric emptying in healthy subjects. In rodents, sucralose stimulates glucose absorption by enhancing apical availability of the GLUT2 transporter. In the study reported in Chapter 11, the effects of exposure of the proximal small intestine to sucralose on glucose absorption and the glycaemic response were evaluated. Cephalosporin antibiotics mimic peptones and stimulate release of cholecystokinin (CCK) in rodents. In

Chapter 12, the acute effects of orally administered cefaclor on CCK release, gastric emptying, and postprandial blood glucose and insulin responses in healthy subjects were assessed.

DECLARATION

Name: Jing Ma   Program: Doctor of Philosophy

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PUBLICATIONS ARISING FROM THIS THESIS

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CHAPTER 1: PHYSIOLOGY OF GASTROINTESTINAL MOTOR FUNCTION AND APPETITE

1.1 Introduction

The gastrointestinal tract, where nutrients are digested and absorbed, plays a central role in energy and glucose homeostasis. This chapter reviews the physiological control of gastrointestinal motor function and appetite in healthy individuals. The role of the gut in insulin secretion will be discussed in Chapter 2.

1.2 Physiology of gastrointestinal motor function

1.2.1 Patterns of motility in the stomach and duodenum

From the functional point of view, the stomach can be divided into proximal and distal regions (Figure 1.1). The proximal part of the stomach is mainly associated with storage of ingested food. It consists of the fundus and the proximal third of the corpus. The characteristic motor activity of the proximal stomach is that of slow and sustained tonic contraction, which modifies the intragastric pressure and, therefore, determines the pressure gradient between the stomach and the duodenum, an important driving force for the emptying of liquids (Cullen and Kelly 1993; Indireshkumar et al. 2000). Proximal gastric tone transiently decreases when food enters the stomach from the oesophagus, allowing an

increase in stomach volume, known as „receptive relaxation" (Cullen and Kelly 1993). This is followed by a more prolonged „accommodative relaxation", which permits the stomach to store ingested volume without a substantial increase in pressure (Horowitz et al. 1994).

The distal stomach consists of the distal two thirds of the corpus and the entire antrum. Contractions of the distal stomach, which are restricted to a maximal rate of 3/min, grind solids into small particles < 2mm in diameter, and pump the chyme across the pylorus to the duodenum, predominantly in a pulsatile manner. Postprandial gastric motility also involves an increase in tonic, and the frequency of phasic, pyloric pressures, acting as a „brake" to gastric outflow (Horowitz et al. 1994). The temporal and spatial patterns of duodenal contractions are complex, and contribute to the regulation of gastric emptying by either facilitating, or resisting the flow of contents in the lumen (Andrews et al. 2001).

Gastric emptying depends on the integration of motor activity in the proximal stomach, antrum and pylorus with the proximal small intestine, and is under the control of electrical signals („slow waves") originating from the interstitial cells of Cajal (ICCs) in the circular and longitudinal muscle layers (see section 1.2.4). This pattern of gastric motility differs between the fasting and postprandial states. During fasting, gastric and small intestinal motility undergoes a cyclical pattern,

termed the „migrating motor complex" (MMC), consisting of phase I (~ 40 minutes; motor quiescence), phase II (~ 50 minutes; irregular contractions) and phase III (~ 5 - 10 minutes; regular contractions at 3/min in the stomach and 10-12/min in the small intestine) (Horowitz et al. 2004). Indigestible solids are emptied from the stomach mainly during phase III. Postprandial motility is characterized by irregular contractions of variable amplitude, which permit mixing and digestion of food (Camilleri 2006). Return of phase III marks the end of the postprandial period and the resumption of the interdigestive state.

The overall rate of gastric emptying is regulated within the relatively broad range of 1-4 kcal/min, varying with the viscosity, texture, particle size, and fat, fibre and energy content of the meal (Camilleri 2007). Non-nutrient liquids empty rapidly in an overall monoexponential pattern (Camilleri 2007). High nutrient liquids are retained in the distal stomach, then empty in a linear fashion at the same caloric rate as homogenized solids (Collins et al. 1991). When liquid is consumed with solids, the liquid empties preferentially. Solids are stored in the proximal stomach (corresponding to an initial „lag phase"), before being redistributed to the antrum to be ground into small particles, prior to their emptying (Collins et al. 1991).

Gastric motility in healthy subjects is modulated by a complex set of neural (both

afferent from the stomach and small intestine, and efferent) and hormonal (predominantly from the small intestine) signals. Feedback arising from the interaction of nutrients with the small intestine acts to regulate the rate of entry of chyme from the stomach to the small intestine by relaxing the fundus, suppressing antral motility, and augmenting pyloric contractions (Heddle et al. 1988a). Small intestinal regulation of gastric activity is dependent on a number of factors, including the length (Lin et al. 1989; Lin et al. 1990) and the region (Azpiroz and Malagelada 1985) of the small intestine exposed to nutrients, and the magnitude of the nutrient load (Pilichiewicz et al. 2007a; Pilichiewicz et al. 2007b), and is regulated by gut hormones including glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) (Horowitz et al. 2004).

1.2.2 Neural regulation of gastrointestinal motility

The stomach and intestines are innervated by the enteric nervous system, distributed within the wall of the gut, as well as extrinsic autonomic nerves, in parasympathetic (predominantly vagus nerve) and sympathetic divisions.

<u>Intrinsic innervation</u>

The gastrointestinal tract is capable of motor function without the input of the central nervous system, by virtue of the enteric nervous system. The enteric nervous system is distributed along the gut, as the myenteric plexus between the

longitudinal and circular layers of muscle, and the submucous plexus in the submucosa between the circular muscle layer and the muscularis mucosae (Goyal and Hirano 1996). The myenteric plexus predominates in the stomach (Schemann et al. 2001), and the submucous plexus is more predominant in the small and large intestine (Furness 2000). Subclasses of neurons within the enteric nervous system include instrinsic primary afferent neurons, interneurons, excitatory or inhibitory motor neurons, vasomotorneurons, and secretomotorneurons (Goyal and Hirano 1996; Di Nardo et al. 2008). Instrinsic primary afferent neurons sense chemical stimuli, and mechanical changes such as distension (Furness 2000). Interneurons connect the instrinsic primary afferent neurons with motor neurons (excitatory or inhibitory). The excitatory neurons utilize acetyl choline, tachykinins and substance P for neurotransmission, whereas the inhibitory transmitters include vasoactive intestinal polypeptide and nitric oxide (Furness 2000).

Extrinsic innervation

The gastrointestinal tract is innervated by the parasympathetic and sympathetic divisions of the autonomic nervous system. The parasympathetic innervation originates from the dorsal motor nucleus of the vagus (DMV) in the medulla (Travagli et al. 2006), while the sympathetic supply derives from the prevertebral ganglia (Furness 2006). Gastric motility is controlled mainly by the vagus nerve,

a mixed motor and sensory nerve. The sensory axons of the vagus receive afferent inputs from gastrointestinal receptors and then project to the nucleus of the solitary tract (Chang et al. 2003). NTS neurons activate vagal motor neurons in the nucleus ambiguus (NA) and the dorsomedial nucleus (DMN) to regulate the smooth muscle contractions in the stomach and duodenum, with these neural loops being known as vagovagal reflexes (Broussard and Altschuler 2000).

Role of interstitial cells of Cajal (ICCs)

In the absence of any neural or hormonal stimulus, most parts of the gastrointestinal tract generate electrical and mechanical activity. Electrical activity originates from a specialized population of cells, the ICCs, which are distributed within the submucosal, intra-muscular and inter-muscular layers of the gastrointestinal tract (Ordog 2008). They serve to generate electrical „slow waves" and coordinate input from the autonomic nervous system to the smooth muscle cells (Ordog 2008). ICCs located between the longitudinal and circular muscle layers of the myenteric plexus (ICC-MY) (Ward and Sanders 2006) are essential for determining the maximal frequency of phasic contractions (Ward et al. 1994). „ICC-IM", lying among the smooth muscles cells in the circular and longitudinal muscles layers of the stomach, are responsible for direct communication with enteric nerve terminals (Ward and Sanders 2006). „ICC-SEP", in septa between muscle bundles, transmit depolarization between

muscle bundles (Horiguchi et al. 2001). In the human small intestine, ICCs are densely located inside the circular layer, forming a deep muscular plexus („ICC-DMP") (Vanderwinden and Rumessen 1999). The ICC-DMP plays a same role in small intestinal motility as the ICC-IM does in the stomach (Ward and Sanders 2006).

1.2.3 Hormonal regulation of gastrointestinal motility

Secretion of hormones, including GLP-1, CCK and PYY, in response to intestinal nutrients, plays an important role in the regulation of gastric emptying; this inhibitory effect on gastric emptying is reduced by octreotide, an inhibitor of peptide hormone secretion (van Berge Henegouwen et al. 1997).

Among the macronutrients, fat empties most slowly from the stomach, in part due to its high energy density. The effects of small intestinal fat on hormone release and gastric emptying are dependent on fat digestion (Feinle et al. 2003; Feinle-Bisset et al. 2005). Compared to isocaloric triacylglycerol, the effects of fatty acids on CCK and PYY release and gastric emptying are markedly greater (Little et al. 2007). When the lipase inhibitor, tetrahydrolipstatin (orlistat), is infused with fat intraduodenally, the stimulation of tonic and phasic pyloric pressure waves, the suppression of antral contractions and the release of satiety hormones are abolished compared to fat alone (Pilichiewicz et al. 2003;

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O'Donovan et al. 2004a). It is not surprising that release of GLP-1, CCK, and PYY are diminished with orlistat, and that energy intake at the following meal compensates for the malabsorbed fat (Feinle et al. 2003; Feinle-Bisset et al. 2005). The action of lipase on the surface of fat droplets is an important determinant of fat digestion. Thus, emulsions of fat with small droplet size have greater effects on hormone release and appetite than those with larger droplets (Seimon et al. 2009). Stimulation of CCK by fatty acids also varies with chain length (McLaughlin et al. 1999). Both C10 and C12 increase plasma CCK with the effect of C12 being greater, and C12, but not C10, stimulates GLP-1 secretion (Feltrin et al. 2004).

Similarly, for carbohydrate, absorption of monosaccharide has been suggested to be necessary to induce GLP-1 release (Sugiyama et al. 1994; Gribble et al. 2003) and to slow gastric emptying in response to intestinal carbohydrate.

Determinants of hormone release by proteins remain less clear, but it has been shown that peptones and certain amino acids are potent stimuli for CCK (Hira et al. 2009a) and GLP-1 secretion (Layer et al. 1995).

GLP-1

GLP-1 is released from L-cells, most densely located in the ileum and colon,

although they have also been found more proximally in the duodenum and jejunum (Theodorakis et al. 2006). Fasting plasma concentrations of total and intact GLP-1 are low (5-10 pmol/L); in response to a meal rich in fat or carbohydrate, circulating levels of GLP-1 increase up to five fold within minutes (Deacon 2005). The peak concentration in the circulation (~ 20 pmol/L) usually occurs at 30-45 min, before a return to basal concentrations by 2-3 h (Vilsboll et al. 2001). GLP-1 is synthesized as an inactive 37-amino acid peptide, derived from a larger proglucagon precursor (Deacon 2005). The half-life of GLP-1 is short (~ 2 min), due largely to rapid degradation by the enzyme, dipeptidyl peptidase-IV (DPP-IV) (Mentlein et al. 1993). DPP-IV, also known as CD26, cleaves dipeptides from the N-terminal to inactivate GLP-1 (Mentlein 1999). DPP-IV is located in diverse organs, including the kidneys, lungs, adrenals, liver, spleen, pancreas and intestines (Baggio and Drucker 2007). A large proportion of GLP-1 is degraded before entering the systemic circulation, by DPP-IV expressed on blood vessels draining the intestinal mucosa (Hansen et al. 1999).

GLP-1 is one of two known incretin hormones, together with glucose-dependent insulinotropic polypeptide (GIP). The insulinotropic effect of GLP-1 is glucose-dependent, via interaction with a specific receptor expressed on the cell membrane of β-cells (Scrocchi et al. 1996). GLP-1 receptors are found on β- and D-cells of the pancreas, parietal cells of the stomach, pylorus, adipose tissue,

lungs and the brain. In animal models, a targeted deletion of the GLP-1 receptor results in glucose intolerance and fasting hyperglycaemia (Scrocchi et al. 1996). GLP-1 has been shown to stimulate β-cell proliferation and induce islet neogenesis, inhibit apoptosis, and enhance the differentiation of new β-cells from progenitors in the pancreatic duct epithelium (Perfetti et al. 2000). GLP-1 also promotes β-cell differentiation, from exocrine ductal cells or immature islet progenitors, towards a more differentiated β-cell phenotype (Drucker 2003).

In healthy subjects, endogenous GLP-1 reduces glucagon release from the α-cells of the pancreas (Schirra et al. 2006), and intravenous administration of GLP-1 lowers glucagon secretion in patients with type 2 diabetes (Meier et al. 2003). Like the insulinotropic action of GLP-1, the inhibition of glucagon is glucose-dependent, ie, it does not occur during euglycaemia, and does not have an impact on the counter-regulation of hypoglycaemia (Nauck et al. 2002). Exogenous GLP-1 lowers fasting glycaemia even in patients with type 1 diabetes (Creutzfeldt et al. 1996), indicating the importance of the glucagonostatic property of GLP-1 in addition to its insulinotropic effect (Baggio and Drucker 2007).

Among the multiple physiological effects of GLP-1 (Figure 1.2), its effect on gastric emptying may outweigh its insulinotropic effect in the regulation of

postprandial glycaemia (Nauck et al. 1997). It has been long established that GLP-1 exerts an inhibitory effect on gastrointestinal motility and secretion. As an enterogastrone, GLP-1 decreases gastrin-induced acid and pancreatic secretion (Schjoldager et al. 1989), as well as the rate of gastric emptying, and may be an important mediator of the „ileal brake" (by which the presence of nutrients in the ileum inhibits upper gastrointestinal transit) (Wettergren et al. 1993). Motor actions of GLP-1 include relaxation of the fundus, inhibition of antral and duodenal contractions and stimulation of tonic and phasic contractions of the pylorus, in response to intraduodenal infusion of nutrient (Schirra et al. 2006). The mechanism underlying the action of GLP-1 on motility is complex and not well understood. In animal models, it has been shown to depend on intact vagal innervation of the stomach (Imeryuz et al. 1997), while in humans, cholinergic pathways regulate the effect of endogenous GLP-1 on fundic accommodation (Schirra et al. 2000), but alternative mechanisms seem to be involved in its effects on antropyloric motility (Schirra et al. 2009). In rats, the action of exogenous GLP-1 on fasting motility in the small intestine appears to be mediated by nitric oxide (Tolessa et al. 1998).

The finding of GLP-1 receptors in the heart and vessels in both rodents and humans has focused recent attention on the potential effects of GLP-1 on the cardiovascular system (Grieve et al. 2009). Administration of GLP-1 increases

systolic and diastolic blood pressure and heart rate in a dose-dependent manner in rodents (Barragan et al. 1994; Bojanowska and Stempniak 2000). In animal models, exogenous GLP-1 protects cardiac myocytes from ischaemic damage and heart failure (Ban et al. 2008; Poornima et al. 2008). However, the mechanism(s) underlying the cardioprotective effects of GLP-1 are not known, and their significance is not yet well understood. Furthermore, it appears that some actions of GLP-1 in the cardiovascular system may be mediated by the „inactive" metabolite, GLP-1-(9-36) (Nikolaidis et al. 2005).

CCK

CCK is secreted from the I-cells in the duodenum and upper jejunum, and is also found in the brain. There are a number of bioactive forms, such as CCK-8, CCK-22, CCK-33 and CCK-58, of which CCK-33 is the major form in human plasma and intestine (Rehfeld et al. 2001). The half-life of CCK is ~1-2 min. In healthy volunteers, the fasting CCK concentration of 1 pmol/L increases to 5–10 pmol/L in response to a mixed meal (Liddle et al. 1985), peaks at about 30 min, and returns to the fasting value after ~ 3-5 h (Moran and Kinzig 2004). Protein, and particularly fat, are strong stimuli for CCK secretion, whereas carbohydrate results in much less stimulation (Liddle 2000).

Small intestinal fat exerts a potent action on slowing gastric emptying which, in

humans, is regulated predominantly by CCK, via CCK-1 receptors, and abolished by the CCK-1 antagonist, loxiglumide (Feinle et al. 1996; Schwizer et al. 1997). CCK slows gastric emptying by relaxing the proximal stomach, increasing basal and phasic pyloric pressures and suppressing antral motility (Schwizer et al. 1997; Rayner et al. 2000a), mediated by a vago-vagal reflex pathway (Glatzle et al. 2003). In contrast to its actions on the stomach, exogenous CCK increases small intestinal motor activity and shortens the intestinal transit time (Gutierrez et al. 1974).

PYY

PYY is co-located with GLP-1 in the L-cells of the distal gut and is involved in the so-called „ileal brake" (Wen et al. 1995). Plasma concentrations of PYY are low in the fasting state, and increase about 30 min after exposure of the small intestine to nutrients (Vincent and le Roux 2008). PYY is secreted in a load-dependent fashion, with fat being the most potent stimulus, followed by carbohydrate and then protein (Degen et al. 2005a; Huda et al. 2006). The secretion of PYY in response to small intestinal fat is mediated partly by CCK (Lin et al. 2000). Because of its capacity to slow gastric emptying, PYY is also likely to improve postprandial glycaemia, although there is no evidence for an insulinotropic effect.

1.3 Gastrointestinal function and appetite control

1.3.1 Peripheral (ie. gastrointestinal) versus central mechanisms

Modulation of appetite and energy intake is essential to maintain energy balance and body weight. Short-term appetite and food intake can be regulated by the hypothalamus, which constitutes a central control mechanism, and by a peripheral mechanism, constituted by signals arising from the gastrointestinal tract (Havel 2001).

The hypothalamus plays a pivotal role in appetite control. It incorporates a satiety centre in the ventromedial nucleus (VMN) and a feeding, or hunger, centre in the lateral hypothalamic nucleus (LHA). Stimulation of the VMN induces satiety, while destructive lesions in this region lead to hyperphagia, whereas stimulation of the LHA induces hunger, and destruction results in anorexia. The arcuate nucleus (ARC) integrates peripheral signals from circulating gut peptides to influence appetite. Two important groups of neurons are found in the ARC: one group expresses proopiomelanocortin (POMC), α-melanocyte-stimulating hormone (α-MSH), and cocaine and amphetamine-regulated transcript, while the other expresses neuropeptide Y (NPY) and agouti-related peptide (AgRP). Hypothalamic nuclei, such as the paraventricular nuclus (PVN), dorsomedial hypothalamus (DMH), LHA and perifornical area, receive NPY/AGRP and POMC/CART neuronal projections

from the ARC. POMC/CART neurons are activated by anorexigenic hormones (CCK, GLP-1, PYY, leptin and others), and inhibit food intake. Orexigenic hormones (ghrelin and orexins) activate NPY/AgRP neurons and stimulate food intake. The nucleus tractus solitarius and area postrema (AP) in the brain stem represent a link between signals from the periphery and the hypothalamus (Konturek et al. 2005).

1.3.2 Gastrointestinal control of appetite

The capacity for intestinal nutrient infusions to suppress food intake at a subsequent meal indicates a role for the gastrointestinal tract in regulating satiation (Ritter 2004). The signals from the gastrointestinal tract arise from the stomach (gastric distension and ghrelin release), proximal small intestine (CCK release) and distal small intestine (GLP-1 and PYY) in response to food (Huda et al. 2006).

Distension is the main „gastric" mechanism of appetite control. In cats (Anand and Pillai 1967), gastric distension activates the hypothalamic satiety centre and suppresses the feeding centre, when the vagus is intact. In humans, volume appears to be a determinant of satiety and subsequent meal intake, independent of food content (de Graaf and Hulshof 1996; Rolls et al. 1998). For example, the volume of isocaloric milk drinks consumed before lunch is related to the

suppression of food intake and an increased sensation of fullness (Rolls et al. 1998). Similarly, water-filled gastric balloons reduce food intake when the balloon volume is ≥ 400 ml (Geliebter 1988; Geliebter et al. 1988). Furthermore, small intestinal nutrient stimulation acts synergistically with gastric distention, converting the resultant sensation to one of „meal-like" fullness (Feinle et al. 1996).

Antral area relates more closely to postprandial fullness than does the area of the proximal stomach (Benini et al. 1994; Jones et al. 1997). Ultrasound studies have yielded insights into the particular importance of antral distension (Hveem et al. 1996); after ingestion of a liquid meal, antral area is related to satiety and accounts for about a third of the variation in energy intake at a subsequent meal (Sturm et al. 2004). The maximum antral distension and the onset of the perception of fullness occur at the same time (~ 5 min), and the correlation between satiation and antral area is much stronger than that between satiation and proximal gastric volume (Mundt et al. 2005).

Ghrelin, a 28-amino acid peptide, is an orexigenic hormone, which is secreted mostly from the gastric mucosa; small quantities can be found in the duodenum and hypothalamus (Tschop et al. 2000). Plasma ghrelin concentrations are high in the fasting state and decrease with nutrient ingestion (Gomez et al. 2004).

Ghrelin declines equally after gastric, intraduodenal and intrajejunal nutrient infusions in rodents and humans, suggesting that gastric distension does not regulate its suppression (Overduin et al. 2005; Parker et al. 2005). Rather, exposure of the small intestine to nutrients is required to suppress ghrelin (Parker et al. 2005). Fasting and postprandial fluctuations of ghrelin are also related to insulin concentrations and sensitivity (Blom et al. 2005). For example, obese individuals with insulin resistance have a blunted suppression of ghrelin in response to nutrients (Greenman et al. 2004).

In both rodents (Tschop et al. 2000) and humans (Wren et al. 2001), exogenous ghrelin administration increases energy intake, while patients with Prader-Willi syndrome, characterized by excessive feeding behaviour, have high levels of circulating ghrelin (Cummings et al. 2002a). Proximal Roux-en-Y gastric bypass decreases ghrelin secretion, which may contribute to weight loss after this procedure (Cummings et al. 2002b). Ghrelin modulates appetite via the ARC of the hypothalamus (Currie et al. 2005); it enhances NPY and AgRY gene expression and release in the LHA, and inhibits the anorexigenic POMC neurons in the ARC (Nakazato et al. 2001). In contrast to ghrelin, leptin, an anorexigenic hormone, inhibits orexigenic neurons containing NPY and AgRY, while stimulating POMC neurons in the ARC (Cowley et al. 2001). Leptin attenuates the activity of ghrelin in the ARC and suppresses food intake. Thus, ghrelin and

leptin interact with each other in the appetite centre, in a so-called „ghrelin-leptin tango"(Cummings and Foster 2003).

CCK was the first gut hormone found to be related to appetite (Gibbs et al. 1973). CCK exerts its effects via two receptors, CCK-1 and CCK-2. The suppressive effect of CCK on energy intake in rats and humans is predominantly mediated by CCK-1 receptors (Moran et al. 1992). CCK-1 receptor antagonists increase meal size, while CCK-1 receptor deficient rats develop hyperphagia and obesity (Moran and Bi 2006). In rats, it has been demonstrated that chronic administration of a CCK antagonist results in weight gain out of proportion to increased feeding (Meereis-Schwanke et al. 1998), but this probably reflects a synergistic effect between CCK and leptin on energy intake and long-term balance of body weight (Wang et al. 2000). In humans, exogenous CCK dose-dependently reduces energy intake at a buffet style meal (Brennan et al. 2008) and the CCK-1 antagonist, loxiglumide, increases energy intake modestly in healthy adults (Beglinger et al. 2001). However, the long term effects of CCK administration in humans and its role in obesity therapy are not clear.

Gastric distension augments the suppressive effect of exogenous CCK on food intake (Kissileff et al. 2003), and CCK-induced satiation is related to the effect on gastric emptying, which favours gastric distension (Moran and McHugh

1982). Suppression of energy intake and slowing of gastric emptying by CCK are both regulated by vagal afferents, mainly capsaicin-sensitive C-type fibres and A-type afferent neurons (van de Wall et al. 2005). CCK increases c-fos expression in the PVN and NTS, which suppresses NPT/AgRP activity in the ARC, also contributing to its anorexigenic actions (Kobelt et al. 2005).

Intracerebroventricular injection of GLP-1 inhibits feeding in rats (Hwa et al. 1998), as does peripheral infusion of GLP-1 and its agonist, exendin-4 (Rodriquez de Fonseca et al. 2000). Peripheral administration of GLP-1 is associated with enhanced satiety and reduction of food intake in healthy humans (Gutzwiller et al. 1999b), the obese (Naslund et al. 1998), and patients with type 2 diabetes (Gutzwiller et al. 1999a). Obese subjects may have slightly lower plasma GLP-1 concentrations than lean individuals (Ranganath et al. 1996). Postprandial GLP-1 secretion increases substantially after Roux-en-Y gastric bypass for morbid obesity, suggesting that enhanced GLP-1 secretion contributes to satiation and weight loss (Morinigo et al. 2006). The mechanism of increased GLP-1 secretion is likely to relate to exposure of more distal small intestinal regions (with a larger density of L-cells) to ingested nutrients. In type 2 diabetic patients, the GLP-1 analogue, exenatide (Byetta, Amylin Pharmaceuticals, San Diego), leads to modest weight loss in prolonged studies (Blonde et al. 2006). GLP-1 analogues, such as exenatide, are approved for the management of

glycaemia control in type 2 diabetic patients, and their utility as an antiobesity therapy is currently under investigation (Chaudhri et al. 2008).

PYY circulates as two main forms, PYY3-36 and PYY1-36, with the former being active in suppressing appetite (Eberlein et al. 1989). In addition to delaying gastric and gall-bladder emptying, inhibiting gastric and pancreatic secretion, and slowing colonic transit (Conter et al. 1987), peripheral administration of PYY3-36 reduces food intake in rats, monkeys and humans (Moran et al. 2005; Neary et al. 2005; Vrang et al. 2006). In humans, both pharmacological and „physiological"intravenous PYY3-36 infusions elicit fullness and suppression of food intake, the latter lasting over 12 hours, despite the plasma PYY concentration falling to baseline (Batterham et al. 2002). In addition, there is a synergistic effect of exogenous PYY3-36 with GLP-17-36 on reduction of energy intake (Neary et al. 2005), whereas exogenous PYY reduces circulating ghrelin concentrations (Batterham et al. 2003). It remains contentious whether the suppressive effect of PYY3-36 on energy intake in humans represents an aversive effect; there are no specific antagonists of PYY which are available for use in humans.

Injection of PYY3-36 into the ARC in rats decreases NPY expression while stimulating expression of POMC, which would account for reduced food intake

(Batterham et al. 2002). The putative pre-synaptic NPY Y2 receptor (Y2R) in the ARC of the hypothalamus appears to mediate these effects (Abbott et al. 2005). Like GLP-1, plasma concentrations of PYY are slightly lower in the fasting and postprandial states and hunger is greater, in obese compared to lean individuals (le Roux et al. 2006b). Low PYY levels predict a trend to overweight, but in obese children, PYY is normalized following weight loss (Roth et al. 2005). However, the obese are as sensitive as lean subjects to suppression of food intake by exogenous PYY3-36 (Batterham et al. 2003). Again, like GLP-1, obese patients have elevated PYY levels after jejunoileal bypass surgery, which may contribute to loss of appetite (Naslund et al. 1997).

1.4 Summary

The crucial roles of gastric emptying/distention and the release of peptide hormones such as CCK, GLP-1 and PYY in response to nutrient intake are increasingly being recognized as important physiological regulators of appetite. A better understanding of the mechanisms of appetite regulation is required to enable the development of effective anti-obesity therapies. Current therapy for obesity is less than ideal, and bariatric surgery is limited to severe cases. After Roux-en-Y gastric bypass, important gut signals such as GLP-1 and PYY are potentiated, which suggests a role for gut hormones in the manipulation of energy homeostasis and diabetes management in the long term (Pournaras et al.

2009), particularly as obese subjects appear to maintain their sensitivity to the actions of gut hormones (Wren 2008). Dietary, or pharmacological strategies based on regulation of gut hormones and motility are likely to be of fundamental importance in the management of obesity.

Studies that address the manipulation of physiological nutrient-gut interactions in health and disease reported in this thesis include:

(i)   Effects of a protein „preload" on gastric emptying, glycaemia, and gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes, both acutely (Chapters 5 and 6) and with more sustained exposure (Chapter 7).

(ii)   Effects of enterically coated, nutrient-containing pellets („CTM#3") on the release of gastrointestinal peptides, and glycaemic control in patients with type 2 diabetes (Chapter 8).

(iii)   Effects of variations in duodenal glucose load on glycaemic, insulin, and incretin responses in type 2 diabetes (Chapter 9).

(iv)   Effect of the artificial sweetener, sucralose, on gastric emptying and incretin hormone release (Chapter 10) and on small intestinal glucose absorption (Chapter 11) in healthy humans.

(v)   Effects of the antibiotic, cefaclor on gastric emptying and cholecystokinin release in healthy humans (Chapter 12).

Figure 1.1 Schematic representation of gastrointestinal motility. Adapted from Koch 1999.
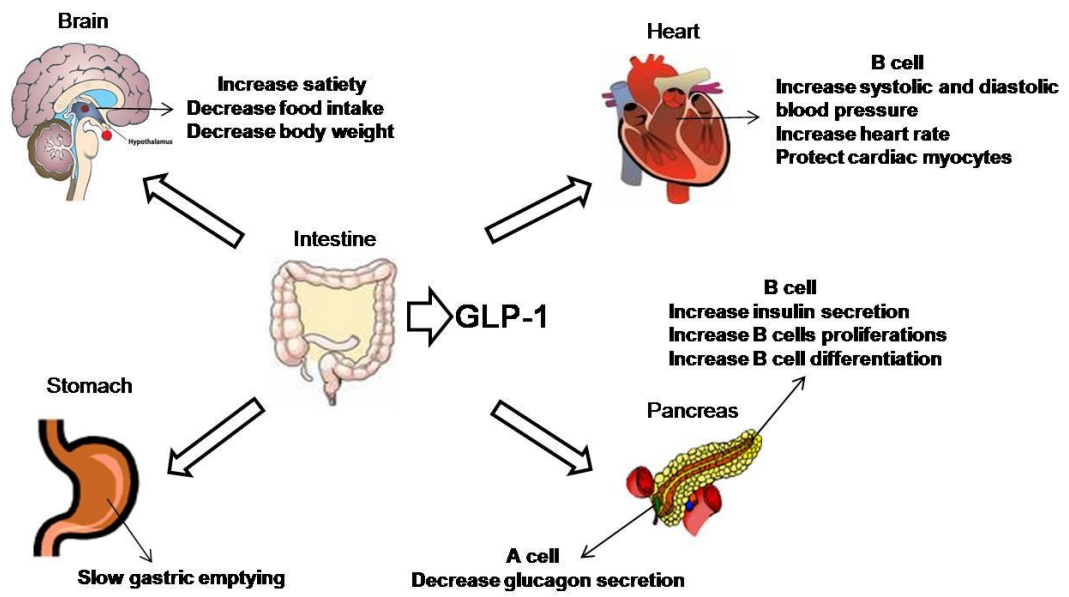
Figure 1.2 Schematic representation of biological effects of GLP-1. Adapted from Drucker 2007.

CHAPTER 2: INSULIN SECRETION IN HEALTHY SUBJECTS AND PATIENTS WITH TYPE 2 DIABETES - ROLE OF THE GASTROINTESTINAL TRACT

Adapted from Ma J et al. Best Pract Res Clin Endocrinol Metab. 23:413-424, 2009.

2.1 Introduction

The gastrointestinal tract stores ingested nutrients in the stomach and empties them, in a closely regulated fashion, into the small intestine, where they are digested, stimulate the release of gut peptides that influence appetite, insulin secretion, and the rate of subsequent gastric emptying, and are ultimately absorbed, resulting in a postprandial excursion in the blood glucose concentration. It is, therefore, logical that the gut plays a central role in glucose homeostasis.

There is increasing recognition that postprandial hyperglycaemia is the major contributor to overall glycaemic control, as measured by glycated haemoglobin (Del Prato 2002; Ceriello et al. 2004), and is therefore pivotal to the development and progression of the micro-, and probably macro-vascular, complications of diabetes (The Diabetes Control and Complications Trial Research Group 1993;

UK Prospective Diabetes Study (UKPDS) Group 1998; Nathan et al. 2005). The peak value of plasma glucose in patients with diabetes (estimated by a 2 hour postprandial measurement), more so than the incremental area under the blood glucose curve, correlates strongly with glycated haemoglobin (American Diabetes Association 2001). This dictates that an understanding of the impact of the gastrointestinal tract on glycaemic control and insulin secretion is of fundamental importance to the development of effective strategies for diabetes management.

This chapter focuses on the factors that regulate emptying of a meal from the stomach, and the mechanisms by which nutrients then interact with the small intestine to determine both blood glucose and insulin concentrations in healthy individuals and those with diabetes.

2.2 The incretin effect

Following a meal, carbohydrate is absorbed and enters the circulation, elevating the blood glucose concentration and stimulating insulin release from the pancreatic beta cells. It has been long established that the insulin response to an oral glucose load is three- to four-fold greater than that observed after an „isoglycaemic" intravenous infusion of glucose (Perley and Kipnis 1967). This phenomenon, known as the „incretin effect", led to the discovery of hormones,

secreted from the gastrointestinal tract in response to nutrients, that stimulate insulin secretion in a glucose-dependent manner. The two known incretin hormones are glucose-dependent insulinotropic polypeptide (GIP), originating predominantly from the proximal small intestine, and glucagon-like peptide-1 (GLP-1), found predominantly in the distal small intestine and colon. GLP-1 and GIP appear to be responsible for over 50 % of the postprandial insulin response in healthy humans (Horowitz and Nauck 2006). Circulating GIP and GLP-1 concentrations are low in the fasting state, but rise within 15 minutes of nutrient ingestion, typically peaking by 30-45 minutes, and returning to basal levels over 2-3 hours (Deacon 2005). The circulating molecular forms of active GLP-1 are GLP-1 (7-36) amide, representing the majority of active GLP-1 found in plasma, and glycine-extended GLP-1 (7-37) (Drucker 2006). These are rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV) to GLP-1 (9-36) amide and GLP-1 (9-37) (Drucker 2006). Similarly, circulating active GIP (1-42) is rapidly degraded to inactive GIP (3-42).

2.3 Gastric emptying and its influence on blood glucose and insulin secretion

Nutrients empty from the stomach at an overall rate of about 2-3 kcal/min (Brener et al. 1983), regulated predominantly by neural and hormonal feedback from the small intestine that slows further emptying by relaxing the fundus, suppressing antral and duodenal contractility, and stimulating tonic and phasic

contractions that are localized to the pylorus (Schirra et al. 2000; Schirra et al. 2006). GLP-1 is one of the peptides involved in this feedback loop; others include cholecystokinin (CCK) and peptide YY (PYY), though not GIP (MacIntosh et al. 1999).

Although the rate of gastric emptying does not affect insulin secretion directly, it regulates the delivery of carbohydrate and other macronutrients to the small intestine, and, therefore, has a major impact on both the timing and magnitude of the blood glucose excursion, and the secretion of the incretin peptides, thereby modulating insulin release indirectly. Indeed, it has been established that variations in the rate of gastric emptying account for some 35 % of the variance in the peak blood glucose concentration after 75 g oral glucose, in both healthy subjects and patients with type 2 diabetes (Figure 2.1) (Horowitz et al. 1993; Jones et al. 1996). Interventions that modify gastric emptying, therefore, have the potential to affect glycaemic control in patients with diabetes. For example, accelerating nutrient entry from the stomach to the small intestine with the potent prokinetic drug, erythromycin, increases the postprandial glycaemic response, whereas slowing gastric emptying with morphine reduces postprandial blood glucose concentrations in patients with type 2 diabetes (Gonlachanvit et al. 2003). The impact of gastric emptying on both the peak and initial rise in blood glucose appears more direct than that on overall glycaemia (Gonlachanvit et al. 2003).

Similarly, the insulin requirement to sustain normoglycaemia after a standard meal is significantly lower in type 1 patients with gastroparesis than those without, with the difference being apparent during the first two hours of the postprandial period (Ishii et al. 1994). Conversely, when gastric emptying is accelerated by giving a meal with smaller particle size in type 1 patients with gastroparesis- a group at risk for postprandial hypoglycaemia- the postprandial blood glucose dip is diminished (Olausson et al. 2008).

The effects of different rates of gastric emptying on glucose homeostasis can be mimicked in the laboratory by infusing glucose through a transnasal catheter, directly into the duodenum (Figure 2.2) (Pilichiewicz et al. 2007a). Using this technique, variations in the rate of delivery of glucose to the small intestine within the physiological range are seen to have differential effects on glycaemia, insulin and incretin hormone release in healthy subjects (Pilichiewicz et al. 2007a). For example, glucose infusion at 2 kcal/min increases the blood glucose concentration more than infusion at 1 kcal/min, but infusion at rates of 2 and 4 kcal/min result in comparable blood glucose responses, reflecting the substantially greater insulin response to the 4 kcal/min load. Under low caloric exposure (1 kcal/min), there is minimal, transient stimulation of GLP-1, compared with sustained elevation in GIP, suggesting a much lesser contribution of GLP-1 towards the „incretin effect" in this circumstance. When intraduodenal

glucose delivery is increased to 2 or 4 kcal/min, progressively increasing GLP-1 secretion, in contrast to apparently capped levels of GIP, appears to be responsible for the marked increase in insulin. Similar studies are required to address the pathophysiology of insulin secretion in type 2 diabetic patients.

Given that the rate of gastric emptying plays such a major role in determining postprandial blood glucose, one could speculate that the initial rate of glucose entry into the small intestine may be particularly important in establishing the response to ongoing glucose exposure over the next hour or two. This may be the case, particularly in type 2 diabetes, where the first phrase of insulin release is delayed, whereas the overall insulin response to a meal may be relatively intact (Bruce et al. 1988; Gerich 1996). Indeed, in both healthy subjects and type 2 patients, initially more rapid delivery of glucose to the small intestine does boost both the incretin and insulin responses when compared to constant delivery of an identical glucose load (O'Donovan et al. 2004b; Chaikomin et al. 2005). Nevertheless, this early increase in insulin cannot compensate for the greater initial rise in absorbed glucose, so that overall glycaemic control is not improved (Figure 2.3) (O'Donovan et al. 2004b; Chaikomin et al. 2005).

2.4 Role of the small intestine

The small intestine, being the site of glucose absorption from the external

environment and the source of numerous regulatory peptides, including the incretins, is central to glucose homeosatasis and postprandial insulin secretion. It is, therefore, remarkable that knowledge regarding many aspects of small intestinal function is rudimentary.

The maximal capacity of glucose absorption from the small intestine is about 0.5 g per minute (or 2 kcal/min) per 30 cm (Duchman et al. 1997). Therefore, it would be expected that changes in small intestinal motor function that act to spread glucose over a larger surface area would increase the rate of glucose absorption, and indeed, absorption of the glucose analogue, 3-O-methylglucose (3-OMG), increases in proportion to the frequency of small intestinal pressure waves and propagated pressure wave sequences in both healthy subjects and patients with type 1 diabetes (Rayner et al. 2002; Schwartz et al. 2002). Recently, the measurement of electrical impedance at multiple sequential sites within the small intestine has yielded information about flow events in the lumen (Nguyen et al. 1995). This technique has been validated against fluoroscopy (Imam et al. 2004) and can be used in studies which are several hours in duration. When duodenal flow events (evaluated by impedance) were suppressed by the anticholinergic drug, hyoscine butylbromide, there was marked attenuation of the rise in blood glucose and 3-OMG, together with a delay in secretion of both the incretins and insulin (Chaikomin et al. 2007). Further studies are required to

examine the relationship between small intestinal flow patterns and glucose absorption in patients with diabetes, many of whom have disturbed small intestinal motility (Camilleri and Malagelada 1984), with slow small intestinal transit in some, but rapid transit in others (Samsom and Verhagen 2004).

It is also unclear to what degree variations in glucose absorption at the level of the mucosa are relevant to postprandial glycaemic control in diabetes. Rodent models of diabetes are associated with increased absorption of glucose across the small intestinal mucosa (Fujita et al. 1998), while acute hyperglycaemia itself appears to enhance glucose absorption (Csaky and Fischer 1977; Csaky and Fischer 1981; Fischer and Lauterbach 1984; Cheeseman and Maenz 1989). While the rate of small intestinal glucose absorption has not been consistently reported to be increased in humans with diabetes (Costrini et al. 1977; Gulliford et al. 1989), expression of monosaccharide transporters SGLT1, GLUT5, and GLUT2 is increased in type 1 and type 2 diabetic humans (Dyer et al. 2002), and a direct relationship has been reported between the rate of intestinal glucose absorption and the ambient blood glucose concentration in type 1 patients and healthy volunteers (Rayner et al. 2002). Further studies are required to clarify the clinical significance of these observations.

Similarly, knowledge about the precise mechanisms and determinants of incretin

hormone release is limited. The size of the nutrient load is clearly important; GIP secretion increases with increasing small intestinal glucose loads within the physiological range of gastric emptying (up to about 4 kcal/min) (Pilichiewicz et al. 2007a). On the other hand, it has been suggested that a threshold of small intestinal glucose delivery of about 1.8 kcal/min needs to be exceeded in order to stimulate GLP-1 release (Schirra et al. 1996), although studies in our laboratory have documented a small, early but transient peak in GLP-1 with a glucose load as low as 1 kcal/min (Kuo et al. 2008). The early rise in GLP-1 after nutrient exposure appears inconsistent with the notion that this hormone is secreted predominantly from the distal intestine, and raises the possibility of a neuroendocrine loop to the distal small intestine that allows simulation of the distal L-cells indirectly (Rocca and Brubaker 1999). GIP appears to mediate such a loop in rodents, but not in humans (Nauck et al. 1993a; Nauck et al. 1993c). Alternatively, the early rise in GLP-1 could reflect direct stimulation of duodenal or jejunal L-cells by luminal glucose (Theodorakis et al. 2006). Moreover, we have shown that distal small intestinal exposure to glucose is required to stimulate GLP-1 release in humans; when exposure was limited to the proximal 60 cm of small intestine by an occluding balloon, there was no rise in GLP-1 (Little et al. 2006a). Macronutrient composition also determines GLP-1 release; in addition to carbohydrate, fat and protein induce GLP-1 and GIP secretion (Elliott et al. 1993; Herrmann et al. 1995), and the GLP-1 response is greater and

more prolonged with the latter two macronutrients (Elliott et al. 1993; Bowen et al. 2006b). Fructose tends to stimulate a similar GLP-1 response to glucose, but does not induce GIP release (Rayner et al. 2000b).

2.5 Gastrointestinal function and the incretin response in type 2 diabetes

Gastric emptying of solids and/or nutrient liquids is abnormally slow in 30-50 % of patients with long standing type 1 and type 2 diabetes, although the magnitude of the delay in emptying is often modest (Horowitz et al. 1996b). Some groups have reported that gastric emptying is abnormally rapid in „early" type 2 diabetes (Phillips et al. 1992; Frank et al. 1995; Schwartz et al. 1996), although this has not been uniformly observed (Jones et al. 1996). The only study to evaluate the natural history of gastric emptying in diabetes has demonstrated that there were no marked changes in the rate of emptying during follow up of about 12 years (Jones et al. 2002). In the absence of symptoms such as nausea, vomiting, or bloating, delayed gastric emptying may actually be beneficial for glycaemic control in type 2 diabetes managed by oral hypoglycaemic drugs or diet, by slowing the absorption of carbohydrate, as discussed earlier. Conversely, in patients treated with insulin, delayed emptying can present a problem if it results in a mismatch between the absorption of nutrients and the action of exogenous insulin.

2.5.1 The incretin effect in type 2 diabetes

Comparisons of the incretin effect in type 2 patients with that in healthy subjects have, to date, all been assessed after oral administration of nutrients, so that potential differences in the rate of gastric emptying have not been accounted for as a confounding factor. A comparison of the incretin response to intraduodenally delivered glucose in type 2 diabetes compared to healthy controls is lacking, and represents a significant gap in current knowledge.

GIP

It has been reported that secretion of GIP is increased, decreased or normal in patients with type 2 diabetes (Krarup 1988). However, it appears clear that the insulinotropic action of GIP is markedly attenuated in these patients (Elahi et al. 1994), particularly during the „late phase" of insulin secretion (Vilsboll et al. 2002). The mechanism of this attenuated response remains uncertain, but defective expression of the GIP receptor has been observed in Zucker diabetic fatty rats (Lynn et al. 2001). About 50 % of glucose-tolerant first-degree relatives of type 2 diabetic patients demonstrate a reduced insulin response to GIP compared with healthy controls, suggesting that the defect might be genetically determined, and could represent a primary abnormality leading to the subsequent development of diabetes (Meier et al. 2001). However, it has also been reported that patients with different aetiologies from typical type 2 diabetes, including

newly diagnosed type 1 diabetes, diabetes secondary to pancreatitis, monogenic diabetes (MODY3), lean type 2 diabetes, and latent autoimmune diabetes in adults, also have diminished insulin responses to GIP when compared to GLP-1, implying that the defect in responsiveness to GIP may be a consequence of the diabetic state (Vilsboll et al. 2003a).

GLP-1

Both total and active concentrations of GLP-1 following a standardized meal are lower in patients with type 2 diabetes when compared to matched controls (Toft-Nielsen et al. 2001). This phenomenon might contribute to impaired postprandial insulin secretion because, in contrast to GIP, the insulin response to exogenous GLP-1 is essentially intact in type 2 diabetes (Nauck et al. 1993c).

2.6 Therapeutic strategies to optimize glycaemia involving modulation of gut function

Potential strategies to optimize postprandial glycaemia in patients with type 2 diabetes that involve dietary or pharmacological modulation of gastrointestinal function include i) slowing gastric emptying to minimize postprandial glucose excursions, ii) inhibiting carbohydrate absorption in the small intestine, iii) augmenting incretin hormone release, and iv) modifying macronutrient composition. It should be recognized that in practice, many of these goals

overlap (Table 2.1). As discussed earlier, the peak blood glucose concentration should be particularly targeted by these interventions. Nevertheless, reduction of the incremental area under the blood glucose curve is also a goal of therapy.

2.6.1 Slowing gastric emptying

As discussed, in patients with type 2 diabetes who are treated with insulin, slowing the absorption of nutrients should be beneficial, as the first phase of insulin secretion is diminished. An increase in soluble fibre (Chandalia et al. 2000), adding the non-absorbable polysaccharide, guar gum (Russo et al. 2003), or combining fat with a carbohydrate-containing meal (Cunningham and Read 1989), all improve blood glucose while lowering insulin responses. Fat is the most potent among the macronutrients to slow gastric emptying, a process that is mediated by the stimulation of a number of gut hormones, including CCK and PYY, as well as GLP-1, and is dependent on lipolysis of triglycerides to fatty acids in the small intestine (Feinle et al. 2003). Therefore, the effect of fat on slowing gastric emptying and releasing gut peptides is attenuated by the lipase inhibitor, tetrahydrolipstatin (orlistat), in healthy subjects and type 2 patients (O'Donovan et al. 2004a). The requirement that triglyceride digestion occurs before gastric emptying can be slowed suggests the concept of giving fat at an interval (eg. 30 min) before the meal. Indeed, this concept of a „preload" does appear more efficacious than incorporation of fat into the meal (Gentilcore et al.

2006a). Furthermore, the „preload" concept could also have the advantage of suppressing appetite and reducing energy intake at the subsequent meal.

Pharmacological agents which slow gastric emptying are also effective at improving postprandial glycaemic control in patients with diabetes. For example, the human amylin analogue, pramlintide, acts predominantly by slowing gastric emptying in patients with type 1 and 2 diabetes (Thompson et al. 1997; Thompson et al. 1998; Singh-Franco et al. 2007), while also suppressing both postprandial glucagon secretion and appetite (Aronne et al. 2007). Analogues of GLP-1, such as exenatide or liraglutide, have been developed primarily with the rationale of stimulating insulin secretion, but in practice, inhibition of gastric emptying by exogenous GLP-1 appears to outweigh its direct insulinotropic effects. Thus, when GLP-1 is administered with a meal in healthy subjects, there is a dose-dependent reduction of, rather than an increase in, postprandial insulin (Nauck et al. 1997; Little et al. 2006b). It has recently been confirmed that slowing of gastric emptying makes a major contribution to the effect of exenatide in reducing postprandial hyperglycaemia in patients with type 2 diabetes (Cervera et al. 2008).

2.6.2 Inhibiting absorption of carboydrate

The alpha-glucosidase inhibitor, acarbose, is routinely used in the treatment of

diabetes, and reduces postprandial plasma glucose excursions by delaying the absorption of carbohydrate (other than monosaccharides) from the small intestine (Bischoff 1994). In healthy volunteers, when sucrose is consumed with acarbose, the delay in absorption allows exposure of the more distal gut to carbohydrate, resulting in greater and more prolonged GLP-1 release than with sucrose alone, which probably accounts for the observed slowing of gastric emptying, representing an additional mechanism contributing to the therapeutic effect of acarbose (Qualmann et al. 1995; Ranganath et al. 1998). However, in type 2 diabetic patients, ingestion of acarbose with a mixed meal failed to enhance GLP-1 release or slow gastric emptying (Hucking et al. 2005). Slowing of small intestinal carbohydrate absorption can also be achieved by diets high in fibre, particularly soluble fibre, and by low glycaemic index diets, which may, for example, contain carbohydrate as starch in the form of amylose, or within kernels that are resistant to digestion (Mourot et al. 1988; Ou et al. 2001; Bjorck and Elmstahl 2003).

2.6.3 Augmenting the incretin response

GLP-1 is rapidly degraded in vivo by the enzyme DPP-IV, as discussed earlier. Analogues of GLP-1 that are resistant to DPP-IV degradation, such as exenatide, have therefore been developed for therapeutic use (Drucker 2007), and appear to

retain all the anti-hyperglycaemic effects of GLP-1 (Fineman et al. 2003). An alternative approach to enhance circulating concentrations of endogenous active GLP-1 is to inhibit DPP-IV. DPP-IV inhibitors, such as vildagliptin and sitagliptin, are available as oral formulations that are effective in improving glycaemic control either as initial monotherapy or in combination with sulfonylureas or metformin (Charbonnel et al. 2006; Ahren 2007), by increasing the insulin to glucose ratio and suppressing glucagon (Ahren 2007). Some data suggest that DPP-IV inhibitors slow gastric emptying (Woerle et al. 2007), while other investigators have not observed a significant effect (Vella et al. 2007). The apparent discrepancy might be accounted for by more modest stimulation of GLP-1 with the meal used in the latter study.

2.6.4 Modifying macronutrient composition

Low carbohydrate and low glycaemic index diets

Lowering the carbohydrate load improves both fasting and postprandial glycaemia in type 2 patients who have failed treatment with conventional diets or sulfonylureas. Conversely, maintaining high carbohydrate ingestion, even with caloric restriction, is associated with poor glycaemic control, and higher levels of glycated haemoglobin (Gutierrez et al. 1998). Low carbohydrate intake improves hyperglycaemia and hyperlipidaemia over follow up of at least 12 months, compared to a conventional diet (Stern et al. 2004).

The glycaemic index ranks the effect of carbohydrate in various foods on postprandial blood glucose, in comparison to a standard of glucose or white bread. While many low glycaemic index foods have a high fibre content, the benefits of which are discussed above, they have beneficial effects on blood glucose control independent of their fibre content (Wolever 1990). In addition to slower rates of gastric emptying, and lower glucose absorption in the small intestine (Bjorck and Elmstahl 2003), low glycaemic index carbohydrates have the potential to increase the length and duration of exposure of the small intestine to nutrients, enhancing the release of distal gut hormones, including GLP-1 (Little et al. 2006a).

Protein supplementation

High protein diets have attracted recent attention in the management of type 2 diabetes; for example, increasing the percentage of dietary protein from 15 % to 30 % for 5 weeks in type 2 patients reduced postprandial glycaemia and achieved a modest (0.4 %) decrease in glycated haemoglobin (Gannon et al. 2003). It has been established for some time that the addition of protein to an oral glucose load lowers the subsequent glycaemic excursion, and, in type 2 patients, stimulates increased secretion of insulin over that of glucose alone (Gannon et al. 1988). Addition of protein to a glucose drink slows gastric emptying of the carbohydrate and stimulates the release of incretin hormones (Karamanlis et al. 2007), while

an additional effect of whey protein, noted in rodents, is the inhibition of small intestinal DPP-IV by digested protein fragments (Gunnarsson et al. 2006). A recent study, adopting the „preload" concept discussed above, demonstrated that whey protein, given 30 minutes before a carbohydrate meal in subjects with type 2 diabetes, profoundly lowered the blood glucose profile when compared to a preload without protein; not only was emptying of the meal slowed, but insulin release was markedly stimulated (Ma et al. 2009c). This approach requires further investigation to develop a dietary strategy that is applicable for long term use.

2.7 Summary and implications

The gastrointestinal tract, which regulates the absorption of carbohydrate and releases the „incretin" peptides that stimulate insulin secretion, is of central importance to glucose homeostasis. Interventions that slow gastric emptying and/or stimulate the incretin effect appear to be effective strategies for the treatment of non-insulin treated type 2 diabetes.

Further information is required to understand the determinants of incretin hormone release in humans, and dietary or pharmacological modulation of gastrointestinal function needs to be evaluated in medium- or long-term clinical trials to establish its benefits for controlling postprandial glycaemia.

Table 2.1 Adapted from Chaikomin et al. 2006.

Therapeutic strategies directed at minimising postprandial glycaemia

Mechanism	Strategy
Slow gastric emptying	• High fibre diets • Adding guar gum, a non-absorbable polysaccharide • Fat „preloads" • Protein „preloads" • Low glycaemic index carbohydrates • Acarbose • GLP-1 analogues • DPP-IV inhibitors • Pramlintide
Inhibit small intestinal carbohydrate absorption	• Acarbose • High fibre diet • Low glycaemic index carbohydrates
Augment the incretin response	• GLP-1 analogues • DPP-IV inhibitors • Acarbose • Fat „preloads" • Protein „preloads"

	• Low glycaemic index carbohydrates
Modify macronutrient composition	• Low carbohydrate diets
	• Low glycaemic index carbohydrate
	• Protein supplementation
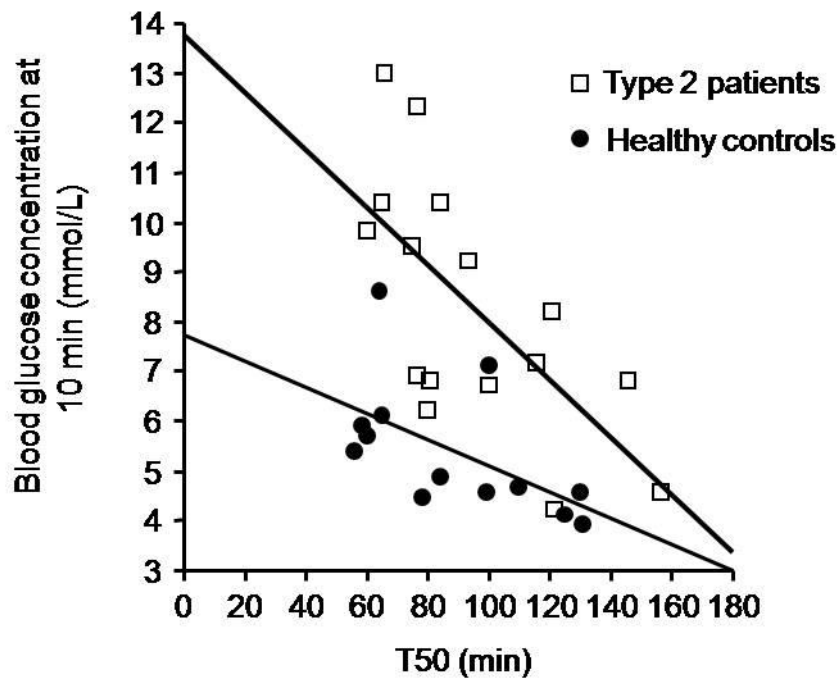
Figure 2.1 Relationship between the blood glucose concentration at 10 minutes and the gastric half-emptying time (T50) of a drink containing 75 g glucose in 300 mL water, in healthy subjects (open circles, r = -0.56, P < 0.05) and patients with type 2 diabetes (filled circles, r = -0.67, P < 0.005). Adapted from Jones et al. 1996.
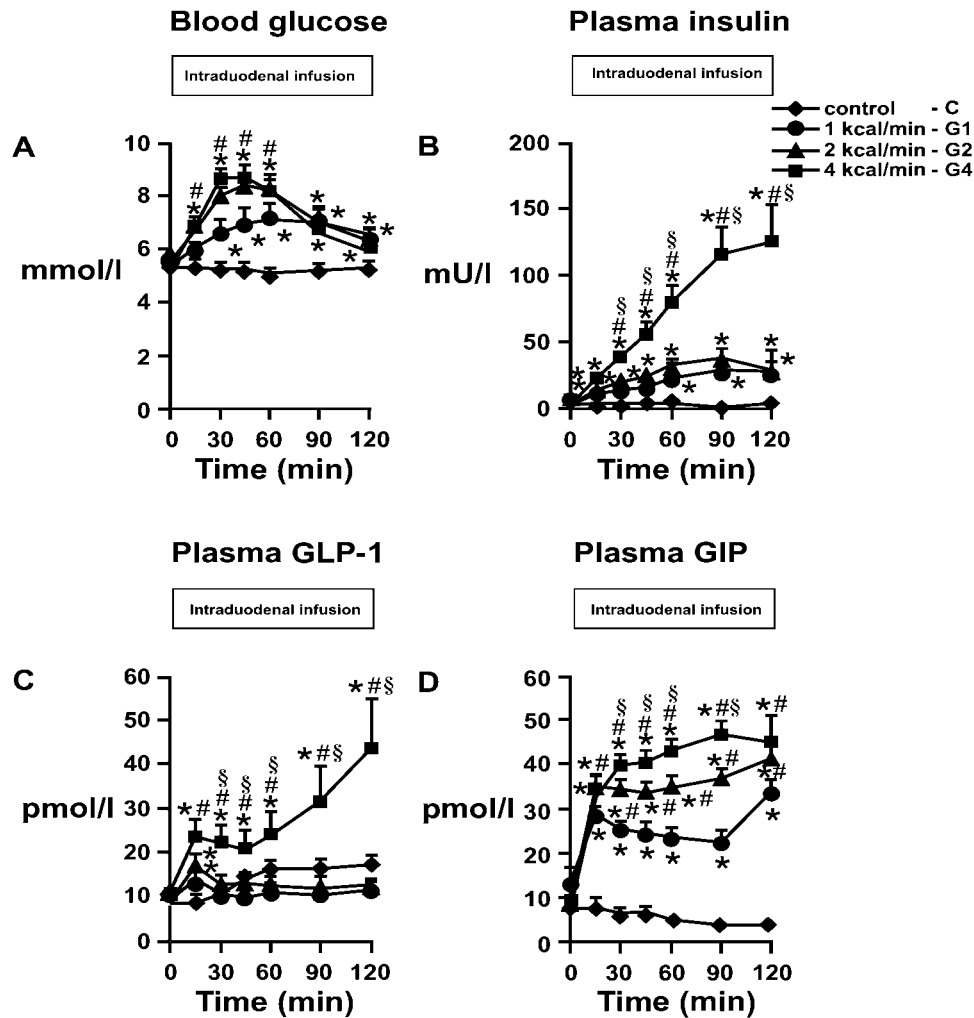
Figure 2.2 Blood glucose (A), plasma insulin (B), GLP-1 (C) and GIP (D) in response to intraduodenal glucose (25 %, 1390 mOsmol/L) infused over 120 minutes at rates of 1 ("G1"), 2 ("G2"), or 4 ("G4") kcal/min, or saline (4.2 %, 1390 mOsmol/L) control ("C"), in 10 healthy males. (A) * vs. control: $P < 0.05$, # 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: $P < 0.05$. (E) * vs. control: $P <$

0.05, # vs. G1: P < 0.01, § vs. G2: P < 0.01. Data are means ± SEM. Adapted

from Pilichiewicz et al. 2007a.
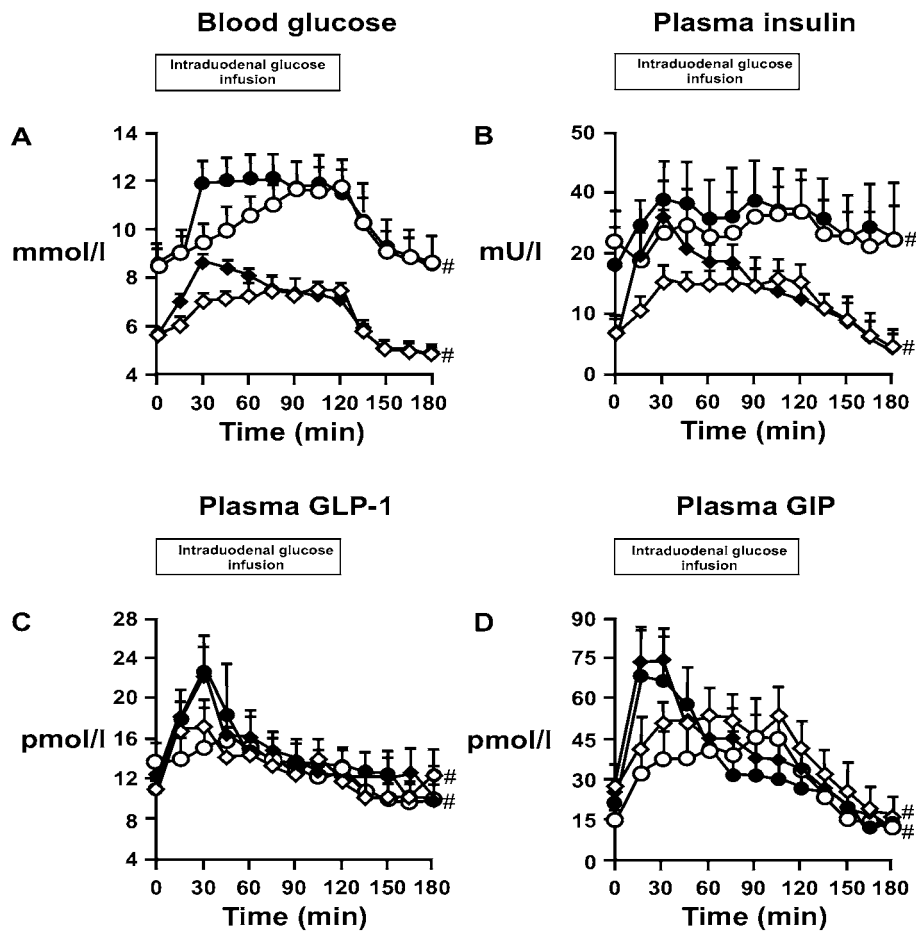
Figure 2.3 Effect of initially more rapid intraduodenal glucose infusion (3 kcal/min between t = 0 and 15 min and 0.71 kcal/min between t = 15 and 120 min) (closed symbols) compared to constant infusion (1 kcal/min between t = 0 and 120 min) (open symbols) in healthy subjects (squares) and patients with type 2 diabetes (circles) on blood glucose, plasma insulin, plasma GLP-1, and plasma GIP. Each pair of curves differs between 0 and 30 min for variable vs. constant intraduodenal infusion (P < 0.05). Adapted from O'Donovan et al. 2004b.

CHAPTER 3: GASTRIC MOTILITY IN PATIENTS WITH TYPE 2 DIABETES- DIAGNOSIS AND MANAGEMENT

Adapted from Ma J et al. Drugs. 69:971-986, 2009.

3.1 Introduction

Gastroparesis refers to the presence of delayed gastric emptying occurring in the absence of mechanical obstruction (Parkman et al. 2004). In the United States, gastroparesis-related hospitalizations doubled from 1995 to 2004 (Wang et al. 2008c). The most common disease complicated by gastroparesis is diabetes mellitus (Abell et al. 2006), accounting for about one third of patients with gastroparesis in a large study from a tertiary referral centre (Parkman et al. 2004). Gastroparesis often occurs in patients with longstanding diabetes and concomitant microvascular complications such as retinopathy, neuropathy, and nephropathy (Camilleri 2007). Most patients with gastroparesis present with upper gastrointestinal symptoms, such as nausea, early satiation, postprandial fullness, vomiting and bloating (Schvarcz et al. 1996; Ricci et al. 2000), although the correlation of symptoms with delayed gastric emptying is relatively weak, and some patients are asymptomatic (Jones et al. 1995). Furthermore, the contribution of disordered gastric emptying to impaired glycaemic control in insulin-treated diabetic patients (type 1 and type 2) has been generally underestimated.

3.2 Prevalence of impaired gastric emptying in diabetes

Gastric motor dysfunction in diabetes was first reported in 1937, when on X-ray examination, stomach contractions were observed to be „slow, lack vigor and die out quickly" compared to those in healthy subjects (Ferroir 1937). More recently, it has been demonstrated that gastric emptying is delayed in 30-50% of long-standing type 1 and type 2 patients (Figure 3.1) (Horowitz et al. 1991; Horowitz et al. 1996b; Horowitz et al. 2001), with the prevalence about equal in both types of diabetes (Moldovan et al. 2005). Gastric emptying of nutrient liquid has been reported to be abnormally rapid in „early" type 2 diabetes (Phillips et al. 1992), although this has not been observed consistently (Jones et al. 1996), and rapid emptying is uncommon in type 1 diabetes (Nowak et al. 1995).

3.2.1 Physiology of gastric emptying

3.2.2 Gastric motor and sensory dysfunction in diabetes

Delayed gastric emptying can potentially result from disordered function of the proximal stomach, antrum, pylorus, and duodenum, or from incoordination of motor activity between different parts of the stomach. Impaired function of the proximal stomach has been found in type 1 patients, with a reduced fasting fundic tone and impaired accommodation to nutrient ingestion (Samsom et al. 1995; Samsom et al. 1998). In the distal stomach, infrequent or low amplitude

contractions of the antrum have been observed (Samsom et al. 1996), as well as incoordination between the antrum and duodenum (Wehrmann et al. 1991; Kawagishi et al. 1994). Prolonged and excessive tonic and phasic contractions of the pylorus have been documented in some patients (Mearin et al. 1986). There is also an increased prevalence of dysrhythmias of the gastric slow wave in diabetes, either abnormally fast (tachygastria) or slow (bradygastria) (Koch 2001), although the association with delayed gastric emptying is imprecise (Nohara et al. 2006). In summary, the motor dysfunctions observed in diabetic gastropathy are heterogeneous, which could explain the mixed success of existing treatments, and may have implications for the need to tailor therapy to individual patients. Furthermore, most studies evaluating disordered gastric function in diabetes have not been done during euglycaemia, so the contribution of a potentially reversible component of motor dysfunction attributable to acute hyperglycaemia, as discussed below, cannot be determined.

The presence of delayed gastric emptying does not appear fully to explain upper gastrointestinal symptoms, as discussed earlier (Jones et al. 1995). Several studies have evaluated perceptions of proximal gastric distension in diabetic patients, and have reported these to be increased when compared with healthy controls (Samsom et al. 1995; Rayner et al. 2000c; Kumar et al. 2008), implying that visceral hypersensitivity potentially contributes to the aetiology of

gastrointestinal symptoms in patients with diabetes.

3.3 Pathogenesis of diabetic gastroparesis

The pathogenesis of gastroparesis is poorly understood but appears to be multi-factorial. Many recent insights have been gained from examination of gastric tissue in animal models and humans with diabetes, while potentially reversible alterations in gastric motor function can occur with acute variations in the blood glucose concentration.

3.3.1 Anatomical and functional observations

While irreversible autonomic neuropathy has been regarded widely as the underlying cause of gastroparesis, recent evidence indicates a heterogeneous picture, with a range of fixed pathology and potentially reversible functional abnormalities. Animal models of diabetes have shown deficiencies of inhibitory neurotransmission, reduced numbers of ICCs, decreased extrinsic autonomic neuron numbers and apoptosis of enteric neurons (Rayner and Horowitz 2006). Diabetic rodents exhibit pathologically distinctive dystrophic axons and dendrites in sympathetic ganglia without neuronal loss (Schmidt 2002), and these abnormalities can be reversed by exogenous insulin or pancreatic islet transplantation (Schmidt et al. 1983). In the NOD mouse model of diabetes,

gastric ICCs are dependent on both insulin and IGF-1 (Horvath et al. 2005), and deficiency of these results in ICC depletion (Horvath et al. 2006). Loss of neuronal nitric oxide synthase (nNOS) expression within myenteric neurons is associated with dysfunction of the antrum and pylorus in diabetic mice (Watkins et al. 2000; Gangula et al. 2007).

Evaluation of gastrointestinal autonomic function in humans is difficult, and tests of cardiovascular autonomic function are widely used as a surrogate marker. However, the correlation between disordered motility and abnormal cardiovascular autonomic function in diabetic patients is weak (Horowitz et al. 2002b; Asakawa et al. 2005), suggesting that autonomic neuropathy is unlikely to be the sole explanation for diabetic gastropathy. Recent studies of tissue from humans with gastroparesis undergoing surgical procedures have provided useful information about the pathogenesis of this condition. Documented changes include loss of neurons in the myenteric plexus of the stomach (Yoshida et al. 1988), while absent or decreased ICCs have been found in about a third of patients with diabetic or idiopathic gastroparesis, correlating with abnormalities of gastric slow waves (Forster et al. 2005). There may be a preferential loss of inhibitory neurotransmission (Pasricha et al. 2008), although changes are heterogeneous when different patients with gastroparesis are compared. Some patients also have abnormalities of gastric smooth muscle, with fibrosis of the muscle layer (Pasricha et al. 2008) and inclusion bodies within myocytes

(Ejskjaer et al. 1999) evident in some reports.

3.3.2 Effects of hyperglycaemia

Regardless of the presence of fixed pathological changes in diabetes, acute variations in blood glucose levels have a major impact on gastric motor function in both healthy subjects and diabetic patients (Rayner et al. 2001). In patients with diabetes, marked hyperglycaemia (16-20 mmol/l) leads to prolongation of the lag phase and half emptying time of solids and liquids, increasing the proportion of patients in the „gastroparetic" range when compared with euglycaemia (5-8 mmol/l) (Fraser et al. 1990). Even in the physiological postprandial glycaemic range, the rate of gastric emptying is slower at a blood glucose of 8 mmol/l than 4 mmol/l in both healthy subjects and patients with uncomplicated type 1 diabetes (Schvarcz et al. 1997). In contrast, insulin-induced hypoglycaemia accelerates gastric emptying, even in type 1 patients with gastroparesis (Schvarcz et al. 1993; Schvarcz et al. 1995; Russo et al. 2005). Acute hyperglycaemia is associated with reduction of fundic tone, suppression of antral waves, increased pyloric contraction (Rayner et al. 2001), and induction of abnormal gastric electrical rhythms (Jebbink et al. 1994; Hasler et al. 1995). Hyperglycaemia also increases the perception of gastrointestinal symptoms in patients with diabetes (Barnett and Owyang 1988; Russo et al. 2005). However, the chronic effects of high blood glucose concentrations on gastric motor and

sensory function are not known. The effects of variations in glycaemia on gastric emptying in type 2 diabetes are less well-documented than for type 1, although cross-sectional data imply similar effects in this group (Horowitz et al. 1989).

3.4 Clinical presentation

Disordered gastric motility in diabetes is associated with upper gastrointestinal symptoms, poor drug absorption, impaired glycaemic control, malnutrition, poor quality of life and a high rate of hospitalization (Gallar et al. 1993; Bell et al. 2002). Gastric bezoar formulation is a rare complication of delayed gastric emptying in diabetic gastroparesis (Whitson et al. 2008), probably reflecting the reduction in gastric phase 3 activity (Horowitz et al. 2002b).

The prevalence of gastrointestinal symptoms, such as fullness, postprandial nausea, vomiting, abdominal pain and bloating (Schvarcz et al. 1996; Ricci et al. 2000), is greater in patients with diabetes compared with nondiabetic groups (Bytzer et al. 2001), although the presence of symptoms correlates poorly with the rate of gastric emptying; only postprandial fullness appears to be a significant predictor of delayed gastric emptying of solids (Jones et al. 2001). Gastrointestinal symptoms seem to be more common in those with worse chronic glycaemic control as assessed by glycated hemoglobin (Bytzer et al. 2001; Quan

et al. 2008) and occur more frequently in patients with markers of psychological disorders (Talley et al. 2001).

The rate of gastric emptying regulates the delivery of carbohydrate and other macronutrients to the small intestine, and therefore has a major impact on the postprandial blood glucose excursion. It has been established that variations in the rate of gastric emptying account for 35 % of the variance in the initial rise of blood glucose after a 75 g oral glucose load, in both healthy subjects and patients with type 2 diabetes (Horowitz et al. 1993; Jones et al. 1996). Even minor changes of initial rate of carbohydrate delivery to the small intestine can have a substantial impact on glycaemia (O'Donovan et al. 2004b; Chaikomin et al. 2005). Thus, dietary and pharmaceutical interventions that modify gastric emptying have the potential to affect postprandial glycaemia in patients with diabetes. Accelerating gastric emptying with erythromycin, increases the postprandial blood glucose concentrations, whereas slowing emptying with morphine reduces postprandial glycaemic response in patients with type 2 diabetes (Gonlachanvit et al. 2003). Similarly, after a standard meal, the insulin dosage to sustain normoglycaemia is substantially less during the first two hours of the postprandial period in type 1 patients with gastroparesis than those without (Ishii et al. 1994). Indeed, delayed gastric emptying has recently been documented as an important cause of otherwise unexplained hypoglycaemia in

insulin-treated patients (of which an increasing proportion have type 2 diabetes) - a phenomenon that has been termed „gastric hypoglycaemia‟ (Horowitz et al. 2006; Ohlsson et al. 2006). Therefore, it is important to match the rate of carbohydrate delivery into the small intestine with the action of exogenous insulin; this may potentially entail accelerating gastric emptying so that nutrient delivery occurs in a more predictable fashion. In contrast, in patients with type 2 diabetes not treated with insulin, slowing the absorption of nutrients is often beneficial for glycaemic control so long as symptoms do not arise. An increase in soluble fibre (Chandalia et al. 2000), adding the non-absorbable polysaccharide, guar gum (Russo et al. 2003), or fat given before (Gentilcore et al. 2006b), or with, a carbohydrate-containing meal (Cunningham and Read 1989) all improve blood glucose acutely by slowing gastric emptying of carbohydrate. Indeed, slowing of gastric emptying is the predominant mechanism by which GLP-1 and its analogues, such as exenatide, reduce postprandial hyperglycaemia in patients with type 2 diabetes managed by diet and/or oral hypoglycaemic drugs (Cervera et al. 2008; Linnebjerg et al. 2008).

Delayed gastric emptying also influences the delivery and absorption of orally administered drugs in the small intestine, generally resulting in later, or fluctuating, maximal serum concentrations (Hebbard et al. 1995). This is particularly important when a rapid onset of drug action is required and has been

documented with oral hypoglycaemic drugs (Groop et al. 1989). Drugs with longer half-lives are less likely to be affected (Horowitz et al. 2002b).

3.5 Diagnosis

Diabetic patients with upper gastrointestinal symptoms suggestive of gastroparesis should be investigated to exclude other causes, and to ascertain whether gastric emptying is indeed delayed. Acute, reversible disorders of gastrointestinal function due to drugs, infection, or electrolyte disorders must be excluded (Horowitz et al. 2001) (Table 3.1). Other disorders that result in similar symptoms, including peptic ulcer disease, gastroesophageal reflux disease, gastric cancer, pancreatic or biliary disease, and gastric outlet or mechanical small bowel obstruction (Parkman et al. 2004), need to be excluded with appropriate investigations, such as endoscopy and contrast radiology studies. Diabetic gastroparesis also needs to be differentiated from chronic gastric stasis due to previous surgery, metabolic and endocrine disease (liver or renal disease, thyroid dysfunction), central nervous system disease (brain tumour, stroke or trauma), malignancy and HIV infection (Horowitz et al. 2002b).

3.5.1 Methods for measuring gastric emptying

Evaluation of solid emptying is more sensitive than that of low-nutrient liquid or

semi-solid meals in the diagnosis of gastroparesis (Wright et al. 1985), and there is debate as to whether liquid emptying should also be studied; some patients will only exhibit delay of the latter. Gastric emptying can be affected acutely by many factors including medications, smoking, and the blood glucose concentration (Camilleri et al. 1998; Maurer and Parkman 2006). Medications which may influence gastric emptying should be withdrawn for 48-72 hours, or for the half-life of the drug, prior to the test (Camilleri et al. 1998), smoking should be avoided on the test day (Abell et al. 2008), and the blood glucose concentration should be monitored, and should ideally be below 10 mmol/L at the beginning of the test (Abell et al. 2008). Failure to demonstrate delayed gastric emptying need not imply that symptoms are not attributable to „diabetic gastropathy‟, of which abnormal visceral sensation may be a component, as discussed earlier, but does help guide the choice of drug therapy.

<u>Scintigraphy</u>

Scintigraphy is regarded as the „gold standard‟ for measurement of gastric emptying of solid and nutrient liquid meals. After meal consumption, a gamma camera is used to monitor scintigraphic counts in various „regions of interest‟. The parameters that may be derived include the half-emptying time of solids and liquids, lag phase for solids, and intragastric distribution of a meal (proximal versus distal retention). The percentage of meal retention at the end of each hour

may be more accurate than the half emptying time (Abell et al. 2008). It has been suggested that extending the study time up to four hours increases the sensitivity for diagnosis of delayed gastric emptying compared with the retention at two hours (Guo et al. 2001), although meal retention at three hours correlates well with the four hour value (Ziessman et al. 2007). Intragastric distribution of the meal is frequently abnormal in diabetes (Jones et al. 1995), but meal retention in the whole stomach is used as the diagnostic measure. The standardization of the meal between centres has been a major limitation, rectified to some extent by recent „consensus" guidelines, which recommend a low fat egg white meal labelled with ^{99m}Tc sulfur colloid, and consumed with jam and toast as a sandwich, with a glass of water (Abell et al. 2008). Despite these guidelines, some issues are unresolved, including whether gastric emptying of nutrient liquid should also be measured. Although criteria of „severity" based on the degree of retention at four hours were suggested in the „consensus" guidelines, these do not include any measure of symptoms and therefore may not be the most appropriate guide.

Other measurement techniques

The limitations of scintigraphy are that patients are exposed to a modest dose of radiation, and the test is relatively expensive and confined to specialist centres. This has made the use of breath tests an appealing option, at least as a screening

tool for delayed gastric emptying. Breath tests employ non-radioactive ^{13}C-acetate or -octanoic acid as a label and are safe, easy to administer and inexpensive (Chew et al. 2003; Bures et al. 2005; Sanaka et al. 2006). After ingestion, the labelled meal passes through the stomach to the small intestine, where the ^{13}C-acetate or -octanoate is absorbed, metabolized into $^{13}CO_2$ in the liver and exhaled via the breath, with the rate of gastric emptying being the rate-limiting step. Breath samples are collected and analyzed for $^{13}CO_2$ by mass spectrometry (Sanaka et al. 2007). Breath tests correlate well with scintigraphy in healthy subjects and patients with diabetes (Pfaffenbach et al. 1995; Zahn et al. 2003), with a sensitivity and specificity $\geqslant$ 80 % for detecting delayed gastric emptying (Viramontes et al. 2001). This method does assume normal intestinal absorption and pulmonary excretion; therefore, further validation is required in various patient groups. It is not valid in those patients with markedly delayed gastric emptying.

Ultrasonography is noninvasive, and 2-dimensional ultrasound has been validated for measuring the emptying of liquids or semi-solids, as well as antral motility and transpyloric flow (Haruma et al. 2008). 3-dimensional ultrasound offers more comprehensive imaging of the whole stomach (Hveem et al. 1996; Gilja et al. 1997; Gentilcore et al. 2006c; Stevens et al. 2008a). However, obesity and abdominal gas, together with the need for an experienced operator, limit the

wide use of ultrasonography.

Electrogastrography noninvasively measures gastric myoelectrical activity by placing surface electrodes on the epigastric skin (Koch 2001). The normal frequency of the gastric slow wave is about 3 cycles per minute. As discussed, gastric dysrhythmia is often associated with disordered gastric emptying and symptoms (Kara et al. 2006), but the relationship between symptoms and dysrhythmia is variable, and electrogastrography should be viewed as a research tool at present.

A barium meal has a place in excluding mucosal lesions or obstruction, but has no role in quantifying gastric emptying.

3.6 Treatment

Treatment of patients with diabetic gastroparesis aims to relieve gastrointestinal symptoms, improve nutritional status, enhance quality of life, and optimize glycaemic control. The latter represents a major goal in the management of diabetes, in order to reduce the risk of micro- and macrovascular complications (UK Prospective Diabetes Study (UKPDS) Group 1998). Careful attention should be paid to improving glycaemic control, which has the capacity to affect gastric motility, as discussed above. Type 2 patients might need insulin therapy

rather than oral medication, while patients with type 1 diabetes might benefit from an insulin pump to optimize blood glucose control (O'Donovan et al. 2003). Patients with unexplained hyperglycaemia or hypoglycaemia should be screened for delayed gastric emptying, in addition to those with upper gastrointestinal symptoms. Patients with severe symptoms may require hospitalization to stabilize their fluid and electrolyte status and provide nutritional support. Many aspects of treatment for gastroparesis have not been evaluated in well–designed controlled trials, and this represents a major limitation.

3.6.1 Dietary management

There have been no published studies evaluating the effects of dietary modification in diabetic gastroparesis. Since fibre and fat in particular have the potential to slow gastric emptying, a low-fat, low-fibre diet is generally recommended. Frequent (4-6 per day), small-volume meals are also advocated (Olausson et al. 2008), with an increased percentage of nutrients in liquid form, as gastric emptying of liquids is often less affected than that of solids (Wright et al. 1985).

3.6.2 Pharmacological interventions

Most patients with gastroparesis require pharmacological therapy to relieve

symptoms and/or accelerate gastric emptying. Given the weak correlation between the presence of symptoms and the degree of impairment of emptying, it is not surprising that the improvement of gastric emptying is not closely related to the relief of gastrointestinal symptoms during treatment (Talley 2003). Prokinetic agents including erythromycin, metoclopramide and domperidone are most commonly used in the treatment of diabetic gastroparesis (Table 3.2). Prokinetic drugs tend to improve gastric emptying and/or symptoms in a dose-dependent fashion, although their mechanisms of action are diverse. The acceleration of gastric emptying is typically greater when emptying is more delayed at baseline, and can be attenuated during acute hyperglycaemia, at least for erythromycin and cisapride (Petrakis et al. 1999; Horowitz et al. 2002a). Some prokinetic drugs have additional effects such as central antiemetic properties or suppression of visceral sensation. The choice of medication is dependent on potential side effects, the nature of the symptoms and concomitant diseases, drug availability, and personal preference of the clinician. In a systematic analysis of clinical trials of prokinetic agents, erythromycin seemed to have the strongest effect on gastric emptying when compared with domperidone, cisapride or metoclopramide (Sturm et al. 1999), while erythromycin and domperidone appeared to be most effective in relieving symptoms (Sturm et al. 1999). Nevertheless, there are few head-to-head comparisons of drugs, and little information about combinations of agents. Furthermore, well-designed double

blind controlled trials generally report more modest effects than open label or single-blind studies (Sturm et al. 1999).

Erythromycin

Erythromycin, a motilin receptor agonist, is one of the most potent gastrokinetic drugs when given by the intravenous route (Janssens et al. 1990; Ehrenpreis et al. 1998); therefore, it has been used as first-line therapy for hospitalized patients with severe gastroparesis (Rayner and Horowitz 2005). Administration of erythromycin is associated with increased antral contractions and accelerated emptying of solids and liquids (Janssens et al. 1990; Richards et al. 1993). Oral erythromycin suspension results in an improvement of gastric symptoms (Dhir and Richter 2004), although in the long term, tolerance frequently develops due to the downregulation of motilin receptors (Richards et al. 1993).

Gastrointestinal symptoms, such as abdominal cramps, nausea and vomiting are common side effects of erythromycin, while the drug can prolong the QT interval, with a consequent risk of sudden death (Ray et al. 2004), especially with given concurrently with CYP3A inhibitors (Ray et al. 2004). Alteration of intestinal flora and fungal infections are further concerns with long-term use. Other macrolide antibiotics, such as clarithromycin and azithromycin, have been reported to have prokinetic properties, but their potential has not been evaluated

sufficiently in clinical trials (Sengupta et al. 2006; Sutera et al. 2008).

Metoclopramide

Metoclopramide acts peripherally and centrally as a dopamine D_2 receptor antagonist and stimulates smooth muscle contraction by release of acetylcholine from enteric cholinergic neurons (5-HT_4 agonist) (Rabine and Barnett 2001). The former is associated with an antiemetic effect, but also entails a risk of adverse effects, including extrapyramidal reactions and hyperprolactinaemia. Metoclopramide in pill or liquid suspension forms, and as a suppository in some countries, is suitable for outpatients with gastroparesis. Subcutaneous administration provides comparable plasma concentrations to the intravenous route and is a useful alternative in patients who cannot tolerate oral medications (McCallum et al. 1991). Metoclopramide appears less effective than cisapride in improving the rate of gastric emptying, but its antiemetic effects provide significant symptom relief (McHugh et al. 1992). Tardive dyskinesia can occur in about 1 % of patients with long term use (Tonini et al. 2004), and may be irreversible.

Domperidone

Domperidone, acting on peripheral D_2 receptors, possesses similar antiemetic and prokinetic effects to metoclopramide. However, domperidone is not

associated with central nervous system side effects due to its poor penetration of the blood-brain barrier (Rayner and Horowitz 2005). It is, therefore, safe for use in Parkinson's disease, but like metoclopramide, can be complicated by hyperprolactinaemia (Rabine and Barnett 2001). Domperidone may be more effective than cisapride in children with diabetic gastroparesis (Franzese et al. 2002), while the combination of domperidone and cisapride was superior to cisapride alone in accelerating gastric emptying and improving gastrointestinal symptoms in patients with functional dyspepsia (Tatsuta et al. 1992). Domperidone is available in the US through the investigational new drug program.

Cisapride

Cisapride used to be the first line oral prokinetic agent in the treatment of gastroparesis, providing long-term symptomatic relief and improvement of gastrointestinal motility (Abell et al. 1991; Veysey et al. 2001). It mainly acts on the $5\text{-}HT_4$ receptors of the myenteric plexus to stimulate smooth muscle contraction, and accelerates solid and liquid emptying from the stomach (Horowitz et al. 1987). It also acts as a $5\text{-}HT_3$ antagonist, providing an antiemetic action. The effects on gastric emptying and improvement in symptoms appear to be sustained with long term use (Kendall et al. 1997; Braden et al. 2002).

Cisapride has been withdrawn from most markets due to its potential to prolong the QT interval, which has been associated with lethal ventricular arrhythmias (Tonini et al. 1999). Predisposing factors include high doses (80 mg/day) and combination with CYP3A inhibitors (O'Donovan et al. 2003). Cisapride is still available in many countries under restricted access arrangements, requiring close patient monitoring for ECG abnormalities and other risk factors. The maximum daily dose of cisapride should be limited to 40mg, and medications which delay cisapride metabolism such as azole antifungals (e.g. ketoconazole) and macrolide antibacterials (e.g. erythromycin), or which prolong the QT interval, should be avoided (Tonini et al. 1999).

Antiemetics

Antiemetic medications may be beneficial in relief of symptoms, particularly when nausea and vomiting are predominant, even though most do not accelerate emptying and some can delay it. The most common antiemetic medications include $5-HT_3$ receptors antagonists (eg. ondansetron), dopamine antagonists (eg. prochlorperazine), tricylic antidepressants (eg. amitriptyline or nortriptyline), cannabinoids (dronabinol) and antihistamines (cyclizine, dimenhydrinate and meclizine), while there are anecdotal reports regarding the neurokinin NK1 antagonist, aprepitant (Hasler 2007).

Other motilin agonists

Motilin is an endogenous hormone that accelerates gastric emptying by induction of phase III-like contractions (Itoh 1997), but its therapeutic use is limited by the need for intravenous administration and its short half life. Orally available motilin analogues have therefore been developed. These include ABT-229, which accelerated gastric emptying acutely (Verhagen et al. 1997), but failed to relieve symptoms in diabetic patients with gastroparesis, possibly due to tachyphylaxis (Tack and Peeters 2001; Thielemans et al. 2005). Unlike ABT–229, mitemcinal induces sustained acceleration of gastric emptying over 4 weeks in diabetic gastroparesis (McCallum and Cynshi 2007a), and shows modest benefit compared to placebo in terms of symptom relief (McCallum and Cynshi 2007b).

Itopride

Itopride acts as a dopamine D_2 receptor antagonist and an acetylcholinesterase inhibitor, and does not cross the blood-brain barrier (Iwanaga et al. 1994). One trial reported symptomatic benefit in patients with functional dyspepsia (Holtmann et al. 2006), but this was not confirmed in a larger study (Talley et al. 2008). Furthermore, itopride (200 mg t.i.d. for 7 days), had minimal effect on the gastric emptying of solids and liquids in patients with longstanding diabetes mellitus; only in those with delayed emptying at baseline did the improvement in liquid emptying reach statistical significance (Stevens et al. 2008b). In this latter

study, symptoms were not improved, but patients were not selected on the basis of being symptomatic.

Ghrelin

Intravenous infusion of ghrelin, an orexigenic hormone, accelerates gastric emptying in both diabetic patients and those with functional dyspepsia (Murray et al. 2005; Tack et al. 2005). Ghrelin receptor agonists are in development, and accelerate gastrointestinal transit in animal models of diabetes (Zheng et al. 2008).

Other agents

Acute and chronic administration of levosulpiride, a dopamine D_2 receptor antagonist, is associated with accelerated gastric emptying and improvement in upper gastrointestinal symptoms in patients with diabetic gastroparesis (Mansi et al. 1995; Melga et al. 1997), but the drug is not available outside Europe. Although tegaserod, a 5-HT$_4$ agonist, improves gastric emptying, it has been withdrawn due to an excess of cardiac events (Morganroth et al. 2002; Degen et al. 2005b). Sildenafil has been reported to accelerate gastric emptying in rodent models of diabetes (Watkins et al. 2000), but this has not been consistently reported in patients with diabetic gastroparesis (Bianco et al. 2002; Dishy et al. 2004). For a subset of patients, where abdominal pain is a major symptom,

gabapentin and pregabalin might be useful agents (Duby et al. 2004; Tolle et al. 2008), but there are no controlled trials of these medications for this indication. The CCK-A receptor antagonist, dexloxiglumide, has been reported to reduce dyspeptic symptoms induced by nutrients in the small intestine (Feinle et al. 2001).

3.6.3 Physical treatment

Several non-pharmaceutical treatments have been the focus of recent attention in the management of gastroparesis, including gastric electrical stimulation and intrapyloric injection of botulinum toxin.

<u>Gastric electrical stimulation</u>

Gastric electrical stimulation shows promise in the treatment of refractory gastroparesis. However, it should be noted that there are very limited controlled data regarding its efficacy (Abell et al. 2003; Jones 2008), and this will need to be addressed before this therapy can be recommended outside the context of clinical trials. Two stimulation parameters, involving long pulses (low frequency/high energy), or short pulses (high frequency/low energy) have been used. Stimulation with long pulses delivers current at about 3 cycles per minute, and in patients with refractory gastroparesis can entrain the gastric slow wave and reverse gastric dysrhythmias, accelerate gastric emptying, and improve

symptoms (Lin et al. 1998; McCallum et al. 1998). However, an implantable device capable of delivering the required energy for this mode of stimulation is not currently available. In contrast, gastric electrical stimulation with short pulses, such as that approved by the US Food and Drug Administration, called „Enterra" Therapy, delivers pulses at about 12 cycles per minute, which do not entrain the slow wave, and probably do not accelerate gastric emptying (Abell et al. 2002; Lin et al. 2004; Lin et al. 2008). Electrical stimulation is performed by inserting a pair of electrodes into the serosa of the greater curvature via laparoscopy or laparotomy (Zhang and Chen 2006); endoscopic placement of electrodes also appears to be feasible (Elfvin et al. 2007; Xu et al. 2007; Pinto et al. 2008; Sallam et al. 2008).

In uncontrolled studies, gastric electrical stimulation has been reported to reduce the severity and frequency of symptoms such as nausea and vomiting significantly, and to enhance quality of life (Mason et al. 2005; McKenna et al. 2008), with improvement maintained over three years (Lin et al. 2006). Patients with diabetic gastroparesis apparently respond better than idiopathic patients, while those with pain (as opposed to nausea and vomiting), or using narcotics at baseline, tend to respond poorly (Maranki et al. 2008).

The mechanism by which gastric electric stimulation acts is unclear. Stimulation

of vagal afferents with increased thalamic activity, increased perception threshold to gastric distention, and improvement in postprandial gastric accommodation have all been reported (McCallum et al. 2006).

Intra-pyloric injection of botulinum toxin

In a manometric study from Mearin et al. (Mearin et al. 1986), a majority of patients with diabetic gastroparesis were found to have prolonged, excessive tonic contractions of the pylorus („pylorospasm"). Botulinum toxin (Botox) blocks the release of neuromuscular transmitter at cholinergic terminals (Jankovic and Brin 1991) and is used widely for treatment of achalasia by injection into the lower oesophageal sphincter (Pasricha et al. 1995). In uncontrolled series, involving patients with diabetic and idiopathic gastroparesis, injection of Botox into the pylorus was associated with acceleration of gastric emptying and reduction in pyloric contractions, paralleled by relief of symptoms (Ezzeddine et al. 2002; Lacy et al. 2002; Miller et al. 2002; Lacy et al. 2004). However, two recent sham-controlled trails, one in patients with predominantly idiopathic gastroparesis (Arts et al. 2007), and the other with a large proportion of diabetic patients (Friedenberg et al. 2008), failed to show superiority of Botox over saline in improving gastric emptying or symptoms (Arts et al. 2007; Friedenberg et al. 2008). Unfortunately, pyloric manometry was not carried out before or after injection in either study. Further evaluation is required before the

therapy should be used widely.

Acupuncture

Acupuncture has been used for gastrointestinal complaints for thousands of years in China, with reported efficacy for symptom relief (Wang 2004) and acceleration of solid gastric emptying in patients with diabetic gastroparesis in a sham-controlled study (Wang et al. 2008a). The underlying mechanisms are unclear and cannot be attributed to differences in hormones such as motilin, CCK or vasoactive intestinal peptide (VIP) (Wang et al. 2007). Further investigations are required to evaluate the benefits of acupuncture in patients with symptomatic diabetic gastroparesis.

3.6.4 Surgical therapy

Most trials of surgical procedures for refractory gastroparesis are small, uncontrolled, or retrospective, with very limited post-surgical follow-up. Therefore, these procedures should be regarded as a last resort, and should ideally be performed in centres with experience in managing such patients (Jones and Maganti 2003). Gastrostomy may be performed to relieve nausea, vomiting, pain and bloating, whereas jejunostomy is indicated to maintain hydration, nutrition and glycaemic control (Jones and Maganti 2003). Subtotal or complete gastrectomy and reconstruction is used as the last resort for severe refractory

gastroparesis and revision of postoperative gastroparesis (Rayner and Horowitz 2005). Pancreatic transplantation is reported to benefit diabetic patients with gastroparesis, with improvement in both gastric emptying and symptoms (Cashion et al. 2004).

3.7 Summary and implications

The prevalence of gastroparesis associated with diabetes is likely to increase in the coming years. Patients may present with a wide spectrum of symptom severity, or may be asymptomatic, but have disordered glycaemic control. Available therapeutic options are limited, while progress in the development of new medications is suboptimal, although there have been some promising developments. The pathogenesis of gastroparesis is complex and incompletely understood, and will need further investigation in order to provide more specific and effective therapy.
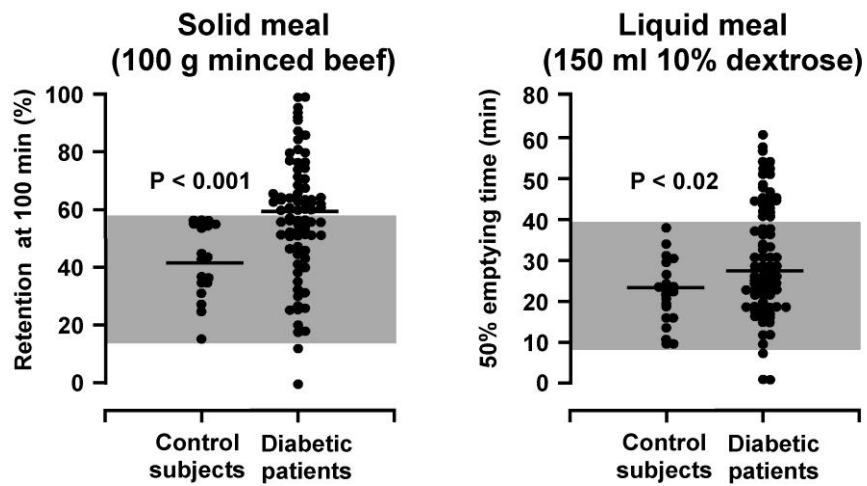
Figure 3.1 Gastric emptying, expressed as retention of a solid meal (minced beef) at 100 minutes, and 50 % emptying time of a nutrient liquid (10 % dextrose), in 87 patients with diabetes and 25 healthy subjects. The range of gastric emptying rates in the healthy subjects is represented by the shaded area. Adapted from Horowitz et al. 1991.

Table 3.1 Potential reversible causes of gastroparesis. Adapted from Horowitz et al. 2001.

- ✧ Drugs (eg. anticholinergics, calcium channel antagonists, opiates, levodopa, octreotide, cannabis and alcohol)
- ✧ Electrolyte or metabolic disturbance (hyperglycaemia, hypokalaemia, hypomagnesaemia, hyperthyroidism, hypothyroidism, hypopituitarism, Addison's disease)
- ✧ Viral infection (gastroenteritis, herpes zoster)
- ✧ Postoperative ileus
- ✧ Critical illness

Table 3.2 Prokinetic drugs used in the diabetic gastroparesis. Adapted from O'Donovan et al. 2003.

Drug	Mechanism	Route	Dose	Adverse effects
Erythromycin	Motilin agonist	IV, oral	50-250mg (t.i.d. or q.i.d)	Nausea, vomiting, abdominal pain
Metoclopramide	D_2 antagonist 5-HT_4 agonist 5-HT_3 antagonist	Oral, SC, IM, IV	10mg (t.i.d. or q.i.d)	Dystonia, tardive dyskinesia, sedation, hyperprolactinaemia
Domperidone	D_2 antagonist	Oral	10-20mg (b.i.d to q.i.d)	Hyperprolactinaemia
Cisapride	5-HT_4 agonist 5-HT_3 antagonist	Oral	10-20mg (b.i.d to q.i.d)	Arrhythmia, abdominal pain, diarrhoea

5-HT:serotonin; D:dopamine; IM:intramuscular; IV: intravenous; SC: subcutaneous;
BID: twice daily; TID: three times daily; QID: four times daily

CHAPTER 4: METHODOLOGIES

4.1 Introduction

The techniques and methods used in the studies documented in this thesis were diverse and included intubation of the upper gut to deliver intraduodenal infusions, measurement of gastric emptying by scintigraphy and breath tests, completion of diet diaries, evaluation of appetite by validated visual analogue scales, measurement of energy intake at a buffet meal, assays for gut hormones and the glucose analogue, 3-O-methylglucose (3-OMG), and assessment of autonomic nerve function.

4.2 Technique for positioning an intraduodenal catheter

In studies involving intraduodenal nutrient infusion (Chapters 9 and 11), a multilumen silicone manometry catheter (Dentsleeve International, Mui Scientic, Mississauga, Canada) incorporating multiple antral and duodenal sideholes and a pyloric sleeve sensor, was inserted through an anaesthetised nostril. The catheter was allowed to pass through the stomach into the duodenum by peristalsis, so that the sleeve sensor straddled the pylorus. The correct position of the catheter was monitored continuously by measuring the transmucosal potential difference (TMPD) at the most distal antral channel ($\sim$ -40 mV) and the most proximal duodenal channel ($\sim$ 0 mV), at either end of the sleeve sensor (Heddle et al.

1988a). For this purpose the two channels were each connected to a calomel half cell (Ionode, Brisbane, Australia) by Dri-Ref^{TM} series reference electrodes filled with 3 M KCl and a small amount of hydrogel (World Precision Instruments, FL, USA) (Heddle et al. 1988a; Heddle et al. 1988b). A reference electrode was connected to a 21G cannula filled with 0.9 % saline, inserted subcutaneously in the forearm. The criteria for correct catheter position were (i) antral TMPD < -20 mV, (ii) duodenal TMPD > -15 mV, and (iii) difference > 15 mV (Heddle et al. 1988a). The catheter included a large diameter channel (0.9 mm diameter), opening ~ 14.5 cm distal to the pylorus, which allowed for intraduodenal infusions via a volumetric infusion pump (Gemini PC-1; IMED Corp, San Diego, CA, USA).

4.3 Techniques for measuring gastric emptying

4.3.1 Scintigraphy

Scintigraphy was used to quantify the rate of emptying and intragastric distribution of a semi-solid high carbohydrate meal (Chapters 5 and 7). In these studies, it was essential to detect even relatively minor differences in gastric emptying. Hence, the „gold standard" technique was used. The meal consisted of 65 g powdered potato (Deb, Epping, Australia) and 20 g glucose (total carbohydrate content: 61 g; 301 kcal), reconstituted with 250 ml water and

labelled with 20 MBq ^{99m}Tc-sulphur colloid (Gentilcore et al. 2006b). After meal consumption, a gamma camera was used to monitor the amount of intraabdominal radioactivity. Images were acquired as 60-sec frames for the first 60 min and 3-min frames between 60 - 180 min. A „region of interest" was drawn around the whole stomach, and was subsequently divided into proximal and distal regions (Collins et al. 1991). Gastric emptying curves for each of these regions, expressed as percent retention over time, were derived. The retention of the meal in the total, proximal and distal stomach at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min was calculated. Data were adjusted for subject movement, radionuclide decay, and gamma-ray attenuation. The lag phase was defined visually as the time period before any radioactivity was present in the proximal small intestine. For the total stomach the half emptying time (T50) was also determined (Collins et al. 1983).

4.3.2 Breath tests

Breath tests are an indirect, but noninvasive and low-cost, measure of gastric emptying of liquid and solid meals. ^{13}C-acetate was used to label a liquid meal (Chapter 10) and ^{13}C-octanoic acid was incorporated into a solid meal (Chapter 12). In these studies, a less precise technique than scintigraphy was used to quantify gastric emptying for logistical reasons, including the potential to compromise the primary endpoint, if the studies were performed in a busy

Nuclear Medicine Department. With the breath test, the labelled meal passes through the stomach to the small intestine where the ^{13}C-octanoic acid, or ^{13}C-acetate, is absorbed. The rate of gastric emptying is the rate-limiting step in the absorption of the ^{13}C isotope and its metabolism to $^{13}CO_2$, which is then excreted in the breath. End-expiratory breath samples were collected at baseline and at pre-determined intervals after the meal and analyzed for $^{13}CO_2$ by isotope ratio mass spectrometry (Europa Scientific, ABCA model 20/20, Crewe, UK) (Sanaka et al. 2007). The ratio of $^{13}CO_2$ to $^{12}CO_2$ was calculated in each breath sample (Sun et al. 2003). Data were corrected for age, gender and body weight (Schofield 1985). T50 and gastric emptying coefficient (GEC) were calculated using the method of Ghoos et al which has been validated against scintigraphy (Ghoos et al. 1993).

4.4 Measurement of appetite and energy intake

4.4.1 Three-Factor Eating Questionnaire

The Three-Factor Eating Questionnaire, a well developed psychometric test, was used to evaluate eating behaviour (Chapters 6 and 7) (Stunkard and Messick 1985). It includes two parts, with 36 items in part 1 and 15 items in part 2. The questionnaire allows the calculation of a score for each of three factors: (i) „cognitive restraint of eating", (ii) „disinhibition" and (iii) „hunger" (Stunkard and

Messick 1985). „Restrained eaters" are defined on those who limit their energy intake in order to control, or reduce, their body weight. In the studies presented in this thesis, volunteers with a score ≥ 11 were considered to be „restrained eaters" and were excluded, in order to evaluate the effects of different dietary inventions on energy intake optimally.

4.4.2 Visual analogues scales (VAS)

VAS questionnaires were used to assess appetite and gastrointestinal sensations in the study reported in Chapter 6. They consisted of 13 different questions (Parker et al. 2004), each followed by a 100 mm horizontal line. For each question, the subject was asked to place a vertical mark at a point along the line to indicate the strength of that sensation eg. „How hungry do you feel?" or „How strong is your desire to eat" within the range from „weak" (to the left) to „strong" (to the right). Scores were determined by measuring the distance from the starting point (on the left) to the mark. Other sensations evaluated in this way included fullness, nausea, bloating, and abdominal discomfort. VAS have been validated as being predictive of food intake in both young and old subjects (Parker et al. 2004).

4.4.3 Energy intake

Food consumption from an *ad libitum* meal, including energy intake and its macronutrient distribution, was used to measure energy intake in the study reported in Chapter 6 (Stubbs et al. 1998). Volunteers were offered a selection of foods in quantities greater than what they would be expected to eat, including whole meal bread, white bread, cheese, chicken, ham, cucumber, lettuce, tomato, yoghurt, custard, apple, banana, iced coffee, water, orange juice, margarine and mayonnaise, and were allowed to eat at will for up to 30 minutes (Lavin et al. 1996). Energy intake was assessed by the difference in weight of each food item before and after consumption, to the nearest 0.1 g. FoodWorks software (version 3.1, Xyris Software, Highgate Hill, Qld, Australia) was used to calculate the macronutrient content of the various foods.

4.4.4 Three-day diet diary

Food diaries were used to assess habitual food intake and eating patterns during various periods of intervention (Bingham et al. 1994). In Chapter 7, a 3-day diet diary was used to assess energy intake and macronutrient consumption of meals for three day periods at baseline, and before and after each 4-week dietary treatment. During these three days, subjects were asked to weigh all food eaten in their main meals and snacks using digital scales, and measure volumes of fluids

and alcoholic drinks using a „measuring" cup. The brand of bread and cereal, type of oil, and whether meat was fatty or lean, were also recorded. Energy intake (calculated as kcal) and macronutrient intake (calculated as g or % of total energy), were analyzed to the nearest 0.1 g or 0.1 kcal by FoodWorks software (version 3.1, Xyris Software, Highgate Hill, Qld, Australia).

4.5 Ambulant glucose monitoring

The continuous glucose monitoring system (CGMS) developed by MiniMed (Medtronic Minimed, CA, USA), incorporating a glucose oxidase based sensor, was used to measure the extracellular fluid glucose concentration in the subcutaneous tissue. In the study reported in Chapter 7, CGMS was performed concurrently with the assessment of energy intake by diet diary. CGMS has the capacity to measure glucose concentrations from 2.2 mmol/L to 22 mmol/L continuously and stores the „average" glucose measurement for every 5 min of sampling for up to 14 days. While wearing the monitor, there are some limitations to lifestyle, such as the inability to bathe or swim, but showering is possible. The CGMS system was calibrated using fingerprick blood glucose levels, measured by a glucometer four times each day (20 min before each of three main meals and once before bedtime). After three days recording, stored data were downloaded via a Medtronic Minimed Com-station (MMT-7301) to a personal computer, for subsequent analysis (Pearce et al. 2008).

4.6 Biochemical/hormone measurements

Venous blood samples were collected in ice-chilled tubes containing EDTA and 480KIU aprotinin (trasyol; Bayer Australia Ltd., Pymble, Australia) per litre of blood. Plasma was separated by centrifugation (3200 rpm, 15 min, 4 °C) and stored at -70 °C for later analysis (Wishart et al. 1998; MacIntosh et al. 1999).

4.6.1 Blood glucose and gut hormones

<u>Blood glucose</u>

Blood glucose concentrations were measured (glucose oxidase method) by a portable glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). The accuracy of this method has been confirmed by the hexokinase technique (Horowitz et al. 1991).

<u>Insulin</u>

Plasma, or serum, insulin was measured by solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity was 2 mU/L, intraassay CV was 3.9 % and interassay CV was 5.0 % (O'Donovan et al. 2004b).

GLP-1

Plasma total GLP-1 was measured by radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and the inter-assay coefficient of variation (CV) was 9.2 % (Ma et al. 2009c).

GIP

Total plasma GIP was measured by RIA. The standard curve was prepared in buffer and the radioiodinated label was provided by ProSearch International (Victoria, Australia). The sensitivity was 2 pmol/L, and both the intra- and inter-assay CVs were 15 % (O'Donovan et al. 2004b).

CCK

Plasma CCK-8 was measured by radioimmunoassay using an adaptation of the method of Santangelo et al. (15; 16). Standards were prepared using synthetic sulphated CCK-8 (Sigma Chemical, St Louis, MO, USA). Intra-assay CV was 6.2 % and inter-assay CV was 14.2 %. Sensitivity was 1 pmol/L (Ma et al. 2009c).

4.6.2 3-OMG

3-OMG, an analogue of glucose, was used to measure initial glucose absorption. It is transported from the gut lumen in the same way as glucose, but is not

metabolized and is excreted in the urine. Accordingly, plasma 3-OMG concentrations represent an index of enteral glucose absorption. Plasma 3-OMG concentrations were measured by liquid chromatography/mass spectrometry (Rayner et al. 2002).

4.7 Autonomic nerve function

Autonomic nerve function was evaluated using standardized cardiovascular reflex tests (Ewing and Clarke 1982). Parasympathetic function was assessed by the change in heart rate (R-R interval) during deep breathing and in response to standing (30:15 ratio); sympathetic function was determined by the decrease in systolic blood pressure in response to standing (Ewing and Clarke 1982). Each test result was scored according to age-adjusted predefined criteria as: 0 = normal, 1 = borderline, and 2 = abnormal, for a total maximum score of 6. A score of 3 or higher was taken as evidence of autonomic dysfunction (Ewing and Clarke 1982).

4.8 Conclusions

All methods used by the author in this thesis have been validated, and were well tolerated by subjects. They represent the optimal techniques to address the hypotheses underlying each study.

CHAPTER 5: EFFECTS OF PROTEIN ON GASTRIC EMPTYING, GLYCAEMIA, AND GUT HORMONES AFTER A CARBOHYDRATE MEAL IN TYPE 2 DIABETIC PATIENTS

5.1 Summary

Both the rate of gastric emptying and incretin hormone secretion are important determinants of the postprandial glycaemic excursion. We evaluated whether a whey protein „preload"could slow gastric emptying, stimulate incretin hormones, and thereby attenuate postprandial glycaemia in type 2 diabetes. 8 diet-controlled type 2 patients were studied on three days each in random order. Subjects ingested beef flavoured soup 30 min before a mashed potato meal; either 55g whey protein was added to the soup („whey preload"), or to the potato („whey in meal"), or no whey was given. Gastric emptying was slowest after „whey preload"(half-emptying time 87.3 $\pm$ 5.4 min), and slower after „whey in meal" (53.0 $\pm$ 8.3 min) than „no whey"(39.0 $\pm$ 6.2 min; P < 0.0005 for each). The incremental area under the blood glucose curve was substantially less after „whey preload" (363.7 $\pm$ 64.5 mmol.min/l) and „whey in meal" (406.3 $\pm$ 85.9mmol.min/l) than „no whey"(734.9 $\pm$ 98.9 mmol.min/l, P < 0.005), and the peak glucose concentration was lower after „whey preload"(11.3 $\pm$ 0.5 mmol/l)

and „whey in meal" (11.7 $\pm$ 0.6 mmol/l) than „no whey" (14.3 $\pm$ 0.5 mmol/l, P = 0.0001). Cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP) and insulin concentrations were higher on both whey days than after „no whey", whereas glucagon-like peptide-1 (GLP-1) was greatest after „whey preload" (P < 0.05). Whey protein consumed before a carbohydrate meal in type 2 diabetes acutely stimulates insulin and incretin hormone secretion, and slows gastric emptying, leading to marked attenuation of postprandial blood glucose. This observation has substantial implications for dietary management of type 2 diabetes.

5.2 Introduction

Optimization of glycaemic control, as evaluated by glycated haemoglobin, represents a major aim in the management of diabetes, in order to reduce the development and progression of micro-, and probably, macrovascular complications (The Diabetes Control and Complications Trial Research Group 1993; UK Prospective Diabetes Study (UKPDS) Group 1998). For most patients with type 2 diabetes, postprandial glycaemia makes a greater contribution to glycated haemoglobin than fasting blood glucose (Monnier et al. 2003). Furthermore, it is important that strategies to „normalize" glycaemia do so without increasing the risk of hypoglycaemia (Holman et al. 2008).

Both gastric emptying, and incretin hormones released from specialized entero-endocrine cells, are known to be major determinants of postprandial blood glucose concentrations (Chaikomin et al. 2006). It has been demonstrated that differences in the rate of gastric emptying account for about one third of the variation in the initial rise in blood glucose after an oral glucose load in healthy subjects and patients with type 2 diabetes (Horowitz et al. 1993; Jones et al. 1996). Indeed, slowing the rate of entry of nutrients into the small intestine may well be the dominant mechanism by which exogenous GLP-1 analogs such as exenatide (Cervera et al. 2008), and the amylin agonist, pramlintide, improve glycaemic control (Edelman 2008).

The incretin hormones, GLP-1 and GIP, account for at least 50 % of the postprandial insulin response in healthy humans (Horowitz and Nauck 2006). Some reports in patients with type 2 diabetes indicate that postprandial GLP-1 concentrations are diminished in this group (Toft-Nielsen et al. 2001; Vilsboll et al. 2001), although this is not uniformly the case (O'Donovan et al. 2004b). Nevertheless, GLP-1 has preserved incretin activity in type 2 diabetes (Nauck et al. 1993c). while the insulinotropic effect of GIP is said to be reduced (Nauck et al. 1993c). Other hormones such as CCK and peptide YY (PYY), secreted from gastrointestinal tract in response to nutrients, also delay gastric emptying (Wen et al. 1995; Schwizer et al. 1997; Rayner et al. 2000a) and are therefore likely to

improve postprandial glycaemia, although there has not been consistent evidence that CCK and PYY enhance insulin secretion (Hasegawa et al. 1996; Hidalgo et al. 2002; Boey et al. 2007).

One strategy to minimize postprandial blood glucose excursions could be to administer a small load of macronutrient at a fixed interval before a meal, so that the presence of nutrients in the small intestine would both induce the release of peptides such as GLP-1, GIP, and CCK, and slow gastric emptying in advance of the main nutrient load (Read 1992b). We previously evaluated this ‚preload" concept by giving 30 ml olive oil 30 minutes before a carbohydrate-rich meal, which resulted in markedly slower gastric emptying, stimulation of GLP-1 secretion, and attenuation in the postprandial glucose, insulin and GIP concentrations, in patients with type 2 diabetes (Gentilcore et al. 2006b). However, despite a delay in peak blood glucose, the duration of measurements was not sufficient to determine whether the overall glycaemic response was lowered.

It has long been established that protein ingestion is not accompanied by an increase in the blood glucose concentration (Conn and Newburgh 1936), and when given together with oral carbohydrate, protein enhances insulin release and slows gastric emptying, compared to carbohydrate alone, in type 2 diabetic

patients (Nuttall et al. 1984). In addition to stimulating the release of GLP-1 and GIP, protein can potentially enhance insulin secretion by direct stimulation of the beta cell by amino acids (Fieseler et al. 1995; Karamanlis et al. 2007). In rodents, digested fragments of whey protein also inhibit activity of dipeptidyl peptidase-IV (DPP-IV), the enzyme responsible for degrading endogenous GLP-1 and GIP, in the upper small intestine (Gunnarsson et al. 2006).

We therefore hypothesized that a protein „preload", in the form of a whey-based drink ingested before a carbohydrate-containing meal, would reduce both the peak blood glucose and overall postprandial glycaemic profile, by slowing gastric emptying and stimulating the release of GLP-1, GIP, CCK and insulin in patients with type 2 diabetes.

5.3 Methods

5.3.1 Subjects

Subjects

Eight patients with type 2 diabetes, diagnosed by World Health Organization criteria and managed by diet alone (7 male; age: 58 ± 3 yr; body mass index: 28.6 ± 1.3 kg/m^2), were recruited by newspaper advertisement. None had a history of significant comorbidities, was a smoker, or was taking medication

known to influence gastrointestinal function. The mean duration of known diabetes was 5.4 ± 1.1 yr, glycated haemoglobin was 6.5 ± 0.2 %, and all had plasma creatinine ≤ 0.12 mmol/L.

The study protocol was approved by the research ethics committee of the Royal Adelaide Hospital, and each subject provided written informed consent. All experiments were carried out in accordance with the Declaration of Helsinki.

5.3.2 Protocol

The study followed a randomized, single-blind, cross-over design. All participants attended the Department of Nuclear Medicine, PET and Bone Densitometry at the Royal Adelaide Hospital at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids) on three occasions, each separated by at least 3 days. A cannula was placed in an antecubital vein for blood sampling, and each subject was seated with his/her back against a gamma camera. On one of three days, subjects consumed a whey-based „preload" (55 g whey protein (Murray Goulburn, Brunswick, VIC, Australia), 4 g non-caloric beef flavoring, and 350 ml water served at 40 °C) within 1 min, 30 min before a mashed potato meal (t = -30). The meal consisted of 65 g powdered potato (Deb, Epping, Australia) and 20 g glucose (total carbohydrate content: 61 g; 1263 kJ),

reconstituted with 250 ml water and labeled with 20 MBq ^{99m}Tc-sulphur colloid, and was consumed within 5 minutes (Gentilcore et al. 2006b). On another day, the preload did not include whey and 55 g whey was mixed into the potato meal. On a third day, neither the preload nor the meal contained whey. Gastric emptying was assessed from the time of ingestion of the meal for 180 min. Venous blood samples were taken immediately before the „preload" (t = -30 min), and at t = -15, 0, 15, 30, 60, 90, 120, 150, 180, 210, 240 and 300 min for measurement of blood glucose and plasma insulin, GLP-1, GIP and CCK concentrations. Blood was also sampled at t = 45, 75, 105, 135, 165, 270 min for measurement of blood glucose only. Samples were placed into tubes containing EDTA and aprotinin on ice and centrifuged at 3200rpm for 15 minutes. Plasma was separated and stored at -70 °C for subsequent hormone analyses, using established assays. On one of the study days, autonomic nerve function was evaluated using standardized cardiovascular reflex tests (Ewing and Clarke 1982), after completion of the other measurements.

5.3.3 Measurements

<u>Gastric emptying and intragastric meal distribution</u>

Radioisotopic data were acquired as 60-sec frames for the first 60 min and 3-min frames between 60-180 min. Data were adjusted for subject movement, radionuclide decay, and gamma-ray attenuation. A „region of interest" was drawn

along the total stomach, which was subsequently divided into proximal and distal regions, and gastric emptying curves (expressed as percent retention over time) were derived (Gentilcore et al. 2006b). The lag phase was defined visually as the period before any radioactivity was present in the proximal small intestine. The retention of the meal in the total, proximal, and distal stomach at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min was calculated; the 50 % emptying time (T50) was also determined.

Blood glucose measurement

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

Plasma Insulin, GLP-1, GIP and CCK assays

Plasma insulin was measured by solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA). The sensitivity was 2 mU/L, intraassay coefficient of variation (CV) was 3.9 % and interassay CV was 5.0 %. Total GLP-1 was measured by radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, Missouri). The sensitivity was 3 pmol/L, and the inter-assay CV was 9.2 %. Plasma GIP was measured by RIA, with a sensitivity of 2 pmol/L, and both intra- and inter-assay CVs of 15 %. CCK-8 was also

measured by RIA with sensitivity of 1 pmol/L, intra-assay CV of 6.2 % and inter-assay CV of 14.2 % (Santangelo et al. 1998).

Cardiovascular autonomic function

Parasympathetic function was assessed by the variation in the heart rate (R-R interval) during deep breathing and the heart rate response to standing („30:15" ratio); sympathetic function was determined by the fall in systolic blood pressure in response to standing. Each test result was scored according to age-adjusted predefined criteria as: 0 = normal, 1 = borderline, and 2 = abnormal, for a total maximum score of 6. A score of 3 or higher was taken as evidence of autonomic dysfunction (Ewing and Clarke 1982).

5.3.4 Statistical analysis

Incremental area under the blood glucose and plasma hormone concentration curves were calculated using the trapezoidal rule, ignoring any negative values (Wolever 2004). Data were evaluated using repeated measures ANOVA, with treatment and time as factors. In the event of a treatment $\times$ time interaction, individual time points were compared with means comparisons. All analyses were performed using StatView (version 5.0, Abacus Concepts, Inc., Berkeley, CA) and SuperANOVA (version 1.11, Abacus Concepts, Inc.). Data are shown as mean values $\pm$ standard error; $P < 0.05$ was considered significant.

5.4 Results

All subjects tolerated the study well. The mean score for autonomic nerve dysfunction was 2.1 ± 0.8; two of the eight subjects had definite autonomic dysfunction.

5.4.1 Gastric emptying (Figure 1)

Total stomach (Figure 1A)

Gastric emptying commenced after a short lag phase; the latter tended to be longer when whey was consumed before the meal („whey preload", 7.1 ± 3.7 min; „whey in meal", 1.8 ± 0.3 min; „no whey", 1.0 ± 0.0 min; P = 0.1). On the „no whey" and „whey in meal" days, emptying was rapid initially, and subsequently slower. In contrast, emptying after the „whey preload" approximated a linear pattern. Gastric emptying was slowest on the „whey preload" day than both the other days (P = 0.0001), and slower when whey was included in the meal compared to „no whey" (P < 0.0005). Accordingly, the T50 was longer with the whey preload (87.3 ± 5.4 min) than both the „no whey" day (39.0 ± 6.2 min; P = 0.0001) and when whey was included in the meal (53.0 ± 8.3 min; P = 0.0001), and was longer with whey in the meal than „no whey" (P < 0.01).

<u>Intragastric distribution</u> (Figure 1B and 1C)

There was a modest increase in meal retention in the proximal stomach with the whey preload compared to the other two days ($P < 0.05$), and when whey was in the meal compared with no whey ($P < 0.05$). In contrast, there was a marked increase in meal retention in the distal stomach after the whey preload compared with the other two days ($P < 0.05$), and distal meal retention was also greater when the whey was in the meal, compared to „no whey" ($P < 0.05$).

5.4.2 Blood glucose concentrations (Figure 2A)

There were no differences between baseline blood glucose concentrations, or between -30 and 0 min on each day. There was a postprandial rise in blood glucose, from 15 min with no whey ($P < 0.001$) or when whey was in the meal ($P < 0.01$), with a later rise at 30 min after the whey preload ($P < 0.005$). The blood glucose concentrations were lower after the whey preload and when whey was in the meal than with no whey ($P < 0.005$ and $P < 0.05$). In particular, the peak blood glucose concentration was markedly less after both the whey preload (11.3 $\pm$ 0.5 mmol/L) and when whey was in the meal (11.7 $\pm$ 0.6mmol/L), compared with no whey (14.3 $\pm$ 0.5 mmol/L; $P = 0.0001$). The incremental area under the curve (iAUC) was also less when whey was given as a preload (363.7 $\pm$ 64.5 mmol.min/l) and in the meal (406.3 $\pm$ 85.9 mmol.min/l), compared to no whey (734.9 $\pm$ 98.9 mmol.min/l; $P < 0.005$). Blood glucose

concentrations had returned to baseline at a mean of 210 min after the whey

preload and when whey was in the meal, and at 240 min without whey. At t =

300 min, blood glucose concentrations were lower than baseline on all three

days.

5.4.3 Plasma insulin concentrations (Figure 2B)

There were no differences in baseline insulin concentrations. Plasma insulin

increased on all 3 days ($P = 0.0001$) with an earlier response after whey preload

($t = 15$ min, $P < 0.005$) than when whey was in the meal ($t = 30$ min, $P < 0.0005$)

or no whey was given ($t = 30$ min, $P < 0.05$). The plasma insulin response was

markedly greater after both the whey preload ($P < 0.05$) and when whey was in

the meal ($P < 0.01$), when compared to no whey. Plasma insulin was higher at 60

min after whey preload than when whey was in the meal ($P < 0.005$), but less at

120 150 and 180 min ($P < 0.01$). iAUC was greater when whey was given as a

preload (32611.781 $\pm$ 9234.9 mU.min/l) or in the meal (37248.8 $\pm$ 10950.0

mU.min/l), compared to no whey (12283.5 $\pm$ 3453.9 mU.min/l; $P < 0.05$ and $P

< 0.005$). Despite an earlier response, the overall iAUC did not differ between the

„whey preload" and „whey in meal" days.

5.4.4 Plasma GLP-1 concentrations (Figure 2C)

There were no differences in GLP-1 concentrations at baseline. Plasma GLP-1 markedly increased on all 3 days (P = 0.0001), and had already risen before the meal after the whey preload (t = -15 min; P = 0.0001). Plasma GLP-1 was markedly greater when whey was given as a preload or in the meal, when compared to no whey (P < 0.05 for each) and greater between -15 min and 90 min with the whey preload when compared with whey in the meal (P = 0.0001). Accordingly, iAUC was greater after whey preload (3965.2 $\pm$ 593.6 pmol.min/l) and whey in the meal (3514.7 $\pm$ 675.6 pmol.min/l), compared to no whey (779.5 $\pm$ 169.2 pmol.min/l; P = 0.0001), but did not differ between the whey preload and whey in the meal.

5.4.5 Plasma GIP concentrations (Figure 2D)

There were no differences in baseline plasma GIP concentrations. There was a rise in plasma GIP on all 3 study days (P = 0.0001), with a rapid response from t = -15 min after the whey preload (P < 0.05). Plasma GIP concentrations were greater after the whey preload and when whey was in meal, compared with no whey (P < 0.05 and P < 0.05 respectively). GIP levels were also greater at t = -15, -5, 15 and 60 min, and lower at t = 180 min, after the whey preload when compared to whey in the meal (P < 0.05). Accordingly, iAUC was greater after

the whey preload (9003.4 $\pm$ 701.2 pmol.min/l) and whey in the meal (8453.9 $\pm$ 1209.2 pmol.min/l), compared to no whey (4493.2 $\pm$ 609.6 pmol.min/l; P = 0.0001), but did not differ between the whey preload and whey in the meal. At t = 300 min, plasma GIP was higher than baseline levels after the whey preload and when whey was in the meal, but lower than baseline on the day without whey.

5.4.6 Plasma CCK concentrations (Figure 2E)

There were no differences in baseline CCK concentrations. There was a rise in CCK on all 3 days (P = 0.0001), with a early increase after the whey preload (t = -15 min, P = 0.0001). The postprandial rise in plasma CCK was slightly greater after the whey preload and when whey was in the meal compared to no whey (P < 0.05 and P < 0.001 respectively). Plasma CCK was also greater at -15, -5 and 15 min on the „whey preload" day than when whey was in the meal (P < 0.05). iAUC for CCK concentrations was greater after whey preload (856.8 $\pm$ 108.5 pmol.min/l) and when whey was in the meal (898.7 $\pm$ 124.1 pmol.min/l), compared to no whey (444.1 $\pm$ 68.7 pmol.min/l; P = 0.0001), but did not differ between the whey preload and whey in the meal.

5.5 Discussion

We demonstrated that whey protein, given either as a „preload" before a high carbohydrate meal, or consumed with the meal, resulted in a substantial reduction in both the peak and overall area of the postprandial blood glucose excursion in patients with diet-controlled type 2 diabetes. The ingestion of protein was associated with stimulation of GLP-1, GIP, and CCK, a markedly greater insulin response, and slower emptying of the potato meal, when compared to control. Whey given as a preload initiated a rise in insulin and gut peptides in advance of meal ingestion, so that the feedback mechanisms regulating gastric emptying and glycaemia were already active at the onset of the meal. Given that the magnitude of the reduction in postprandial glycaemia was at least what would be hoped for with pharmacological therapy such as the sulphonylureas, these data have considerable implications for the further development of nutritional strategies in the treatment of type 2 diabetes.

The role of the gastrointestinal tract in determining postprandial blood glucose has often been overlooked in the past, but is now rising to prominence, at least in part due to the development of gut peptide based therapies for diabetes, such as the GLP-1 analogue, exenatide (Cervera et al. 2008), and the amylin analogue, pramlintide (Thompson et al. 1998). Both the regulation of entry of carbohydrate from the stomach into the small intestine, and the release of peptide hormones

from the small intestine that stimulate insulin secretion, make the upper gastrointestinal tract central to glycaemic control.

Our group has previously demonstrated that olive oil, given as a preload, modified the glycaemic response to a subsequent high carbohydrate meal in patients with type 2 diabetes (Gentilcore et al. 2006b). Like whey protein, oil had a profound effect in slowing the emptying of the meal from the stomach, which was associated with stimulation of GLP-1. After the oil preload, however, the GIP and insulin responses were diminished and delayed in contrast to the current study, and the effect on blood glucose was less marked. This implies that with oil, slowing of gastric emptying was the major mechanism for attenuating postprandial glycaemia. Given that the whey preload had comparable effects on gastric emptying, but a much greater effect on blood glucose levels, it is likely that the stimulation of insulin by whey, in contrast to the delayed insulin response after oil, played the dominant role in improving glycaemia in the current study.

Potential mediators of the stimulation of insulin by whey protein include the incretin hormones, GIP and GLP-1, and direct stimulation of the beta cells by amino acids absorbed after digestion of whey (Fieseler et al. 1995). While there is no direct evidence that CCK enhances insulin secretion, this hormone was likely to have played a role in the slowing of gastric emptying induced by the

preload (Liddle et al. 1988; Schwizer et al. 1997). Whey certainly stimulated the release of both GIP and GLP-1, and when given as a preload, plasma levels of these peptides had risen even before ingestion of the subsequent meal. The latter observation might account for the fact that the insulin profile showed an earlier increase after the whey preload than when whey was consumed with the meal. Subsequent to the meal, the magnitude of the GIP and insulin responses was otherwise similar on both whey days, in contrast to the greatly enhanced initial stimulation of GLP-1 when whey was given as a preload. The fact that insulin was not stimulated even more with the whey preload may be accounted for by the action of GLP-1 to retard gastric emptying, and thereby limit further carbohydrate absorption, since the insulinotropic action of GLP-1 is glucose-dependent. In contrast to previous reports that GLP-1 secretion is deficient in type 2 diabetes (Toft-Nielsen et al. 2001; Vilsboll et al. 2001), we have observed both previously (O'Donovan et al. 2004b) and in the current study that diet-controlled type 2 patients can mount a strong GLP-1 response, and it is also established that the insulin response to GLP-1 remains intact in type 2 diabetes (Nauck et al. 1993c). In contrast, type 2 patients become unresponsive to GIP, although recent evidence suggests that this can be reversed by tight glycaemic control (Hojberg et al. 2008). In support of the importance of the incretin hormones in stimulating insulin in the current study is that the time course of insulin release seemed to follow that of GLP-1 and GIP. In addition to

increasing the rate of glucose disposal, it is possible that the stimulation of insulin results in suppression of glucagon; however, we did not measure plasma glucagon concentrations.

It appears that the choice of macronutrient is an important factor in the efficacy of the „preload" strategy. In addition to any benefit in glycaemic response, protein preloads are attractive, as they may promote satiety and therefore have the potential to reduce total energy intake (Bowen et al. 2006b; Bowen et al. 2006c). The type of protein consumed also appears to be important; whey results in greater increases in GLP-1, GIP and CCK, more delayed gastric emptying, and lower energy intake when consumed as a preload, compared with casein (Hall et al. 2003). An additional advantage of whey is the capacity for digested protein fragments to inhibit DPP-IV in the upper small intestine, resulting in greater concentrations of the active forms of GIP and GLP-1, at least in rodents (Gunnarsson et al. 2006). Our assays for GIP and GLP-1 measured only total peptide concentrations; it would be of interest to evaluate the concentration of intact peptides to see whether this effect of whey is evident in humans. It would also be of interest to study the effects of a combination of whey protein with a DPP-IV inhibitor, in an effort to optimize the effects of incretin stimulation.

Our study involved a relatively small number of subjects who had

well-controlled, predominantly uncomplicated type 2 diabetes. Despite evidence of autonomic neuropathy in two of our patients, none had abnormally slow gastric emptying on the control day, and therefore we cannot be certain that our results would apply to patients with diabetic gastroparesis. Further evaluation would also be required in order to generalise the outcome to patients with poorly controlled type 2 diabetes and those using pharmacological antidiabetic agents, and to determine whether the acute benefits that we observed are sustained in the longer term. The dose of whey protein was chosen on the basis of previous data that it was sufficient to stimulate gut hormones (Bowen et al. 2006c). It would be important to confirm whether the effects we observed are maintained with a smaller load of protein, in order to minimize the additional energy load. Nevertheless, despite the small sample size, the improvement in postprandial glycaemia was marked, and consistent between subjects, and the concept of using dietary manipulations to treat type 2 diabetes, based on our knowledge of the contribution of gastric emptying and gut peptides to postprandial glycaemic responses, therefore holds much promise.
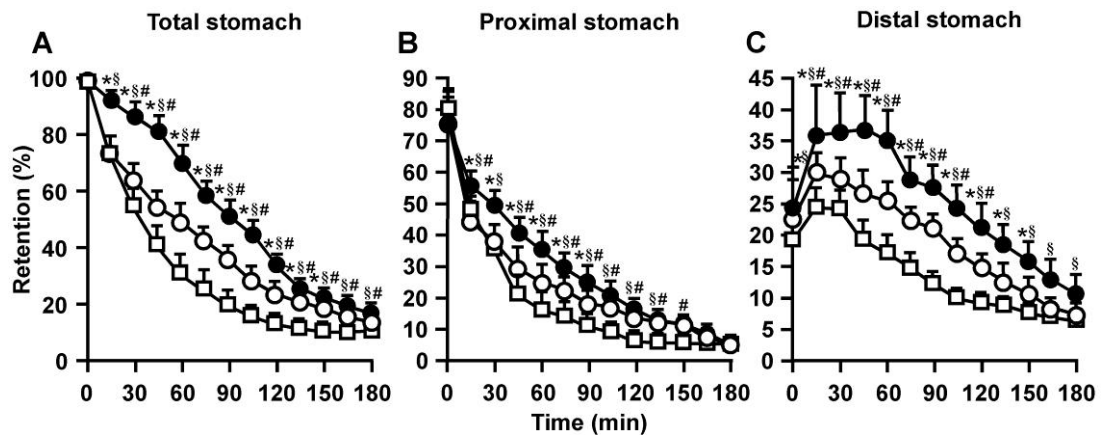
Figure 5.1 Meal retention in the total stomach (A), proximal stomach (B) and distal stomach (C) on three study days in 8 type 2 diabetic patients. On each day, subjects ingested 350 ml beef flavoured soup 30 min before a radiolabeled mashed potato meal; either 55 g whey protein was added to the soup („whey preload"), or the whey was added to the potato („whey in meal"), or no whey was given („no whey"). Data are mean ± standard error. * $P < 0.05$, whey preload vs. whey in meal; # $P < 0.05$, whey in meal vs. no whey; § $P < 0.05$, whey preload vs. no whey.

Blood glucose

Plasma insulin

Plasma GLP-1

Plasma GIP
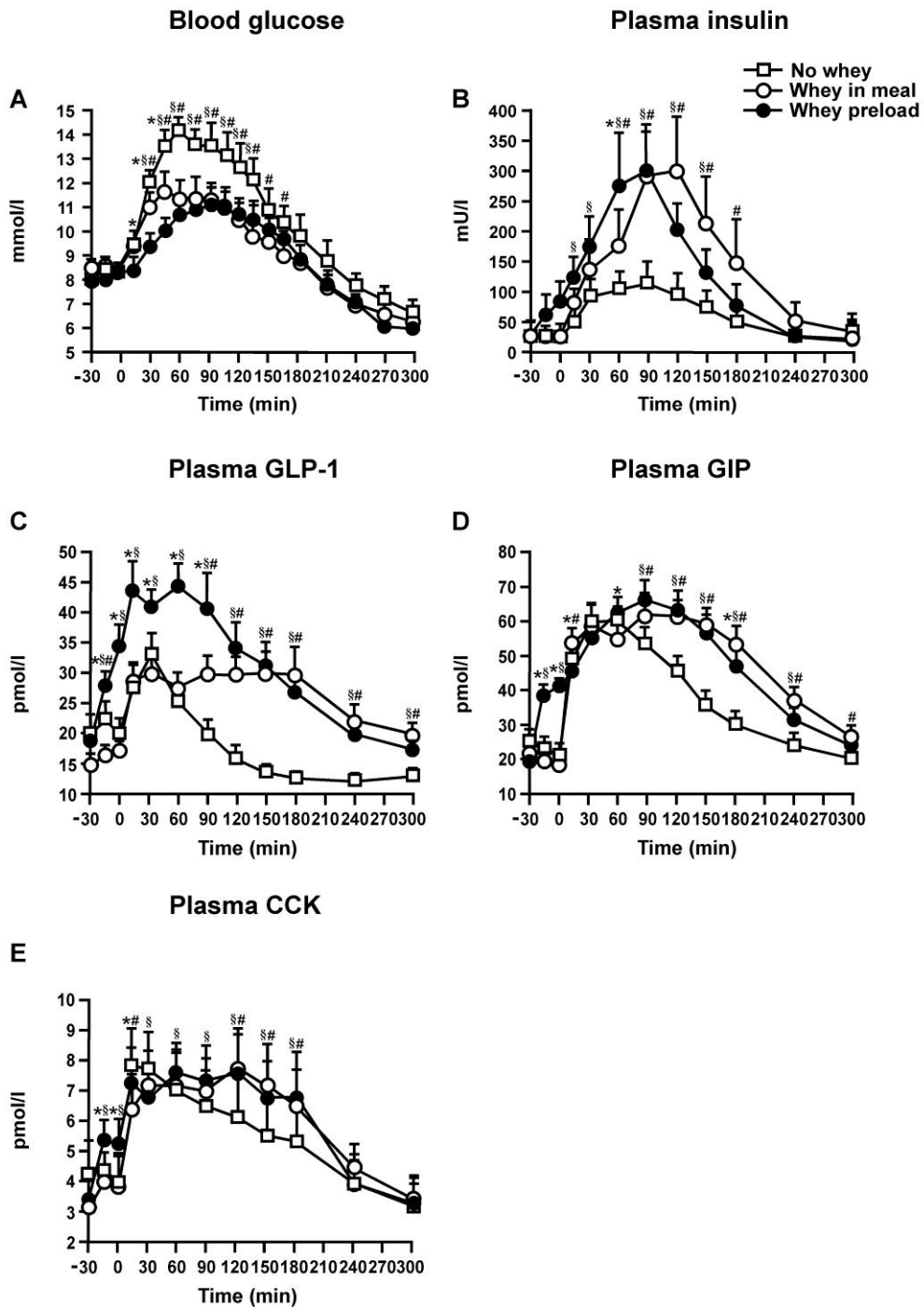
Plasma CCK

Figure 5.2 Concentrations of blood glucose (A), plasma insulin (B), plasma

GLP-1 (C), plasma GIP (D) and plasma CCK (E) in response to a mashed potato

meal in 8 diabetic patients. On each of study day, subjects ingested 350 ml beef flavored soup 30 min before a radiolabeled mashed potato meal; either 55 g whey protein was added to the soup („whey preload"), or the whey was added to the potato („whey in meal"), or no whey was given („no whey"). Data are mean ± standard error. * 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.

CHAPTER 6: EFFECTS OF TWO PROTEIN 'PRELOADS' ON ENERGY INTAKE, GLYCAEMIA AND GASTROINTESTINAL HORMONES IN TYPE 2 DIABETES

6.1 Summary

A whey protein „preload" of 55 g was shown, in the study reported in Chapter 5, to reduce markedly the glycaemic response to a high carbohydrate meal in type 2 diabetes, associated with stimulation of glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK), release, and slowing of gastric emptying. Both GLP-1 and CCK reduce appetite and energy intake. The primary aim of the current study was to determine whether whey, when ingested before a buffet-style meal, would dose-dependently suppress hunger and increase fullness, and reduce energy intake in type 2 diabetes, while concurrently improving glycaemia by stimulating gut peptides including GIP, GLP-1, and CCK. Nine patients with diet-controlled type 2 diabetes were studied on three separate days in randomized order. On each day, subjects consumed a chocolate-flavoured liquid „preload" (100 ml water mixed with either 25 g „diet" chocolate sauce (8 kcal), or with 25 g (89 kcal) or 55 g (195 kcal) chocolate-flavoured whey protein isolate. 30 minutes later, each subject was offered a cold buffet style meal from which they ate ad libitum for up

to 30 minutes. The peak postprandial blood glucose was lower with 25 g and 55 g whey protein than with placebo (12.0 ± 0.5 and 12.0 ± 0.6 vs. 12.7 ± 0.7 mmol/L; $P < 0.05$). Both preloads also stimulated GIP and CCK before the meal (all $P < 0.05$); the stimulation of CCK was greater ($P < 0.05$) with 55 g whey. Both whey preloads increased postprandial fullness slightly ($P < 0.05$), but neither effected energy intake. In conclusion, a whey protein „preload" dose-dependently increased fullness, stimulated CCK and GIP and reduced postprandial glycaemia, but had no effect on energy intake in patients with diet-controlled type 2 diabetes.

6.2 Introduction

Excessive energy intake and obesity are strongly associated with diabetes (Wang et al. 2008b); the majority of patients with type 2 diabetes are obese or overweight, and the latter is associated with insulin resistance (Zeyda and Stulnig 2009). Reduction of energy intake leading to weight loss, accordingly, represents an important goal in the management of diabetes. Therefore, there is substantial interest in dietary strategies to reduce energy intake and facilitate weight loss in this group.

Exposure of the small intestine to nutrients plays a major role in the regulation of appetite and energy intake, mediated by slowing of gastric emptying and the

secretion of gastrointestinal hormones, including CCK, and GLP-1 (Read 1992a).

The „preload" concept is a dietary strategy involving administration of a specific macronutrient in advance of a subsequent meal, in order to stimulate gut hormone release prior to ingestion of the main meal. We have demonstrated that fat (Gentilcore et al. 2006b), and particularly whey protein (Ma et al. 2009c), preloads have the capacity to reduce markedly the glycaemic response to a high carbohydrate meal in type 2 diabetes. For example, ingestion of 350 ml beef-flavoured soup with 55 g whey protein before a carbohydrate (mashed potato) meal markedly potentates the secretion of GIP, GLP-1 and CCK, associated with slowing of gastric emptying, stimulation of insulin, and attenuation of the peak and overall glycaemic response in patients with type 2 diabetes (Ma et al. 2009c).

Among the macronutrients, protein is probably the most satiating (Porrini et al. 1995; Poppitt et al. 1998a; Latner and Schwartz 1999). Protein „preloads" have the capacity to reduce energy intake at a subsequent meal in lean and overweight subjects (Poppitt et al. 1998b; Bowen et al. 2006a), and may be associated with more sustained elevation of satiety hormones incuding CCK and GLP-1, and suppression of ghrelin (which stimulates appetite), and slower gastric emptying, than with carbohydrate (Bowen et al. 2006d). We therefore aimed to determine whether the whey protein „preload" that we had demonstrated to be effective for

lowering postprandial glycaemia would also increase fullness, suppress hunger and reduce energy intake when given in advance of an ad libitum meal. Since 55 g whey represents a significant caloric burden, we also aimed to investigate whether a smaller (25 g) whey protein preload would be effective in reducing appetite and energy intake, as well as for reducing glycaemia. Our hypothesis was that whey preloads, when ingested before a buffet-style meal, would result in a dose-dependent suppression of hunger and increase in fullness as well as improving glycaemia, with stimulation of GIP and CCK, and a reduction in energy intake at a subsequent meal in patients with type 2 diabetes.

6.3 Methods

6.3.1 Subjects

Nine patients with type 2 diabetes, diagnosed by World Health Organization criteria and managed by diet alone (3 female; age: 61 ± 3 yr; body mass index: 30.0 ± 1.7 kg/m^2), were recruited by newspaper advertisement. The mean duration of known diabetes was 4 ± 1 yr, glycated haemoglobin was 6.7 ± 0.3 %, and in all the plasma creatinine was ≤ 0.12 mmol/L. None had a history of significant comorbidities, was a smoker, or was taking medication known to influence gastrointestinal function. The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital, and all subjects

provided written informed consent. All experiments were carried out in accordance with the Declaration of Helsinki.

6.3.2 Protocol

The study followed a randomized, single-blind, cross-over design. All participants attended the Discipline of Medicine at the Royal Adelaide Hospital at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids) on three occasions, each separated by at least 3 days, when an intravenous cannula was inserted into an antecubital vein for blood sampling. On each of the study days, subjects consumed, within 60 sec, a chocolate-flavoured liquid „preload" (100ml water mixed with either 25 g „diet" chocolate sauce (Cottee's, Southbank, VIC, Australia, 8 kcal), or with 25 g (Ascend, Murray Goulburn, Brunswick, VIC, Australia, 89 kcal) or 55 g (Ascend, Murray Goulburn, Brunswick, VIC, Australia, 195 kcal) chocolate-flavoured whey protein isolate) served at 40 °C at t = -30 min (Pilichiewicz et al. 2007a). The order of the three days was randomized and subjects were asked whether they could distinguish between the preloads. Following the „preload" drink, subjects were offered a cold buffet style meal at t = 0 and were allowed to eat ad libitum for up to 30 minutes ie t = 0-30 min (Bowen et al. 2006a). The buffet meal comprised various food items, including bread, cheese, chicken, ham, yoghurt, fruit and juices (Sturm et al. 2004). Venous blood samples, for measurement of blood glucose and plasma

hormones, were taken immediately before the preload (t = -30 min), and at predetermined time points until t = 270 min. Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). The remainder of each sample was placed in a prechilled EDTA tube containing aprotinin on ice and centrifuged at 3200rpm for 15 minutes. Plasma was separated and samples stored at -70 °C for subsequent analysis of plasma hormones using established assays (19; 20). Perceptions of appetite were assessed by visual analogue scales (VAS) immediately after blood was sampled (Bowen et al. 2006d).

6.3.3 Measurements

Blood glucose

Blood glucose concentrations were measured at t = -30, -15, 0, 30, 45, 60, 90, 120, 150, 180, 210, 240 and 270 min, using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA) (Horowitz et al. 1991).

Plasma GIP and CCK

These hormones were measured at t = -30, 0, 30, 45, 60, 90, 150, 210 and 270 min. Plasma GIP was measured by radioimmunoassay (RIA), with a sensitivity of 2 pmol/L, and both intra- and inter-assay CVs of 15 % (Ma et al. 2009c). CCK-8 was also measured by RIA with sensitivity of 1 pmol/L, intra-assay CV

of 6.2 % and inter-assay CV of 14.2 % (Santangelo et al. 1998). Plasma is stored awaiting GLP-1 and insulin assays; hence these data are not yet available.

Appetite and energy intake

Hunger and fullness were assessed using validated VAS (Parker et al. 2004). Each of VAS consisted of a 100-mm horizontal line; the subject was asked to place a vertical mark to indicate the strength of each sensation. Nausea and bloating were also quantified in this way.

Energy intake was assessed by food consumption from the buffet meal and determined by weighing each item before and after consumption and calculating the energy intake consumed using commercially available software (Foodworks 3.01; Xyris Software, Highgate Hill, QLD, Australia) (Brennan et al. 2005).

6.3.4 Statistical analysis

Data were evaluated using repeated measures ANOVA, with treatment and time as factors. In the event of a treatment × time interaction, individual time points were compared with means comparisons. Peak blood glucose and plasma hormones levels were also compared using ANOVA. All analyses were performed using StatView (version 5.0, Abacus Concepts, Inc., Berkeley, CA,

USA) and SuperANOVA (version 1.11, Abacus Concepts, Inc., Berkeley, CA, USA). Data are shown as mean values ± standard error; P < 0.05 was considered significant.

6.4 Results

All subjects tolerated the study well and they were unable to distinguish between the preloads.

6.4.1 Blood glucose concentrations (Figure 1A)

Fasting blood glucose (t = -30 min) did not differ among the three study days, nor was there any change in blood glucose after the preload and before the meal (t = 0 min). Blood glucose concentrations increased after the meal (P < 0.001) with a significant treatment × time interaction (P = 0.0001). Blood glucose was lower at t = 30, 45, 60, 75, and 90 min, and higher at t = 180, 210 and 240 min, with the 55 g whey preload than placebo (P < 0.05). Blood glucose was also lower at t = 60 and 75 min (P < 0.05) with the 25 g whey protein preload compared to placebo. After the 55 g whey preload, blood glucose was lower at t = 45 min and higher at t = 150, 180, 210, 240 min than after the 25 g whey preload (P < 0.05). The peak blood glucose was lower with the 25 g and 55 g whey preload than with placebo (12.0 ± 0.5 and 12.0 ± 0.6 vs. 12.7 ± 0.7 mmol/L;

P < 0.05), without any difference between the two whey preloads. At t = 270 min, blood glucose was less than baseline for placebo and the 25 g whey preload (P < 0.05).

6.4.2 Plasma GIP concentrations (Figure 1B)

Baseline plasma GIP concentrations (t = - 30 min) did not differ. After both whey preloads there was a rise in GIP at t = 0 min (P < 0.0005 for 55g whey and P < 0.05 for 25 g whey, versus placebo), but without any difference between the two. After the meal, there was no difference in plasma GIP between placebo and either of the two whey preloads. At t = 270 min, plasma GIP was greater than baseline on all three days (P = 0.0001).

6.4.3 Plasma CCK concentrations (Figure 1C)

Baseline plasma CCK concentrations (t = -30 min) did not differ. After both whey preloads, there was a rise in CCK at t = 0 min (P < 0.0005 for each versus placebo), with a greater rise after 55 g than 25 g whey protein (P < 0.05). After the meal, there was a treatment × time interaction (P = 0.0001). Plasma CCK was greater at t = 60 min after the 25 g and 55 g whey protein preloads than placebo (P < 0.05 for both), and was also greater at t = 30, 150 and 270 min after the 55 g compared to the 25 g whey preload (P < 0.05 for all). At t = 270 min, plasma

CCK was greater than baseline on all three days (P = 0.0001).

6.4.4 Perceptions (Figure 2)

Baseline scores (t = -30 min) for fullness were low and did not differ between the three days, nor did fullness change significantly after the preloads (t = 0 min). There was a significant treatment × time interaction for fullness scores after the meal (P < 0.0005). Fullness was slightly greater (P < 0.05) at t = 30, 45, 60, 75, 150, 180, 210, 240, 270 min after the 55 g whey preload than placebo, and greater (P < 0.05) after the 25 g whey preload at t = 150, 180, 210, and 270 min than placebo. Fullness was also greater after the 55 g whey protein compared to the 25g whey at t = 30, 45, 240 and 270 min (P < 0.05).

There were no differences in hunger, desire to eat or nausea between the three days (data not shown).

6.4.5 Energy intake (Figure 3)

Energy intake was 1159 ± 170 kcal after placebo, 1095 ± 194 kcal after the 25 g whey preload and 1078 ± 186 kcal after the 55 g whey preload. These differences were not significant. Similarly, there was no difference in total energy intake when calculated to include the preload (1168 ± 170 kcal after placebo, 1184 ±

194 kcal after 25 g whey and 1273 ± 186 kcal after 55 g whey).

6.5 Discussion

This study has demonstrated that whey protein, when given as a preload before a buffet style meal, dose-dependently increased fullness and reduced postprandial blood glucose concentrations in patients with diet-controlled type 2 diabetes, associated with stimulation of GIP and CCK, but had no significant effect on energy intake.

Both gastric emptying and the incretin hormones are important determinants of postprandial blood glucose excursions. In our previous study, 55 g whey protein, when given as a preload, slowed gastric emptying of a high carbohydrate meal, which probably contributed to the lowering of postprandial blood glucose concentrations (Ma et al. 2009c). Although we did not measure gastric emptying in the current study, slowing of emptying is likely to explain, at least in part, the lowering of postprandial blood glucose, particularly as the initial rise in blood glucose was diminished. Another important effect of whey protein is the stimulation of insulin secretion, both by the release of incretin hormones, GIP and GLP-1, in a glucose- dependent manner (Drucker 2006), and by direct stimulation of the pancreas by amino acids. Furthermore, in rodents, whey protein has been shown to inhibit dipeptidyl peptidase-IV (DPP-IV) activity in

the proximal small intestine, which would potentially prolong the local action of GLP-1 and GIP (Gunnarsson et al. 2006). GLP-1 lowers postprandial glycaemia in patients with type 2 diabetes by stimulating insulin secretion, slowing gastric emptying and suppressing glucagon (Nauck and Meier 2005). GLP-1 receptor agonists, such as exenatide, and DPP-IV inhibitors, such as sitagliptin, are now used in the clinical management of diabetes (Gilbert and Pratley 2009). In contrast to GLP-1, the insulinotropic action of GIP is abolished in patients with type 2 diabetes (Nauck et al. 1993c). However, this resistance to the actions of GIP can be reversed by good glycaemic control (Hojberg et al. 2009), so the stimulation of GIP by whey preloads in the current study might have contributed to the improvement in the glycaemic response. At the time of writing this thesis, the insulin and GLP-1 data were not available, so conclusions regarding the mechanism of glucose-lowering by whey should be circumspect. An important implication from the current study, however, is that a smaller protein load than used in our initial acute whey preload study (ie 25 g versus 55 g) would be suitable for more prolonged studies in patients with type 2 diabetes if the primary aim is to improve glycaemic control.

Potential mediators of the suppression of appetite and energy intake by protein include diet-related thermogenesis, the central effects of absorbed amino acids (Anderson 1979), the release of gut hormones such as CCK, GLP-1 (Hall et al.

2003) and PYY (Batterham et al. 2006) and the suppression of ghrelin. The effect is dependent on the type of protein, with whey protein reducing energy intake more than protein from soya or egg (Anderson et al. 2004). Whey protein is rapidly digested to branched chain amino acids, including leucine, isoleucine and valine, which are potent in suppressing energy intake (Anderson 1979), and administration of whey has been reported to result in prolonged stimulation of CCK and GLP-1, as well as GIP (Hall et al. 2003). In the current study, both whey protein preloads stimulated fullness, compared to placebo. The effect was modest, and evident soon after meal ingestion.

We have previously observed that plasma GLP-1, GIP and CCK concentrations increased when 55 g whey was given as a „preload", and that these effects were evident before ingestion of a subsequent meal (Ma et al. 2009c). The importance of gut hormones in the regulation of appetite is highlighted by the observation following gastric bypass surgery (Pournaras et al. 2009), that increased satiety and weight loss are associated with enhanced PYY and GLP-1 secretion. Plasma GLP-1 data for the current study are not yet available, and PYY was not measured.

A number of studies indicate that CCK is a physiological regulator of energy intake in humans. For example, exogenous CCK, infused at a rate designed to

mimic „physiological" postprandial plasma concentrations, reduces energy intake (Ballinger et al. 1995; Lieverse et al. 1995), while the specific CCK-A receptor antagonist, loxiglumide, increases hunger and energy intake (Beglinger et al. 2001). It is, however, uncertain whether CCK plays an important role in protein induced-satiety. We observed an increase in CCK concentrations after whey preloads compared to placebo, but no significant difference in energy intake. This is in accordance with a previous study in which stimulation of CCK release by whey was associated with increased satiety, but no difference in food intake, although the meal was given later after the preload in that report (Bowen et al. 2006b). It should be recognised, however, that the magnitude of CCK stimulation by whey was not marked, at least at the time the meal was ingested. Hence, both timing of the meal and the extent of CCK stimulation may be relevant to our observations.

It has been suggested that plasma ghrelin levels are related to meal initiation (Cummings et al. 2001), and an increase in satiety after whey protein could potentially be mediated by prolonged ghrelin suppression, with a delay in the subsequent timing of a meal and a sustained increase in fullness (Bowen et al. 2006b). Plasma ghrelin has been reported to remain lower than the fasting value for 3-4 hours after consumption of whey protein (Bowen et al. 2006b). Plasma ghrelin was not measured in the current study.

It is perhaps surprising that despite stimulation of CCK and increased fullness after whey preloads, there were no differences in energy intake. The number of subjects we studied was relatively small and it is possible that the failure to demonstrate suppression of energy intake represented a type 2 error. It is likely that the variability of energy intake would be higher in type 2 diabetic patients than healthy controls, and there is little information about the effects of dietary or pharmacological interventions on energy intake in this group (Chapman et al. 2005). The interval between the preload and the meal was selected to match our previous study, in which there was a marked effect of whey on glycaemia, and as discussed, this may have influenced the outcome. Of note, it has been reported that the suppression of energy intake by CCK is sustained for less than 30 min (Karhunen et al. 2008). Furthermore, since the interval between the preload and the meal was fixed, we were not able to evaluate whether consumption of the whey preload might delay the initiation of eating. It should also be recognised that breakfast may not be the ideal meal at which to detect differences in energy intake. In the current study, fullness was higher after whey preloads than with placebo for at least 4 hours and it would be of interest to evaluate the effects in energy intake at a second meal.
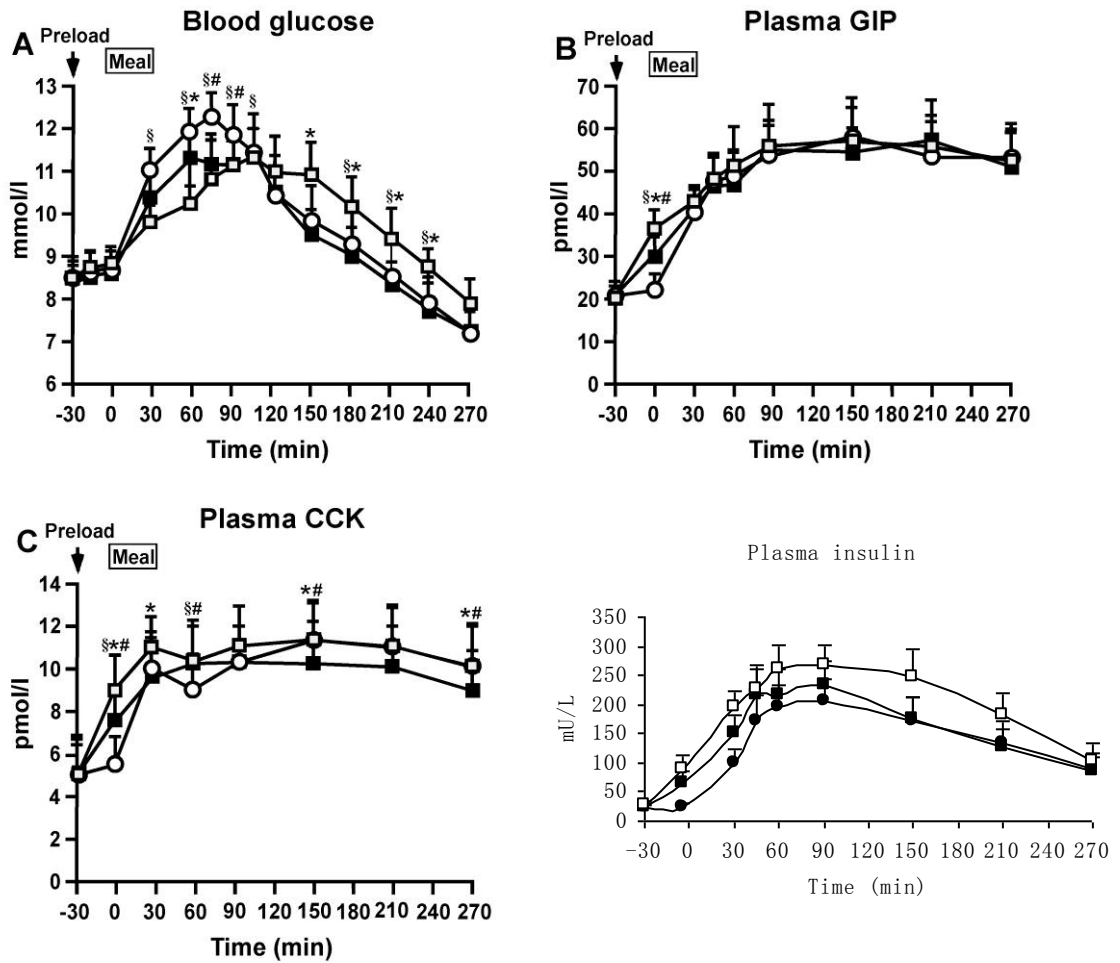
Figure 6.1 Concentrations of blood glucose (A), plasma GIP (B) and CCK (C) before and after the meal. On each study day, subjects ingested 125 ml chocolate flavoured drink (100 ml water mixed with either diet chocolate-flavoured sauce, 25 g or 55 g chocolate-flavoured whey protein isolate) 30 min before a buffet style meal. Data are mean ± SE. * $P < 0.05$, 25 g whey preload vs. 55g whey preload; # $P < 0.05$, 25 g whey preload vs. placebo; § $P < 0.05$, 55 g whey preload vs. placebo. n = 9.
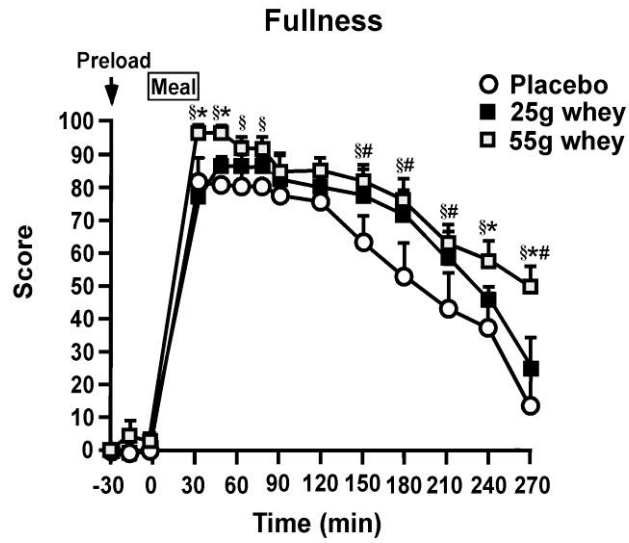
Figure 6.2 Scores for fullness before and after the meal. On each study day, subjects ingested 125 ml chocolate flavoured drink (100 ml water mixed with either diet chocolate-flavoured sauce, 25 g or 55 g chocolate-flavoured whey protein isolate) 30 min before a buffet style meal. Data are mean ± SE. * $P < 0.05$, 25 g whey preload vs. 55 g whey preload; # $P < 0.05$, 25 g whey preload vs. placebo; § $P < 0.05$, 55 g whey preload vs. placebo. n = 9.
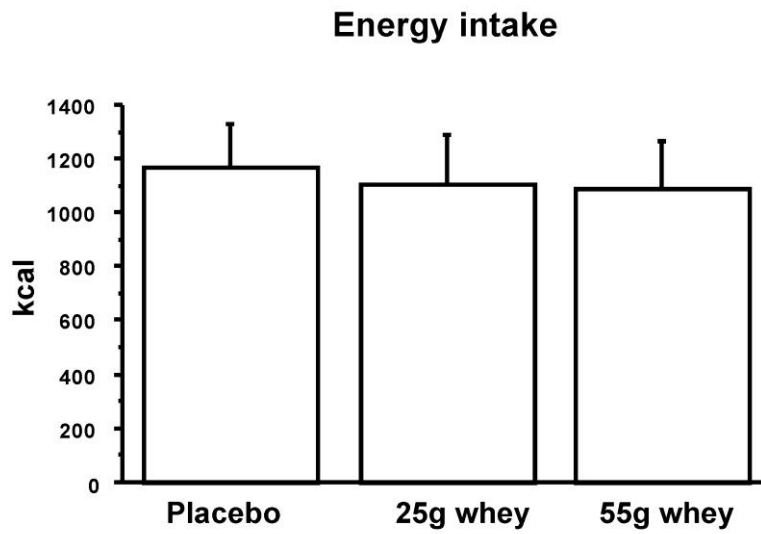
Energy intake

Figure 6.3 Energy intake at the buffet style meal. On each study day, subjects ingested 125 ml chocolate flavoured drink (100 ml water mixed with either diet chocolate-flavoured sauce, 25 g or 55 g chocolate-flavoured whey protein isolate) 30 min before the meal. Data are mean ± SE. n = 9

CHAPTER 7: ACUTE AND CHRONIC EFFECTS OF A PROTEIN 'PRELOAD' ON GASTRIC EMPTYING, GLYCAEMIA, GASTROINTESTINAL HORMONE RELEASE AND ENERGY INTAKE IN TYPE 2 DIABETES

7.1 Summary

In Chapters 5 and 6, it was reported that whey protein, when given as a „preload‟, acutely reduced the glycaemic response to a subsequent meal in type 2 diabetes, associated with slowing of gastric emptying and the stimulation of incretin hormone release. However, adaptive changes in small intestinal feedback inhibition of gastric emptying and gut hormone release might reduce the efficacy of this therapy in the long term. The aim of the current study was to evaluate whether the effects of a protein preload on gastric emptying, glycaemia and incretin hormone release are sustained with „chronic‟ (4 weeks) administration and impact on 72-hour glycaemic profiles and food intake. Seven patients with diet-controlled type 2 diabetes participated in the study. Each consumed a chocolate-flavoured „preload‟, (containing either 25 g whey protein or placebo), 30 min before each of the three main meals for 4 weeks, followed by a „washout‟ period of 2 weeks, and then the alternative preload for 4 weeks, in a randomized crossover design. Gastric emptying (scintigraphy) and the glycaemic response

after a standard meal 30 min after the preload, were measured at the beginning and end of each 4 week period. Diet diaries and glucose monitoring were undertaken concurrently for 3 day periods before and during the last week of each 4 week treatment period. The 25 g whey preload slowed gastric emptying and reduced postprandial blood glucose compared to the placebo, and the effects on glycaemia ($P < 0.05$) and gastric emptying ($P < 0.05$) were sustained after 4 weeks administration. Fructosamine tended ($P = 0.06$) to be lower after 4 weeks whey than 4 weeks placebo, but there was no difference in energy intake. In conclusion, a whey preload remains effective in reducing postprandial glycaemia and slowing gastric emptying after sustained exposure.

7.2 Introduction

The risk of microvasular, and probably macrovascular, complications of diabetes is related to control of blood glucose concentrations, as assessed by glycated haemoglobin. It is now recognized that postprandial glycaemia is a key determinant of overall glycaemic control (Heine et al. 2004). Therapeutic strategies directed to minimizing postprandial blood glucose excursions therefore represent a major focus of the management of type 2 (as well as type 1) diabetes (Nauck and Meier 2005).

Recent efforts to control postprandial blood glucose have been aimed at both

slowing the rate of gastric emptying and stimulating the incretin response (Chaikomin et al. 2006). Indeed, glucagon-like peptide-1 (GLP-1) analogues and GLP-1 receptor agonists have been developed for the treatment of diabetes. Slowing the rate of entry of nutrients into the small intestine may be the dominant mechanism by which exogenous GLP-1 analogues such as exenatide (Cervera et al. 2008), and the amylin agonist, pramlintide (Edelman 2008), improve glycaemic control.

One dietary strategy to stimulate incretin hormones and slow gastric emptying, as discussed in Chapter 5, is to administer a small load of macronutrient as a „preload" so that the presence of nutrients in the small intestine both induces the release of peptides such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), and slows gastric emptying, in advance of a meal (Gentilcore et al. 2006b; Ma et al. 2009c). In the study reported in Chapter 5, ingestion of 55 g whey protein, 30 minutes before a high carbohydrate meal, slowed gastric emptying, stimulated incretin hormone secretion, and markedly attenuated the postprandial rise in blood glucose in patients with type 2 diabetes (Ma et al. 2009c). In Chapter 6, a smaller load of protein (25 g whey) was shown also to lower the glycaemic response to a subsequent meal. However, adaptive changes in gastric emptying might occur in response to sustained changes in dietary protein intake, presumably as a result of altered small intestinal feedback, as has

been reported after a sustained increase in carbohydrate intake (Horowitz et al. 1996a; Beckoff et al. 2001). Therefore, the aim of the current study was to determine whether the effects of a whey preload on gastric emptying, glycaemia and incretin hormone release are sustained with „chronic" (4 weeks) administration, and whether this impacts on the glycaemic profile and on food intake.

7.3 Methods

7.3.1 Subjects

Seven patients with type 2 diabetes, diagnosed by World Health Organization criteria and managed by diet alone (3 male; age: 60 ± 2 yr; body mass index: 31 ± 2 kg/m^2), were recruited by newspaper advertisement. None had a history of significant comorbidities, was a smoker, or was taking medication known to influence gastrointestinal function. The mean duration of known diabetes was 4 ± 1 yr, glycated haemoglobin was 6.0 ± 0.2 %, and all had plasma creatinine $\leq$ 0.12mmol/L.

The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital, and all subjects provided written informed consent. All experiments were carried out in accordance with the Declaration of Helsinki.

7.3.2 Protocol

The study followed a randomized, single-blind, cross-over design. Each subject consumed a chocolate flavoured preload (100 ml water with either 25 g chocolate flavoured „diet" sauce (Cottee's, Southbank, VIC, Australia, 8 kcal), or with 25 g whey protein isolate (Ascend, Murray Goulburn, Brunswick, VIC, Australia, 89 kcal)), 30 min before each of three main meals for 4 weeks (weeks 1-4), immediately followed by a „washout" period of 2 weeks (weeks 5-6), and then the alternative preload for 4 weeks (weeks 7-10). The odour, taste, palatability, consistency and sweetness of the preloads were similar and both contained comparable, and relatively small, amounts of carbohydrate (3.1 g vs. 0.1 g), and fat (0.1 g vs. 0.4 g).

All participants attended the Department of Nuclear Medicine, PET and Bone Densitometry at the Royal Adelaide Hospital at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids) on four occasions, each separated by at least 3 days, at „baseline" and during weeks 4, 6, and 10. An intravenous cannula was inserted into an antecubital vein for blood sampling at frequent intervals. Subjects were seated with their back against a gamma camera. On each of the four days, subjects consumed the preload corresponding to the treatment period to which they were assigned. The preload was consumed within one minute, 30 min before a mashed potato meal, consisting of 65 g powdered potato

(Deb, Epping, NSW, Australia) and 20 g glucose, reconstituted with 250 ml water (total carbohydrate content: 62 g; 314 kcal (Gentilcore et al. 2006b)) and labelled with 20 MBq ^{99m}Tc-sulphur colloid, which was consumed within 5 minutes. Gastric emptying was assessed from the time of ingestion of the meal and for 240 min afterwards. Blood glucose concentrations were measured immediately by glucometer. The remainder of each blood sample was placed in EDTA tubes containing aprotinin on ice, and was centrifuged at 3200rpm for 15 minutes. Plasma was separated and stored at -70 °C for subsequent hormone analyses, using established assays.

Food consumption (by diet diary) and blood glucose data (by continuous glucose monitoring) were collected concurrently for 3 day periods at baseline and in the second half of weeks 4, 6 and 10. Body weight, height, fructosamine and glycated haemoglobin were measured at baseline and at the end of weeks 4, 6, and 10. Compliance was monitored by assessing a checklist which the subject marked after consuming each „preload", and by weekly telephone contact.

7.3.3 Measurements

<u>Gastric emptying</u>

Radioisotopic data were acquired as 60-sec frames for the first 60 min and 3-min frames between 60-240 min. Data were adjusted for subject movement,

radionuclide decay, and gamma-ray attenuation. A „region of interest" was drawn around the total stomach, which was subsequently divided into proximal and distal regions, and gastric emptying curves (expressed as percent retention over time) were derived (Gentilcore et al. 2006b). The lag phase was defined visually as the period before any radioactivity was present in the proximal small intestine. The retention of the meal in the total, proximal, and distal stomach at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 225 and 240 min was calculated; the 50% emptying time (T50) was also determined (Collins et al. 1983).

Blood glucose measurement

Blood glucose concentrations on the four study days were measured immediately using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA).

Plasma insulin, GLP-1, GIP and CCK assays

At the time of writing, the assays have not yet been completed. Plasma insulin will be measured by a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity is 2 mU/L, intraassay coefficient of variation (CV) is 3.9 % and interassay CV is 5.0 %. Total GLP-1 will be measured by

radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, MO, USA). The sensitivity is 3 pmol/L, and the inter-assay CV is 9.2 %. Plasma GIP will be measured by RIA, with a sensitivity of 2 pmol/L, and both intra- and inter-assay CVs of 15 %. CCK-8 will also be measured by RIA with sensitivity of 1 pmol/L, intra-assay CV of 6.2 % and inter-assay CV of 14.2 % (Santangelo et al. 1998).

Energy intake

A 3 day diet diary was used to assess energy intake for three day periods in the week before, and the last week of, each 4 week dietary treatment (Bingham et al. 1994). During these three days, subjects were asked to weigh all food eaten in their main meals and snacks using digital scales, and measure volumes of fluids and alcoholic drinks using a „measuring" cup. The brand of bread and cereal, type of oil, and whether meat was fatty or lean, were also recorded. Energy intake (calculated as kcal) was analyzed to the nearest 0.1 kcal by FoodWorks software (version 3.1, Xyris Software, Highgate Hill, Qld, Australia).

72-hour continuous glucose monitoring

A continuous glucose monitoring system (CGMS, Medtronic Minimed, Northridge, CA, USA) was used to monitor glucose levels over 72 hours, at the same time as the 3 day diet diary. Subjects were asked to input fingerprick blood

glucose levels, measured by a glucometer four times each day (20 min before each of three main meals and once before bedtime) in order to calibrate the device (Pearce et al. 2008).

7.3.4 Statistical analysis

Blood glucose concentrations, gastric emptying data, fructosamine and glycated haemoglobin were analyzed by repeated measures ANOVA (StatView, version 5.0, Abacus Concepts, Inc., Berkeley, CA, USA and SuperANOVA version 1.11, Abacus Concepts, Inc. Berkeley, CA, USA) with treatment and time as factors. In the event of a significant treatment × time interaction, individual time points were compared with means comparisons. Data are shown as mean values ± standard error; $P < 0.05$ was considered significant. The number of subjects was based on power calculations derived from our previous work.

7.4 Results

All subjects completed the study and tolerated the protocol well. Analysis of the checklists for compliance indicated that all prelaods were taken, with the exception of two doses in one subject and three in another.

7.4.1 Blood glucose concentrations (Figure 1)

At baseline and week 6, there were no differences in blood glucose concentrations, either fasting (t = -30 min), or before the meal (t = 0 min). There was a postprandial rise in blood glucose from 15min with both placebo ($P < 0.0005$) and whey preloads ($P < 0.05$). Blood glucose concentrations were lower at t = 15, 30, 45, 60, 75, 90, and 105 min and greater at t = 165 min after the whey preload than with placebo (treatment × time interaction, **P < 0.05**). The peak blood glucose concentration was also reduced after the whey preload (12.5 ± 0.8 mmol/L vs. 13.8 ± 0.5 mmol/L, P = 0.005).

After 4 weeks administration of each preload, there was again no difference in blood glucose concentrations, either fasting (t = -30 min) or before the meal (t = 0 min) between whey preload and placebo. There was a postprandial rise in blood glucose from t = 15 min with placebo ($P < 0.005$) and from t = 5 min with whey ($P < 0.05$), and blood glucose concentrations were lower at t = 30, 45, 60, 75, and 120 min and greater at t = 165 min after the whey preload than with placebo (treatment × time interaction, **P < 0.05**), although peak blood glucose did not differ between the placebo and whey preloads. There was no difference in blood glucose between the two whey days, nor between the two placebo days.

7.4.2 Gastric emptying (Figure 2)

At baseline and week 6, gastric emptying commenced after a short lag phase; the latter tended to be longer after whey preload than placebo (7.7 $\pm$ 2.4 vs. 4.9 $\pm$ 1.4 min; P = 0.10). On all days, emptying was more rapid initially, and subsequently slower. Gastric emptying was slower after the whey preload compared to placebo (treatment $\times$ time interaction, P < 0.0005). Meal retention was greater at t = 30, 45, 60, 75, 90 and 105 min after the whey preload than placebo (P < 0.05). Accordingly, the T50 was longer with the whey preload (81.3 $\pm$ 5.8 vs. 67.7 $\pm$ 8.4 min; P = 0.057).

After 4 weeks exposure to each preload (weeks 4 and 10), the lag phase again tended to be longer after the whey preload than placebo (7.7 $\pm$ 3.0 vs. 3.9 $\pm$ 2.0 min, P = 0.09). Gastric emptying was slower after the whey preload compared to placebo (treatment $\times$ time interaction, P < 0.0005), with greater meal retention at t = 30, 45, 60, 75 and 90 min (P < 0.05). Again, the T50 was longer with the whey preload than placebo (76.5 $\pm$ 6.4 vs. 59.7 $\pm$ 7.5 min; P < 0.05). There was no difference in the rate of gastric emptying or T50 between the two whey days, nor between the two placebo days.

7.4.3 Fructosamine and glycated haemoglobin (Figure 3)

At baseline and week 6, fructosamine did not differ between the whey and placebo preloads (259.3 ± 16.4 vs. 257.3 ± 11.2 μmol/L). After 4 weeks exposure (week 4 and 10), fructosamine tended to be lower after the whey preload than placebo (252.9 ± 15.2 vs. 279.4 ± 9.6 μmol/L, P = 0.06). However, there was no significant difference between the values before and after whey, or before and after placebo.

At baseline and week 6, glycated haemoglobin did not differ significantly between the whey and placebo preloads (6.0 ± 0.3 vs. 6.0 ± 0.2 %). After 4 weeks exposure (week 4 and 10), glycated haemoglobin again did not differ between whey preload and placebo (5.9 ± 0.2 vs. 6.0 ± 0.2 %). There was no difference between the values before and after either the whey or placebo treatment periods.

7.4.4 Energy intake (Figure 4)

Energy intake did not differ significantly between the whey and placebo preload, either before the intervention (baseline and week 6) (7818 ± 549 vs. 8535 ± 613 kcal), or after 4 weeks exposure to preload (week 4 and 10) (7818 ± 354 vs. 7939 ± 673 kcal). There was no difference between energy intake before and after

either whey or placebo treatment.

7.4.5 Body weight

Body weight did not differ significantly between the whey and placebo preload either before the intervention (baseline and week 6) (89.7 ± 6.8 vs. 89.4 ± 6.8 kg), or after 4 weeks exposure to preload (week 4 and 10) (88.8 ± 7.1 vs. 89.3 ± 7.0 kg). There was no difference between body weights before and after either the whey or placebo treatments.

7.5 Discussion

This study has established that 25 g whey protein, when given as a preload before a carbohydrate meal, slowed gastric emptying and reduced the postprandial blood glucose excursion in patients with diet-controlled type 2 diabetes, and importantly, that these effects were sustained after four weeks exposure to the preload. It had already been demonstrated in the study reported in Chapter 5 that a whey preload attenuated the glycaemic response to a subsequent high carbohydrate meal in patients with type 2 diabetes by stimulating GLP-1, GIP and insulin secretion and slowing gastric emptying (Ma et al. 2009c), and in the study reported in Chapter 6, that 25 g whey induced a similar, acute reduction in postprandial blood glucose after a buffet style meal. The current study has,

accordingly, established the feasibility and efficacy of the preload concept with more sustained administration. During a four week period, the preload was well tolerated and compliance was excellent.

The mechanisms by which whey protein lowers glycaemia include an increase in insulin secretion, both by stimulating GLP-1 and GIP, and by absorbed amino acids such as leucine, isoleucine, valine and lysine released after digestion of whey (Fieseler et al. 1995; Nilsson et al. 2007). The latter appear responsible for the majority of insulin stimulation (Nilsson et al. 2007), although the two mechanisms may potentially interact (Fieseler et al. 1995). Moreover, it has been demonstrated that whey protein consumption is associated with reduced hepatic insulin extraction and enhanced C-peptide clearance (Lan-Pidhainy and Wolever 2009). At the time of writing this thesis, the hormone data were not available. However, the fact that the slowing of gastric emptying and decrease in glycaemia were unchanged over 4 weeks in the current study suggests that the gut hormones would also be unchanged and that the dominant mechanism accounting for glucose-lowing is incretin stimulation.

It has been increasingly recognised that gastrointestinal motor function, in particular the rate of gastric emptying, is a major determinant of the postprandial glucose excursion in healthy subjects and patients with type 2 diabetes. In the

study reported in Chapter 5, a whey protein preload acutely attenuated the glycaemic response in patients with type 2 diabetes, in part by slowing gastric emptying (Ma et al. 2009c). However, the rate of gastric emptying can be influenced by dietary modifications (Cunningham et al. 1991b; Corvilain et al. 1995). For example, short-term supplementation of the diet with glucose accelerates gastric emptying of both glucose and fructose in healthy subjects (Horowitz et al. 1996a). Similarly, dietary supplementation with fat leads to more rapid gastric emptying of a high fat meal (Cunningham et al. 1991a). There is little information about the chronic effects of protein supplementation, but in the current study, no adaptive changes in gastric emptying were evident after giving the protein preload for four weeks. This might support the use of protein, rather than other macronutrient preloads, for long term administration in the control of glycaemia. It is possible that gastric emptying did not adapt after 4 weeks because the test meal was relatively low in protein, as adaptation of gastric emptying appears to be macronutrient-specific (Beckoff et al. 2001).

In the current study, the chronic effects of protein on glycaemic control were assessed by glycated haemoglobin, fructosamine and CGMS. Glycated haemoglobin is regarded as the gold standard for monitoring blood glucose control over the preceding 12 weeks, and is widely used for this purpose in the clinical management of diabetic patients (Landgraf 2006). In present study, a

four week treatment period was probably insufficient to detect a difference in glycated haemoglobin. The capacity for detecting a change was also diminished by the good glycaemic control at baseline (glycated haemoglobin 6.0 ± 0.3 vs. 6.0 ± 0.2 %). Fructosamine correlates with glycated haemoglobin and can be altered by shorter-term changes in glycaemia (Youssef et al. 2008). After treatment with whey preload for 4 weeks, there was a non-significant trend for fructosamine to be lower than after 4 weeks exposure to placebo. CGMS has been developed recently as a noninvasive measure of dynamic glucose changes in the interstitial fluid every 5 min for 72 h (Pearce et al. 2008). It has been shown that CGMS recordings correlate closely with capillary blood glucose in detecting asymptomatic hypoglycaemia and glycaemic fluctuations (Caplin et al. 2003) and represent a more responsive index for assessing glycaemic control than glycated haemoglobin and fructosamine in some patients, such as those on haemodialysis (Riveline et al. 2009). The analysis of the CGMS data will be of considerable interest, but has not been completed at the time of writing this thesis.

Chronic modulation of dietary protein intake could potentially affect energy intake, as well as glucose homeostasis (Farnsworth et al. 2003). Whey protein preloads are associated with secretion of satiety hormones such as GLP-1 and CCK (Ma et al. 2009c) (Chapter 5). However, no acute effects of a 25 g or 55 g

protein preload were observed on energy intake from a buffet style meal in the study reported in Chapter 6. Accordingly, it is not surprising that there were no differences in energy intake in the 3 day diet diary analysis in the current study. In both studies the number of subjects was relatively small. Hence, a type 2 error cannot be excluded. It is also possible that the whey preload will affect patterns of energy intake, as opposed to total energy intake; this remains to be determined.

In conclusion, there are sustained, beneficial effects of a small protein preload (25 g) on glycaemia and gastric emptying in patients with diet-controlled type 2 diabetes. These observations indicate that dietary strategies, involving whey protein to modulate postprandial glycaemic excursions, are feasible in patients with type 2 diabetes and should be pursued. It should be recognized that this study involved a relatively small number of patients with well controlled diabetes. Longer-term studies involving larger numbers of type 2 patients, including those with poor glycaemic control, are needed to determine whether this approach can achieve sustained improvement in glycaemia.

Blood glucose concentration
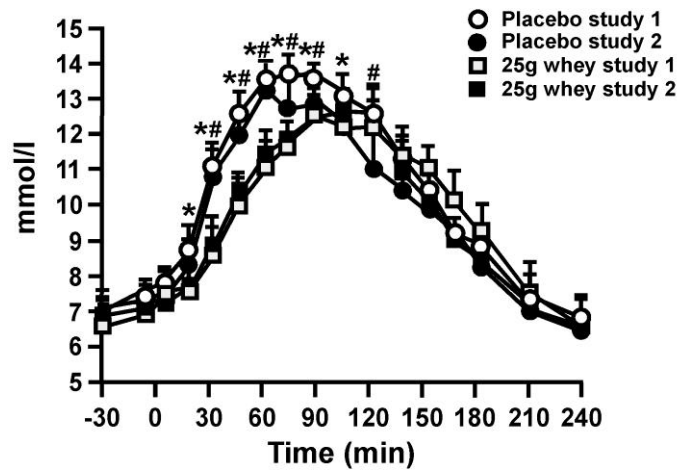
Figure 7.1 Blood glucose concentrations on the four study days, at „baseline" and during weeks 4, 6, and 10, in 7 type 2 diabetic patients. On each study day, subjects ingested 125 g chocolate flavoured drink (100 ml water with either chocolate flavoured „diet" sauce or with 25 g whey protein isolate) 30 min before a radiolabeled mashed potato meal. Data are mean ± standard error. * P < 0.05, 1st whey day vs. 1st placebo day; # P < 0.05, 2nd whey day vs. 2nd placebo day.

Gastric emptying
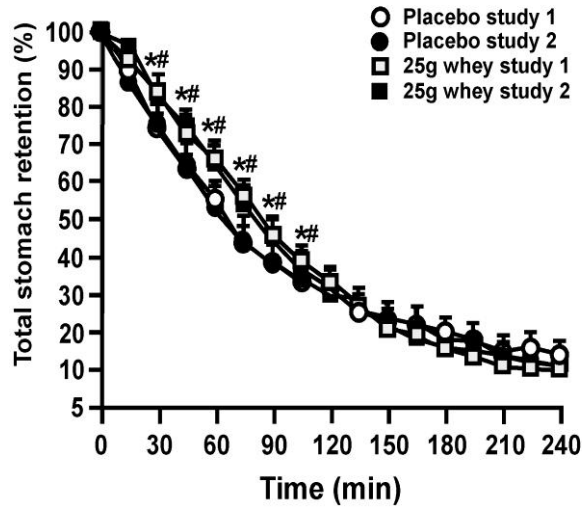
Figure 7.2 Gastric emptying on the four study days, at „baseline" and during weeks 4, 6, and 10, in 7 type 2 diabetic patients. On each study day, subjects ingested 125 g chocolate flavoured drink (100 ml water with either chocolate flavoured „diet" sauce or with 25 g whey protein isolate) 30 min before a radiolabeled mashed potato meal. Data are mean ± standard error. * $P < 0.05$, 1st whey day vs. 1st placebo day; # $P < 0.05$, 2nd whey day vs. 2nd placebo day.

Fructosamine

Glycated haemoglobin
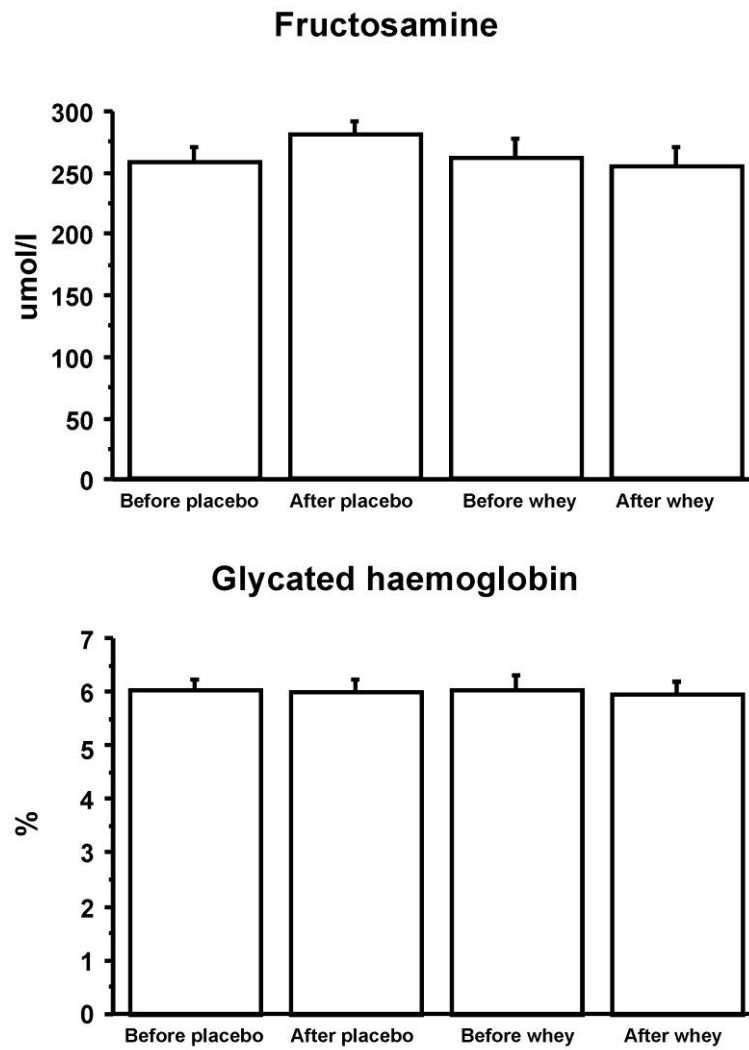
Figure 7.3 Fructosamine and glycated haemoglobin at „baseline" and during weeks 4, 6, and 10, in 7 type 2 diabetic patients. These represent the values before and after 4 weeks treatment with 25 g whey or placebo preload, taken before each main meal.
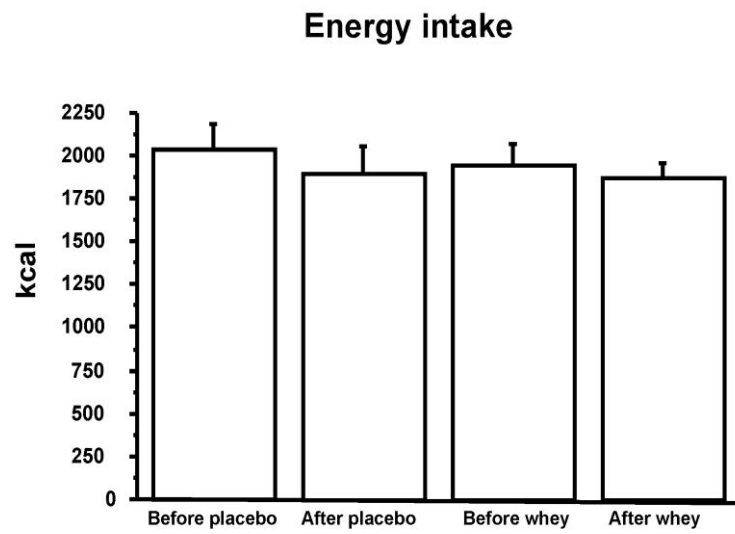
Energy intake

Figure 7.4 Energy intake over 72 hours at „baseline" and during weeks 4, 6, and

10, in 7 type 2 diabetic patients.

CHAPTER 8: EFFECTS OF ENTERICALLY COATED, NUTRIENT-CONTAINING PELLETS ON GLYCAEMIA AND THE RELEASE OF GASTROINTESTINAL PEPTIDES IN PATIENTS WITH TYPE 2 DIABETES

8.1 Summary

Dietary and pharmacological strategies to increase endogenous glucagon-like peptide-1 (GLP-1) secretion are attracting much interest in the management of diabetes. The study presented in this chapter evaluates the effects of enterically-coated pellets designed to release lauric acid in the ileum („Clinical Testing Material #3" or „CTM#3") on GLP-1 secretion and the glycaemic response after both the „carrier meal" (breakfast), and a subsequent lunch. Ten patients with type 2 diabetes, managed by diet alone, were studied on two separate days. On each study day, they consumed a „carrier meal" (breakfast) containing either 10 g CTM#3 or placebo pellets. A second meal (lunch) was consumed at t = 240 min. Blood samples were taken to measure blood glucose, serum insulin, and plasma glucose-dependent insulinotropic polypeptide (GIP) and GLP-1. Blood glucose concentrations were lower, and plasma GLP-1 concentrations higher after CTM# 3 than placebo, after both breakfast and lunch (P < 0.05 for each). The rise in serum insulin after breakfast and lunch did not

differ between CTM#3 and placebo. In conclusion, there was a glucose lowering effect of CTM#3 after both breakfast and lunch, associated with prolonged stimulation of GLP-1 in patients with type 2 diabetes. Exposure of the distal small intestine to a small quantity of nutrient can have profound effects on gut hormone release and glycaemic control.

8.2 Introduction

Achieving good postprandial glycaemic control appears to be of relatively greater importance than targeting fasting blood glucose to optimize glycated haemoglobin and reduce the onset and/or progression of microvascular, and probably macrovasular, complications of diabetes (Ceriello et al. 2008). Both the rate of gastric emptying, and the action of the incretin hormones, GLP-1 and GIP, are major determinants of postprandial glucose excursions (Chaikomin et al. 2006). In healthy subjects, GLP-1 and GIP account for up to 70 % of postprandial insulin secretion (Holst et al. 2009), while differences in the rate of gastric emptying are responsible for about one third of the variation in the initial rise in blood glucose after an oral glucose load in healthy subjects and patients with type 2 diabetes (Horowitz et al. 1993; Jones et al. 1996). Therefore, dietary and pharmacological strategies that act on the incretin axis, in particular focusing on GLP-1, have risen to prominence in the management of diabetes.

Dietary strategies that aim to stimulate endogenous GLP-1 release require

interaction of macronutrients, or other GLP-1 secretagogues, with sensors located in the small intestine, that regulate both gastric emptying and hormone release. The degree of nutrient-mediated small intestinal „feedback" is dependent on the length of contact with stimulating nutrient (Lin et al. 1989; Lin et al. 1990), and the region of small intestine exposed (Pilichiewicz et al. 2006). For example, gastric emptying is strongly inhibited by delivering nutrients to the ileum (Lin et al. 1989; Lin et al. 1990), an effect modulated by release of peptides including GLP-1 and polypeptide YY (PYY) from L-cells, which are located most densely in the ileum and colon (Baggio and Drucker 2007).

One effect of the bariatric operation, Roux-en-Y gastric bypass (RYGB), is to accelerate transit of ingested nutrients to the distal small intestine. It is increasingly recognized that such surgery ameliorates type 2 diabetes before significant weight loss has occurred (le Roux et al. 2006a). The mechanism(s) underlying this phenomenon appear to be related to increased GLP-1 secretion from L-cells in the ileum or colon (Morinigo et al. 2006), such that after RYGB, glucose-stimulated concentrations of GLP-1 are increased up to four fold compared to pre-operative values (Laferrere et al. 2007).

Meyer Nutriceuticals (Santa Monica, CA, USA) has designed enterically-coated pellets („clinical testing materials") to release macronutrients in the distal small

intestine. Clinical testing material number 3 (CTM#3) consists of pellets of a diameter of 1-3 mm containing 47 % lauric acid by weight, enterically coated to release lauric acid in the ileum and colon. CTM#3 also contains 4.7 % paracetamol, as a pharmacokinetic marker that is rapidly absorbed throughout the small and large intestine, and can be assayed in peripheral blood to yield information about the timing of lauric acid release. When given with a „carrier meal" of 415 kcal, the entry of pellets into the small intestine is regulated so that they start to arrive in the ileum with one hour of meal ingestion and continue to do so over another 2 – 3 h. Dissolution and discharge of nutrient from the pellets begins in < 30 min and takes up to 6 h to complete (unpublished in vitro observations), so that the ileum is exposed continuously to lauric acid for > 3 h following meal ingestion. For several more hours, the colon is also exposed to lauric acid. The capacity of CTM#3 to release nutrient in the ileum and colon in this fashion has been validated in healthy volunteers (unpublished observations).

In addition to inducing release of the incretin hormone GLP-1, nutrient exposure of the ileum could also slow the rate of gastric emptying of a subsequent meal. It is increasingly accepted that gastric emptying is a major determinant of the postprandial glycaemic response, and patients with type 2 diabetes can benefit from slowing of gastric emptying (Chapter 5), which is the main action of some pharmacological therapies such as exenatide (Gilbert and Pratley 2009). Gastric

emptying could be regulated by secretion of GLP-1 and PYY, as well as by stimulation of neural pathways.

The hypothesis underlying the present study was that a small load of lauric acid, released in the ileum so as to provide a long length of intestinal exposure, would induce sufficient release of GLP-1 to reduce the glycaemic response after both the „carrier meal", given as a breakfast, and after a subsequent lunch, in patients with type 2 diabetes.

8.3 Methods

8.3.1 Subjects

Ten patients with type 2 diabetes, diagnosed by World Health Organization criteria and managed by diet alone (5 male; age: 62 ± 2 yr; body mass index: 28.9 ± 0.1 kg/m^2), were recruited by newspaper advertisement. None had a history of significant comorbidities, was a smoker, or was taking medication known to influence gastrointestinal function. The mean duration of known diabetes was 4.6 ± 0.6 yr, glycated haemoglobin was 5.9 ± 0.2 % (normal range: < 6.0 %), and all had plasma creatinine ≤ 0.12 mmol/L.

The study protocol was approved by the Research Ethics Committee of the Royal

Adelaide Hospital, and each subject provided written informed consent. All experiments were carried out in accordance with the Declaration of Helsinki.

8.3.2 Protocol

Each subject was studied on two occasions, separated by at least 3 days, in double-blinded, randomized fashion. On the evening preceding each study day (~1900 h), subjects were given a standardized meal (McCains frozen beef lasagne (McCain Foods Proprietary Ltd, Australia, 656 kcal), consumed with bread, a non-alcoholic beverage and one piece of fruit. Following the meal, subjects were asked to fast from solids and liquids (14 h for solids, 12 h for liquids, other than water) until the following morning.

Subjects attended the Discipline of Medicine at approximately 0800h. An intravenous cannula was inserted into an antecubital vein for blood sampling. The „carrier meal" („breakfast", 415 kcal; 71 g carbohydrate, 12 g fat, 4.3 g protein) comprising 3 pikelets (Golden, North Ryde, NSW, Australia), coated with 9 g butter (Westin Star, Rowville, VIC, Australia), 15 g golden syrup (Smith's, Richlands, NSW, Australia), and 140 g pureed apple (Goulburn Valley, Shepparton, VIC, Australia), and containing either 10 g CTM#3 or placebo pellets, was consumed with 100 ml water at t = 0. A second meal („lunch", 708 kcal; 89 g carbohydrate, 26 g fat, 29 g protein) was consumed at t = 240 min and

it contained one sandwich (60 g white bread, 50 g ham, 10 g margarine), 300 ml orange juice, 200 g strawberry yogurt, 20 g cheese portion and 10 g crackers. Subjects were seated comfortably in a chair for the duration of the study. Blood samples were obtained at frequent intervals for measurements of blood glucose, serum insulin and plasma GLP-1 and GIP. Blood samples were placed in prechilled EDTA tubes containing 400 kIU aprotinin (Trasylol, Bayer Australia Ltd, Pymble, NSW, Australia) per litre blood for incretin hormone assays and pre-chilled serum tubes with clotting activator for insulin assays, both stored on ice, and centrifuged at 3200 rpm and 4 ºC for 15 min. Samples were stored at -70 °C until analyzed. On one of the study days, autonomic nerve function was evaluated using standardized cardiovascular reflex tests (Ewing and Clarke 1982), after completion of the other measurements.

8.3.3 Measurements

Blood glucose concentrations

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. 1991).

Serum insulin, plasma GLP-1 and GIP

Serum insulin was measured by a solid-phase, two-site chemiluminescent immunometric assay (Ma et al. 2009b). The sensitivity was 2 mU/L, intra-assay coefficient of variation (CV) 3.9 % and inter-assay CV 5.0 %. Total GLP-1 was measured by radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, MO, USA) (Ma et al. 2009c). The sensitivity was 3 pmol/L, and inter-assay CV 9.2 %. Plasma GIP was measured by RIA, with a sensitivity of 2 pmol/L, and both intra- and inter-assay CVs of 15 % (Ma et al. 2009c).

Cardiovascular autonomic function

Parasympathetic function was assessed by the variation in heart rate (R-R interval) during deep breathing and the heart rate response to standing („30:15‟ ratio); sympathetic function was determined by the fall in systolic blood pressure in response to standing. Each test result was scored according to age-adjusted predefined criteria as: 0 = normal, 1 = borderline, and 2 = abnormal, for a total maximum score of 6. A score of 3 or higher was taken as evidence of autonomic dysfunction (Ewing and Clarke 1982).

8.3.4 Statistical analysis

Incremental areas under the blood glucose and plasma hormone concentration curves (iAUC) were calculated using the trapezoidal rule, ignoring any negative

values (Wolever 2004). Data were analyzed in two periods, after breakfast (t = 0-240 min) and after lunch (t = 240-480 min) using repeated measures ANOVA, with treatment and time as factors. In the event of significant treatment × time interactions, individual time points were compared with means comparisons. Baseline and peak values were compared using student's paired test. All analyses were performed using StatView (version 5.0, Abacus Concepts, Inc., Berkeley, CA, USA) and SuperANOVA (version 1.11, Abacus Concepts, Inc., Berkeley, CA, USA). Data are shown as mean values ± standard error; P < 0.05 was considered statistically significant.

8.4 Results

All subjects tolerated the study well. The mean score for autonomic nerve dysfunction was 1.8 ± 0.4; four of the ten diabetic subjects had definite autonomic dysfunction.

8.4.1 Blood glucose concentrations (Figure 1A)

Fasting blood glucose did not differ between the two study days. After breakfast (t = 0 − 240 min), blood glucose concentrations increased on both days (P < 0.0005), but were lower after CTM#3 than placebo (P < 0.05, treatment × time interaction). The differences were evident after t = 45 min up to t = 150 min. The

peak blood glucose concentration was also lower after CTM#3 than placebo (10.0 ± 0.6 vs. 10.9 ± 0.7 mmol/L; $P < 0.01$).

Before lunch (t = 240 min), blood glucose values did not differ between the study days, but was lower than before breakfast at t = -2 min on the placebo day (7.9 ± 0.5 vs. 7.0 ± 0.5 mmol/L; $P < 0.05$), but not with CTM#3. After lunch (t = 240 - 480 min), blood glucose increased on both days ($P < 0.005$), again was lower after CTM#3 than placebo ($P < 0.05$, treatment $\times$ time interaction) with a significant difference at t = 330 min. The peak blood glucose was also lower after CTM#3 (9.0 ± 0.5 mmol/L vs. 9.7 ± 0.5 mmol/L, $P < 0.05$).

The peak blood glucose level was lower after lunch than after breakfast on both study days (placebo: 9.7 ± 0.5 vs 10.9 ± 0.7 mmol/ L, $P < 0.05$; CTM#3: 9.0 ± 0.5 vs. 10.0 ± 0.6 mmol/L, $P < 0.01$).

8.4.2 Serum insulin concentrations (Figure 1B)

Fasting serum insulin concentrations did not differ between the two days. After breakfast, serum insulin increased on both days ($P < 0.0005$), with no difference between CTM#3 and placebo.

Insulin concentrations before lunch (t = 240 min) did not differ between CTM#3

and placebo, but were higher before lunch than before breakfast at t = -2 min for both CTM#3 and placebo (P < 0.05 and P < 0.005 respectively). After lunch (t = 240-480 min), serum insulin increased on both days (P < 0.0005 for both), again with no difference between CTM#3 and placebo.

The insulin response to lunch was greater than to breakfast on both days (iAUC 14062.6 ± 4106.5 vs. 9046.8 ± 2366.4 mU.min/L after placebo, and 10922.4 ± 2928.7 vs. 8688.3 ± 2755.1 mU.min/L after CTM#3; P < 0.05 for both).

8.4.3 Insulin:glucose ratio (Figure 1C)

The insulin:glucose ratio did not differ between CTM#3 and placebo, either after breakfast or after lunch.

The insulin:glucose ratio after lunch was greater than after breakfast on both days (iAUC: 918.3 ± 241.5 vs. 1524.6 ± 477.3 mU.min/mol after placebo, and 1264.8 ± 364.2 vs. 961.4 ± 315.2 mU.min/L after CTM#3; P < 0.05 for both).

8.4.4 Plasma GLP-1 concentrations (Figure 1D)

Fasting GLP-1 concentrations did not differ between the two study days. After breakfast (t = 0-240 min), GLP-1 increased on both days (P < 0.05) with greater

concentrations after CTM#3 than placebo ($P < 0.01$, treatment × time interaction), with significant differences at $t = 90$, 150, 180 and 240 min.

Plasma GLP-1 concentrations did not differ after CTM #3 v. placebo before lunch, at 240 min, but were higher before lunch than before breakfast ($t = -2$ min) with CTM #3 (19.3 ± 2.9 vs. 12.2 ± 2.5 mmol/L; $P < 0.05$), but not with placebo. After lunch ($t = 240$-480 min), GLP-1 concentrations increased on both days and were higher after CTM#3 than placebo ($P < 0.05$, treatment effect). The peak GLP-1 concentration was also greater after CTM#3 than placebo (39.2 ± 5.3 vs. 29.0 ± 3.5 pmol/L; $P < 0.05$).

On both days, iAUC GLP-1 was greater after lunch than after breakfast (1524.6 ± 477.3 vs. 918.3 ± 241.5 pmol.min/L after placebo, and 1268.4 ± 364.2 vs. 961.4 ± 315.2 pmol.min/L after CTM#3; $P < 0.05$ for both).

8.4.5 Plasma GIP concentrations (Figure 1E)

Fasting plasma GIP concentrations did not differ between the two days. After breakfast ($t = 0$-240 min), there was a rise in plasma GIP on both days ($P < 0.05$), with no difference between CTM#3 and placebo.

Plasma GIP concentrations before lunch ($t = 240$ min) did not differ, but were

higher before lunch than before breakfast (t = -2 min), with both CTM#3 and placebo (P < 0.001 and P < 0.005 respectively). On both days, plasma GIP increased after lunch (P < 0.0005), again with no difference between CTM#3 and placebo.

8.4.6 Plasma paracetamol concentrations (Figure 1F)

Plasma paracetamol concentrations increased progressively to a peak occurring at 320 ± 16.4 min, indicating sustained release from CTM#3 throughout most of the study.

8.5 Discussion

This study establishes that there is a glucose lowering effect of a dose of CTM#3 given with breakfast, compared to placebo, observed after both breakfast and lunch, and associated with prolonged stimulation of GLP-1 release. This represents ‚proof of concept"that even a small nutrient load (4.7 g lauric acid, ~ 40 kcal), when delivered over a long length of ileum and colon, can stimulate a relatively large amount of GLP-1. The magnitude of reduction in blood glucose was ~ 1 mmol/L, which although modest, is likely to be clinically significant in these well-controlled patients who had modest glycaemic excursions on the placebo day. The glucose-lowering effect would likely be greater if blood glucose

levels had been higher. The difference in blood glucose was less marked after lunch than after breakfast, but the lunch contained proportionally less carbohydrate, and more protein and fat, which would have contributed to greater insulin secretion after lunch, and would in itself would have limited the rise in blood glucose after lunch on the placebo day (Karamanlis et al. 2007), making it difficult to demonstrate any additional effect of CTM#3.

GLP-1 is secreted from L-cells mainly in the ileum and colon, with fewer L-cells found in the duodenum and jejunum (Theodorakis et al. 2006). Digestion of fat to yield fatty acids is required for GLP-1 secretion (Feinle et al. 2003), and the secretion of GLP-1 is also length-dependent, with greater release when > 60 cm of small intestine is exposed to glucose (Little et al. 2006a). It would, therefore, be expected that GLP-1 secretion in response to nutrients in the ileum would be greater than in the proximal small intestine (Little et al. 2006a; Hira et al. 2009b). In healthy subjects, we have observed that CTM#3 consumed with breakfast potentiated the release of polypeptide YY (PYY), a hormone co-located with GLP-1 in the L-cells of the distal gut (Wen et al. 1995) (unpublished observations). The current study confirmed that CTM#3 taken with breakfast stimulates the release of GLP-1 more than placebo, in patients with type 2 diabetes. The fact that the timing of the peak in plasma GLP-1, which occurred after lunch, was similar to the peak plasma concentration of paracetamol,

indicates that the pellets continued to release lauric acid slowly over about 6 hours.

The incretin hormones, GLP-1 and GIP, contribute to postprandial insulin secretion approximately equally in healthy subjects (Vilsboll et al. 2003b). However, in type 2 diabetic patients, while the insulinotropic effect of GLP-1 is preserved, that of GIP is abolished (Nauck et al. 1993b). Nevertheless, despite marked stimulation of GLP-1 by CTM#3 in the current study, neither serum insulin nor the insulin:glucose ratio was greater than with placebo, indicating that other mechanisms are likely to be involved in the observed lowering of blood glucose concentrations.

Among the multiple actions of GLP-1 on glucose homeostasis, inhibition of gastric emptying (the „ileal brake") represents an important effect, which may outweigh the insulinotropic action of GLP-1, as demonstrated in studies involving „physiological" infusions of exogenous GLP-1 (Nauck et al. 1997), or the GLP-1 antagonist, exendin(9-39) (Deane et al. 2009). Slowing of gastric emptying also plays a dominant role in the action of the GLP-1 analogue, exenatide, to improve postprandial glycaemia (Cervera et al. 2008). It is possible that the blood glucose lowering effect of CTM#3 is partly explained by the action of GLP-1 on gastric emptying. However, we have previously observed that

emptying of CTM#3 pellets given with the carrier meal was not slower than that of placebo pellets, when evaluated by scintigraphy in healthy subjects (unpublished data). This is consistent with that the absence of any difference in the initial rise in blood glucose (after breakfast or lunch) in the current study. Nevertheless, we did not measure the gastric emptying of either breakfast or lunch in this group of patients.

GLP-1 also has the capacity to suppress glucagon secretion (Meier et al. 2003), and intravenous administration of GLP-1 in physiological concentrations has been shown to lower plasma glucagon in patients with type 2 diabetes. The glucagonostatic action of GLP-1 is glucose-dependent (Baggio and Drucker 2007), but is not dependent on the stimulation of insulin (Nauck et al. 1997). However, we did not measure plasma glucagon levels in the present study.

Extrapancreatic influences on glucose homeostasis, such as paracrine and portal effects of GLP-1, may be at least as important as its endocrine effects. In rodents, the GLP-1 receptor is present on visceral afferent neurons in the hepatic portal region (Vahl et al. 2007), and both GLP-1 and glucose in portal venous blood act synergistically to decrease peripheral blood glucose levels (Burcelin et al. 2001; Ionut et al. 2005). Indeed, intraportal infusion of GLP-1 can increase glucose disposal without any change in pancreatic hormone secretion, by increasing both

hepatic and extra-hepatic glucose uptake, a response controlled by autonomic nerves (Johnson et al. 2007). Another possibility is that stimulation of vagal afferent nerves in the small intestinal villi by GLP-1, in a paracrine manner, participates in the glucose lowering effects of GLP-1, especially since postprandial GLP-1 concentrations in the small intestine lymph are much greater than in peripheral blood (D'Alessio et al. 2007). However, this remains speculative.

We did not show any differences in plasma GIP between CTM#3 and placebo. This reinforces the idea that the amelioration of the postprandial blood glucose was due to GLP-1, and supports the concept that CTM #3 was releasing lauric acid in the ileum rather than in the proximal small intestine.

The peak blood glucose value after lunch was lower than after breakfast despite a greater load of carbohydrate being consumed in the lunch, on both the CTM#3 and placebo days. This „second meal effect" could be due to both the greater amount of protein and fat in the lunch than in the breakfast, and to the presence of residual nutrients in the distal small intestine at the time when lunch was consumed, reflected in higher GLP-1 and insulin levels at this time even on the placebo day.

In conclusion, we showed that CTM#3, consumed with breakfast, decreased postprandial glycaemia and stimulated GLP-1 release in patients with type 2 diabetes. The current dose of CTM#3 was chosen on the basis of previous data in healthy subjects, and the optimum dose needs to be clarified. A higher dose may not necessarily be more effective, because limited data from healthy subjects indicate that higher doses induce more rapid small intestinal transit and less prolonged ileal contact (unpublished observations). Conversely, it remains unknown whether a lower dose would suffice, particularly if the dose were split and given before each meal. For example, a dose of 5 g CTM#3 may be as effective as 10 g in lowering blood glucose, if local concentrations released from the 5 g dose were greater than the threshold for release of GLP-1 from ileal L-cells between 0 and 240 min. Likewise, subsequent GLP-1 release from residual CTM#3 entering the colon at 240-360 min may then also be as effective for a 5 g as for a 10 g dose. Moreover, a second dose of 5 g ingested with lunch might boost GLP-1 after lunch, caused by the presence concurrently of the first 5 g dose in the colon and the second 5 g dose in the ileum, thus enhancing the post-lunch effect. Finally, the combination of CTM#3 with a DPP-IV inhibitor could potentially have synergistic effects on the glycaemic and incretin hormone responses to a meal in patients with type 2 diabetes, and warrants evaluation in future studies.
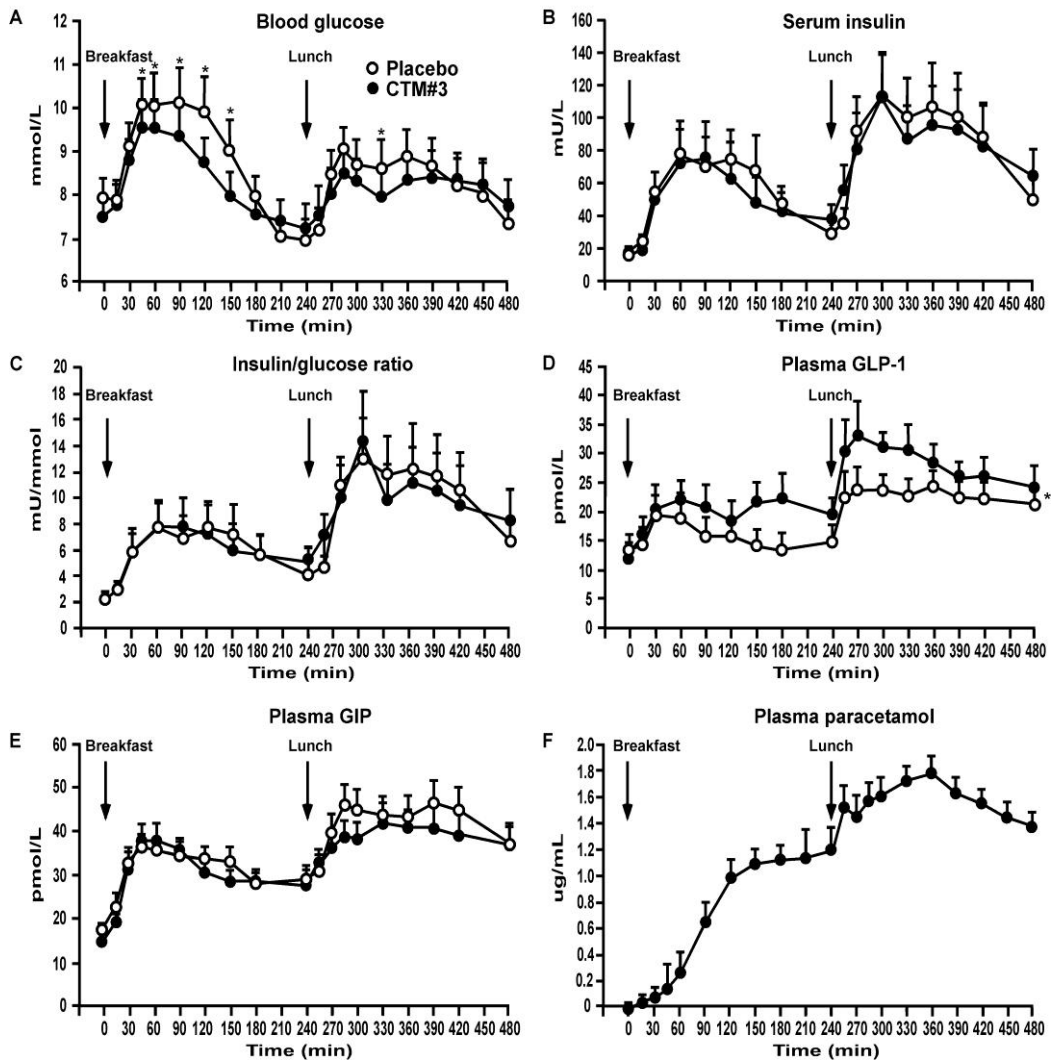
Figure 8.1 Concentrations of blood glucose (A), serum insulin (B), insulin/glucose ratio (C), GLP-1 (D) and GIP (E) in response to breakfast with CTM#3 or placebo at t = 0 min and a standard lunch at t = 240 min in 10 patients with type 2 diabetes, and plasma paracetamol concentrations (F) with CTM#3. *CTM#3 vs. placebo, $P < 0.05$. Data are means ± SE. There was a treatment effect between CTM#3 and placebo for plasma GLP-1 ($P < 0.05$). There was no significant difference in serum insulin and plasma GIP between CTM#3 and placebo.

CHAPTER 9: EFFECTS OF VARIATIONS IN DUODENAL GLUCOSE LOAD ON GLYCAEMIC, INSULIN AND INCRETIN RESPONSES IN TYPE 2 DIABETES

9.1 Summary

The postprandial incretin response has been reported to be deficient in type 2 diabetes, but most studies have not controlled for variations in the rate of gastric emptying. We evaluated the blood glucose, serum insulin, and plasma glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) responses to intraduodenal (ID) glucose in patients with type 2 diabetes, and compared these to our previous data from 10 healthy controls. 8 males with well-controlled type 2 diabetes, managed by diet alone, were studied on four occasions in single-blind, randomized order. Blood glucose, serum insulin and plasma hormones were measured during 120 min ID glucose infusions at (i) 1 ("G1"), (ii) 2 ("G2"), and (iii) 4 ("G4") kcal/min, or (iv) saline control ("S"). Type 2 diabetic patients had higher basal ($P < 0.0001$) and incremental ($P < 0.0001$) blood glucose responses to G2 and G4, when compared with healthy controls. In both groups, the stimulation of insulin and GLP-1 by increasing glucose loads were not linear; responses to G1 and G2 were minimal, whereas responses to G4 were much greater ($P = 0.0001$ for each). In both healthy subjects and patients with type 2 diabetes, there was a load dependent

increase in plasma GIP. In patients with well-controlled type 2 diabetes, blood glucose, insulin, and GLP-1 responses are critically dependent on the duodenal glucose load. GLP-1 responses are not deficient, when compared to healthy controls.

9.2 Introduction

In healthy subjects, the incretin hormones, GLP-1 and GIP, account for up to 70 % of postprandial insulin secretion (Nauck et al. 1986b; Shuster et al. 1988). In type 2 patients, the incretin effect is reported to be substantially attenuated, which potentially leads to insufficient insulin secretion and potentiates postprandial hyperglycaemia (Nauck et al. 1986a). The secretion of GIP appears relatively normal in these patients (Krarup 1988), whereas its insulinotropic activity is markedly diminished (Elahi et al. 1994), particularly during the „late phase" of insulin secretion (Vilsboll et al. 2002). In contrast to GIP, the insulin response to exogenous GLP-1 remains intact in type 2 diabetes (Nauck et al. 1993c), but there has been some controversy as to whether endogenous GLP-1 secretion is impaired. Some studies have reported that plasma concentrations of both total and active GLP-1 are lower in response to a standardized meal in diabetic patients (Toft-Nielsen et al. 2001; Vilsboll et al. 2001), but others have failed to show a difference between healthy subjects and patients with type 2 diabetes (Ryskjaer et al. 2006; Vollmer et al. 2008). Furthermore, postprandial

GLP-1 concentrations are not impaired in patients with impaired glucose tolerance (Vollmer et al. 2008), women with gestational diabetes (Meier et al. 2005), and first-degree relatives of type 2 patients (Nauck et al. 2004b), arguing against GLP-1 deficiency in the primary pathogenesis of diabetes.

The rate of gastric emptying regulates the entry of glucose and other nutrients into the small intestine in the range of 1-4 kcal/min (Brener et al. 1983; Schirra et al. 1996; Gentilcore et al. 2006c), and is, therefore, an important factor in determining incretin hormone secretion in both healthy subjects and patients with diabetes. Even minor changes in the initial rate of gastric emptying of glucose substantially influence the glycemic and incretin responses (O'Donovan et al. 2004b; Chaikomin et al. 2005). For example, incretin concentrations were greater when glucose was infused intraduodenally at an initially rapid, and subsequently slower rate, when compared to a constant infusion providing the same total load, in both healthy subjects and type 2 patients (O'Donovan et al. 2004b). While gastric emptying is known to be delayed in 30-50 % of patients with long-standing diabetes (Horowitz et al. 1991), it is noteworthy that all comparisons of the incretin effect between type 2 patients and healthy subjects have involved oral administration of nutrients, and potential differences in the rate of gastric emptying have not been considered as a confounding factor. A direct comparison of the incretin response to intraduodenally delivered glucose

between type 2 patients and healthy subjects has not previously been undertaken.

In our previous study in healthy subjects (Pilichiewicz et al. 2007a), a low duodenal glucose load (~ 1 kcal/min) caused minimum rise in blood glucose and plasma insulin, while GLP-1 was only substantially elevated with a high duodenal glucose load (4 kcal/min). A better understanding of the incretin response to increasing duodenal glucose loads in patients with type 2 diabetes is fundamental to developing strategies to regulate the postprandial glucose excursion.

The aims of this study were i) to evaluate glycaemia, insulinaemia, and incretin hormone concentrations in response to duodenal glucose loads, spanning a physiological range, in patients with type 2 diabetes, and ii) to compare these with our previous data from 10 healthy controls (Pilichiewicz et al. 2007a).

9.3 Methods

9.3.1 Subjects

8 male patients with type 2 diabetes (age 57 ± 4 yrs), as diagnosed by World Health Organization criteria and managed by diet alone, were recruited by newspaper advertisement. None had a history of significant comorbidities,

gastrointestinal symptoms, or was taking medication known to influence gastrointestinal function. The mean duration of known diabetes was 5.4 ± 1.1 yr, glycated haemoglobin was 6.2 ± 0.3 %, and body mass index (BMI) was 26.5 ± 1.4 kg/m^2. Data were compared to healthy subjects (age 32 ± 4 yrs; BMI 25.1 ± 0.4 kg/m^2) (Pilichiewicz et al. 2007a).

9.3.2 Protocol

Following an overnight fast (14 h for solids and 12 h for liquids), subjects attended the laboratory at 0830 h. Each subject underwent four studies, in single-blind, randomized fashion, separated by 4 - 7 days. On each day, a manometric assembly (diameter 3.5 mm; Dentsleeve, International Ltd., Mui Scientific, Ontario, Canada) was inserted through an anesthetised nostril and allowed to pass through the stomach into the duodenum by peristalsis (Pilichiewicz et al. 2007a). The manometric assembly contained an infusion channel, located ~ 14.5 cm distal to the pylorus. There were also two other saline perfused sideholes on either side of the pylorus, used to maintain the correct position of the catheter by continuous measurement of the transmucosal potential difference (TMPD), using established criteria (antral TMPD < -20 mV, duodenal TMPD > -15 mV, difference > 15 mV) (Heddle et al. 1988a; Heddle et al. 1989). This required the insertion of a 20G saline-filled cannula subcutaneously in the forearm as a reference (Pilichiewicz et al. 2007a). An intravenous cannula was

placed into a forearm vein for blood sampling.

When the catheter was positioned correctly, subjects received an intraduodenal infusion of glucose at either (i) 1 kcal/min (G1), (ii) 2 kcal/min (G2) or (iii) 4 kcal/min (G4), or (iv) saline control, for 120 min. By varying the mixture of glucose and saline, the osmolarity (1,390 mosmol/L) and volume (4 ml/min) of all solutions were kept identical (Pilichiewicz et al. 2007a). At baseline and at frequent intervals throughout the infusion period, blood samples were taken for measurement of blood glucose, serum insulin and plasma GLP-1 and GIP. Blood samples were placed in both a prechilled EDTA tube containing 400 kIU aprotinin (Trasylol, Bayer Australia Ltd, Pymble, Australia) per liter blood for GLP-1 and GIP assays and a pre-chilled serum tube with clotting activator for insulin assay, both stored on ice, and centrifuged at 3200 rpm and 4 ºC for 15 min. Plasma and serum were separated and samples were stored at -70 ºC for subsequent analysis using established assays. At t = 120 min, the catheter was removed and subjects were allowed to leave. On one of the study days, autonomic nerve function was evaluated using standardized cardiovascular reflex tests (Ewing and Clarke 1982), after completion of the other measurements.

9.3.3 Measurements

Blood glucose concentrations

Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). We have previously validated the accuracy of this method against the hexokinase technique (Horowitz et al. 1991).

Serum insulin and plasma GLP-1 and GIP assays

Serum insulin was measured by a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity was 2 mU/L, intra-assay coefficient of variation (CV) 3.9 % and inter-assay CV 5.0 %. Total GLP-1 was measured by radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and inter-assay CV 9.2 %. Plasma GIP was measured by RIA, with a sensitivity of 2 pmol/L, and both intra- and inter-assay CVs of 15 % (Pilichiewicz et al. 2007a).

In the study with healthy controls, total GLP-1 was measured by a different RIA with intra-assay CVs of 17 %, inter-assay CVs of 18 % and sensitivity of 1.5 pmol/liter (Pilichiewicz et al. 2007a). Antibody was supplied by Professor S. R. Bloom (Hammersmith Hospital, London, UK) (Pilichiewicz et al. 2007a), and

was no longer available for the current study. Although it is likely that the different assays would give similar measurements, we did not compare these data statistically. However, we believe that valid qualitative comparisons could be made.

Cardiovascular autonomic function

Parasympathetic function was assessed by the variation in heart rate (R-R interval) during deep breathing and the heart rate response to standing („30:15" ratio); sympathetic function was determined by the fall in systolic blood pressure in response to standing. Each test result was scored according to age-adjusted predefined criteria as: 0 = normal, 1 = borderline, and 2 = abnormal, for a total maximum score of 6. A score of 3 or higher was taken as evidence of autonomic dysfunction (Ewing and Clarke 1982).

9.3.4 Statistical analysis

Data were evaluated using repeated measures ANOVA, with „glucose load" and „time" as factors within the diabetic group, and „glucose load" and „group" as factors when comparing data with healthy controls. In the event of a glucose load × time interaction, individual time points were compared with means comparisons. Baseline blood glucose and hormone concentrations were compared using paired t-tests within groups, and unpaired t tests between

controls and diabetic patients. All analyses were performed using StatView (version 5.0, Abacus Concepts, Inc., Berkeley, CA, USA) and SuperANOVA (version 1.11, Abacus Concepts, Inc., Berkeley, CA, USA). Data are shown as mean values ± standard error; $P < 0.05$ was considered significant. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin sensitivity, and was calculated by the formula: fasting glucose (mmol/L) × fasting insulin value (pmol/L)/22.5 (Matthews et al. 1985). The incremental glucose (ΔG_{30}) and insulin (ΔI_{30}) (in pmol/L) responses at $t = 30$ min were used to calculate beta cell function corrected for insulin sensitivity, using the formula ($\Delta I_{30}/\Delta G_{30}$)/HOMA-IR (Jensen et al. 2002).

9.4 Results

All subjects tolerated the study well. The mean score for autonomic nerve dysfunction was 2.1 ± 0.8; two of the eight diabetic subjects had definite autonomic dysfunction. GLP-1 concentration data were available from only 9 of 10 control subjects.

9.4.1 Blood glucose concentrations (Figure 1A)

Fasting blood glucose did not differ among the four study days in the type 2 patients. Blood glucose concentrations were higher from $t = 30$ to 120 min during

all glucose infusions, compared with saline (P < 0.05, treatment × time interaction, P = 0.0001). There was a time-dependent increase in blood glucose during G1, G2 and G4 infusions (P < 0.05). The increase in blood glucose between t = 30 min and 120 min was greater during G2 and G4 compared with G1 (P < 0.05) and was greater from t = 45 min to 90 min during G4 compared with G2 (P < 0.0005). The peak blood glucose concentration (t = 120 min) was higher during G2 infusion than that during G1 (P < 0.0005). The peak blood glucose concentration was higher during G4 infusion than during G2 (15.6 ± 0.9 vs. 13.8 ± 1.0 mmol/L; P < 0.005). Blood glucose fell thereafter despite ongoing intraduodenal infusion, such that there was no difference in blood glucose concentrations at 120 min between G2 and G4 infusions.

Compared to healthy subjects, type 2 diabetic patients had higher basal (P < 0.0005) and incremental (P < 0.0005) blood glucose responses to G2 and G4, but not G1 glucose loads. However, in the diabetic patients, the blood glucose continued to rise from baseline during G1, whereas it plateaued in the controls. There was no significant difference between the glycemic response to G2 and G4 in healthy subjects, in contrast to the patients with type 2 diabetes. Nevertheless, even in type 2 patients, the increase in blood glucose with increasing duodenal glucose loads was not linear.

9.4.2 Serum insulin concentrations (Figure 1B)

The baseline serum insulin concentrations were not different among the four days in type 2 patients. There was a treatment × time interaction (P = 0.0001); the rise in insulin with G2 and G4 infusions (P < 0.05 for both), but not G1, was greater than saline, between t = 45 min to 120 min for G2 and t = 30 min to 120 min for G4 (P < 0.05). Insulin concentrations did not differ between saline and G1 infusions. During G4 infusion, insulin concentrations were markedly greater, compared with G1 infusion from t = 30 min (P < 0.05), and G2 infusion from t = 45 min (P < 0.05).

Fasting insulin levels were higher in diabetic patients compared with healthy subjects (10.5 ± 0.2 mU/L vs. 2.7 ± 0.1 mU/L; P < 0.05), and the HOMA-IR score indicated that the type 2 patients were insulin resistant (G1: 21.6 ± 8.0 pmol.mmol, G2: 21.7 ± 8.0 pmol.mmol; G4: 23.4 ± 7.2 pmol.mmol in type 2 patients and G1: 4.9 ± 0.7 pmol.mmol, 4.4 ± 0.6 pmol.mmol and G4: 4.2 ± 0.4 pmol.mmol in healthy controls). In both groups, the insulin responses to increasing glucose loads were not linear, with minimal insulin response to G1 and G2, but a much greater response to G4. Incremental increases to each glucose load did not differ statistically between patients with diabetes and healthy subjects.

9.4.3 Plasma GLP-1 concentrations (Figure 1C)

Fasting GLP-1 concentrations did not differ among the four study days in type 2 patients. GLP-1 increased markedly from t = 15 min to 120 min during G4 infusion (P < 0.005, treatment × time interaction), compared with G1, G2 and saline, during which GLP-1 concentrations did not differ.

As discussed, the difference in the GLP-1 assay between type 2 patients and healthy subjects precluded statistical comparison between the groups. Nevertheless, the GLP-1 responses to each load appeared similar in the two groups, and were not grossly deficient in the type 2 patients. However, in healthy subjects, there was an early, transient increase at t = 15 min (P < 0.05) in response to each glucose load, which was not observed with G1 and G2 infusions in the type 2 patients.

9.4.4 Plasma GIP concentrations (Figure 1D)

Fasting levels of GIP were not different among the four study days. There were rapid rises in GIP during all glucose infusions until t = 30 min, with a plateau thereafter to 120 min. There was a treatment × time interaction (P = 0.0001), such that plasma GIP concentrations were higher during G4 than G2 and G1 (P < 0.0005 for both), and higher during G2 than G1 (P < 0.0005).

Fasting plasma GIP was higher in type 2 patients compared with healthy subjects (18.2 ± 0.3 pmol/L vs. 9.2 ± 1.4 pmol/L; $P < 0.05$). However, in both groups, there was a load-dependent increase in GIP, which was approximately linear, although in healthy subjects the difference between G2 and G4 did not reach statistical significance. The incremental increase in GIP in response to each glucose load did not differ between the two groups.

9.4.5 Beta cell function (Figure 2)

Compared with healthy controls, $(\Delta I_{30}/\Delta G_{30})$/HOMA-IR was markedly reduced in response to each glucose load in type 2 diabetic patients ($P < 0.005$). This index of beta cell function increased with increasing glucose loads ($P < 0.01$) in healthy controls, but not in type 2 patients, so that the difference between groups become greater as the glucose load increased ($P < 0.05$, group × load interaction).

9.5 Discussion

Our study evaluated the effect of duodenal glucose loads delivered at 1, 2 and 4 kcal/min on blood glucose, serum insulin, and plasma GIP and GLP-1 concentrations in patients with type 2 diabetes, and compared these with our previous data from healthy controls. We demonstrated that the increments in blood glucose, plasma insulin and GLP-1 concentrations with increasing glucose

loads were not linear. Furthermore, the incremental increases in GIP and GLP-1 were qualitatively similar in patients with type 2 diabetes and healthy subjects, implying that the secretion of incretin hormones is not grossly impaired in patients with relatively well-controlled type 2 diabetes managed with diet alone, compared with healthy subjects.

We measured only total GLP-1 and GIP concentrations, which are of greatest relevance in a study focusing on secretion, rather than action, of the incretins. We infused glucose intraduodenally and, therefore, eliminated differences in the rate of gastric emptying as a source of variation in the incretin responses. Moreover, we studied rates of glucose infusion that spanned the physiological range of gastric emptying (Brener et al. 1983; Schirra et al. 1996; Gentilcore et al. 2006c).

In previous studies, a reduction of 20-30 % in postprandial GLP-1 concentrations was reported in patients with type 2 diabetes compared with healthy subjects (Toft-Nielsen et al. 2001; Vilsboll et al. 2001). A deficient GLP-1 response would provide a hypothetical explanation for the development of diabetes; however, this was not observed in subsequent studies (Ryskjaer et al. 2006; Vollmer et al. 2008). Moreover, the presence of apparently impaired GLP-1 release does not appear to correlate with early phase insulin deficiency (Vilsboll et al. 2001). None of these studies has controlled for differences in the rate of gastric

emptying, particularly in patients with long-standing diabetes, of whom it is established that 30–50 % have delayed emptying (Horowitz et al. 1991). Rapid gastric emptying has been also reported in some „early" type 2 patients (Phillips et al. 1992), although this has not been consistently observed (Jones et al. 1996). Furthermore, the rate of gastric emptying can be affected by acute variations in the blood glucose concentration. In both healthy subjects and patients with diabetes, marked hyperglycaemia (16–20 mmol/L) leads to prolonged emptying times for solids and liquids, compared with euglycaemia (5–8 mmol/L) (Fraser et al. 1990), and even small variations in blood glucose, within the physiological range (~ 8 mmol/L versus 4 mmol/L), have an appreciable effect on the rate of gastric emptying in healthy and diabetic subjects (Schvarcz et al. 1997). A recent report indicated that GLP-1 and GIP responses to a mixed meal were attenuated by acute hyperglycaemia in healthy humans (Vollmer et al. 2009). While the mechanisms of this effect were not evaluated, it seems likely that delayed gastric emptying would have been a major contributing factor (O'Donovan et al. 2004b). The effect of chronic hyperglycaemia on gastric emptying and incretin hormone release in patients with type 2 diabetes has not been investigated, and further studies evaluating the effects of chronic hyperglycaemia are, therefore, indicated.

There has been much recent interest in the effects of exogenous GLP-1 and its analogs in patients with diabetes, whereas the mechanisms of endogenous GLP-1

secretion remain relatively unclear. While the greatest density of GLP-1 secreting L cells is found in the distal small intestine and colon, plasma GLP-1 concentrations normally rise within minutes of glucose entry to the small intestine (Deacon 2005). It has been suggested that a threshold load of glucose delivery (~ 1.8 kcal/min) needs to be exceeded in order to stimulate GLP-1 release (Schirra et al. 1996). However, our group has observed an early, albeit transient, peak in GLP-1 secretion, at relatively low rates of glucose administration (1 kcal/min) in healthy volunteers (Kuo et al. 2008). One explanation for this phenomenon is that a significant number of L-cells are present in the duodenum and jejunum (Theodorakis et al. 2006). Within the limitations of a change in our assay for GLP-1, we did not observe gross differences in GLP-1 secretion between type 2 patients and healthy subjects. However, the early rise in GLP-1 that we have observed in healthy subjects appeared to be absent in type 2 patients. The mechanism underlying this deficiency is not clear, nor is its functional significance.

We did not show any differences in incremental GIP responses to each glucose load between type 2 patients and healthy subjects, which is consistent with previous reports (Nauck et al. 2004a; Vollmer et al. 2008). Interestingly, we observed higher fasting GIP concentrations in patients with type 2 diabetes, compared with healthy subjects, also in agreement with previous data showing

that fasting GIP levels were markedly greater in diabetic patients and subjects with impaired glucose tolerance (Mazzaferri et al. 1985; Theodorakis et al. 2004). It has been suggested that GIP hypersecretion in the fasting state might be associated with hyperinsulinaemia, and might contribute to the pathogenesis of early type 2 diabetes (Mazzaferri et al. 1985; Theodorakis et al. 2004). It is not clear whether hyperinsulinaemia could also influence the postprandial GIP response; our diabetic subjects did have higher fasting insulin levels than healthy controls, but the postprandial insulin concentrations were comparable. It has been reported that glucose absorption via SGLT1 is involved in stimulating GIP secretion (Wachters-Hagedoorn et al. 2006). Given the proximal location of K-cells (Schirra et al. 1996), it is not surprising that there was a dose-dependent increase in the plasma GIP response in both healthy subjects and patients with type 2 diabetes as the rate of duodenal glucose infusion increased.

In the present study, fasting blood glucose and incremental increases with increasing glucose loads in patients with type 2 diabetes were greater than in healthy subjects, which is not unexpected. There are several potential explanations for these differential responses. Beta cell failure is probably the major factor for higher blood glucose levels in the diabetic patients, since an established index of beta cell function (($\Delta I_{30}/\Delta G_{30}$)/HOMA-IR) (Jensen et al. 2002) was markedly lower in diabetic patients compared to healthy controls

across all glucose loads, with the difference being magnified as duodenal glucose delivery increased. Secondly, given that the maximal capacity of glucose absorption is about 0.5 g per 30 cm of small intestine per minute in healthy humans (Duchman et al. 1997), both 2 and 4 kcal/min infusions might reach, or exceed the absorptive capacity of the most proximal small intestine (Pilichiewicz et al. 2007a). In type 2 patients, the glycemic response to intraduodenal glucose was not linear, but the overall blood glucose response to 4 kcal/min was greater than during 2 kcal/min. It is not known whether glucose absorption is increased in patients with type 2 diabetes, although increased absorption has been reported in rodent models of diabetes (Fujita et al. 1998), and expression of the monosaccharide transporters SGLT1, GLUT5 and GLUT2 is enhanced in type 1 and 2 diabetic humans (Dyer et al. 2002). Thirdly, the diabetic patients were insulin resistant on the basis of their HOMA-IR values, and this would have impaired glucose disposal. Fourthly, hepatic glucose release would potentially be greater than in healthy subjects (Rayner et al. 2001), due to mechanisms that include a failure of suppression of glucagon secretion, although we did not measure plasma glucagon concentrations.

We have demonstrated that the secretion of incretins, particularly GLP-1, is critically dependent on the rate of small intestinal glucose entry in both healthy and type 2 patients. If glucose entry is at the low end of the physiological range

(about 1 kcal/min), the incretin response appears relatively less important. Moreover, we showed that beta cell failure is less evident at low than high rates of duodenal glucose delivery. This might indicate that an appropriate strategy in the management of type 2 patients who are not on insulin is to slow the emptying of carbohydrate from the stomach, such as by a high fibre diet or fat „preload" (Ray et al. 1983; Gentilcore et al. 2006b).

In conclusion, we have demonstrated that impaired GLP-1 is not an early defect in type 2 diabetes. We studied patients with a short duration of diabetes who were well-controlled by diet alone. Therefore, studies in patients with longstanding diabetes, or who are less well controlled, are indicated. Although defective release of incretin hormones in previous studies involving patients with type 2 diabetes may not be fully accounted for by delayed gastric emptying, it is clear that this variable must be taken into account in future studies of the incretin response.

Type 2 patients

Healthy controls

Blood glucose

A

Legend:
- ○ S (Saline)
- ■ G1 (1kcal/min)
- △ G2 (2kcal/min)
- ● G4 (4kcal/min)

Plasma insulin

B

Plasma GLP-1

C

Plasma GIP

D

188
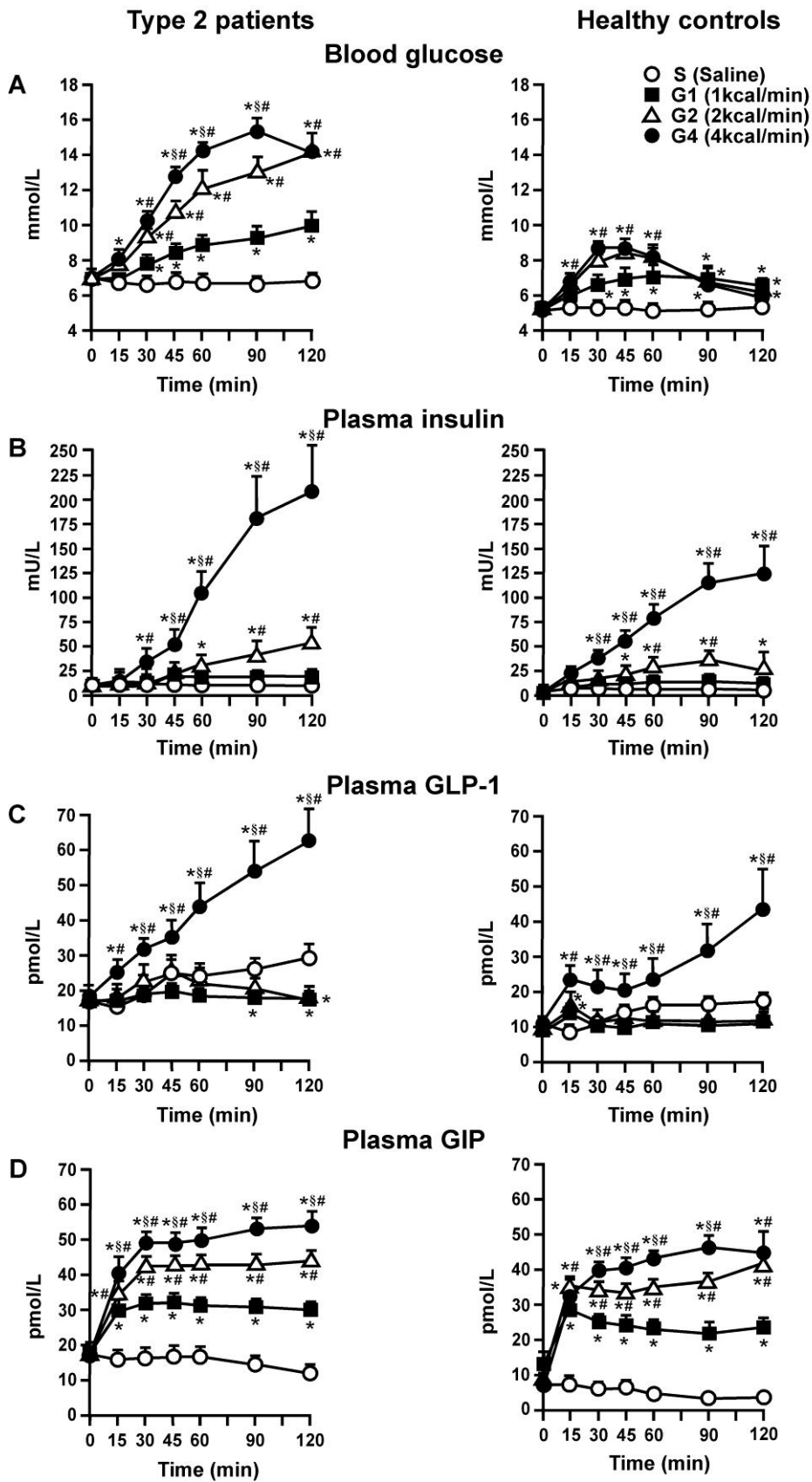
Figure 9.1 Blood glucose (A), plasma insulin (B), GLP-1 (C) and GIP (D) concentrations in response to 120 min intraduodenal glucose infusion at (i) 1 ("G1"), (ii) 2 ("G2"), and (iii) 4 ("G4") kcal/min, or (iv) saline control ("S") in 10 healthy subjects and 8 type 2 patients. Data are presented as mean ± SEM. * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$.

(ΔI30/ΔG30)/HOMA-IR
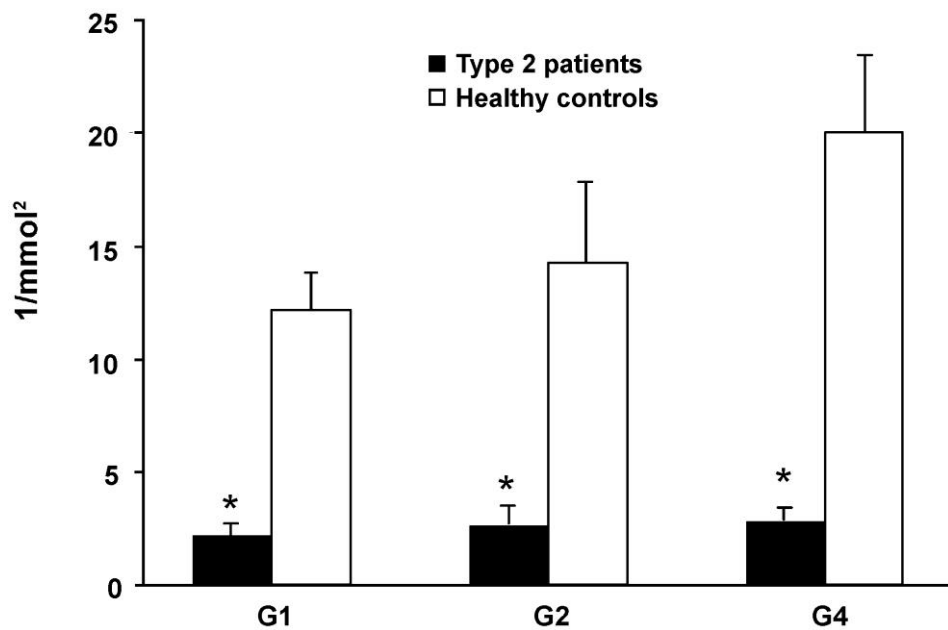
Figure 9.2 Beta cell function as measured by $(\Delta I_{30}/\Delta G_{30})$/HOMA-IR, in response to intraduodenal glucose infusions delivered at (i) 1 ("G1"), (ii) 2 ("G2"), and (iii) 4 ("G4") kcal/min, in 10 healthy subjects and 8 type 2 patients. This index of beta cell function was markedly lower for each glucose load in type 2 diabetic patients, compared to healthy subjects (* $P < 0.005$), with the difference increasing as the glucose load increased ($P < 0.05$, group × load interaction).

CHAPTER 10: EFFECT OF THE ARTIFICIAL SWEETNER, SUCRALOSE, ON GASTRIC EMPTYING AND INCRETIN HORMONE RELEASE IN HEALTHY SUBJECTS

10.1 Summary

The incretin hormones, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP), play an important role in glucose homeostasis in both health and diabetes. In mice, sucralose, an artificial sweetener, stimulates GLP-1 release via sweet taste receptors on enteroendocrine cells. We studied blood glucose, plasma levels of insulin, GLP-1 and GIP and gastric emptying (by a breath test) in 7 healthy humans after intragastric infusions of (i) 50 g sucrose in water to a total volume of 500 ml (~ 290 mosmol/L), (ii) 80 mg sucralose in 500 ml normal saline (~ 300 mosmol/L, 0.4 mM sucralose), (iii) 800 mg sucralose in 500 ml normal saline (~ 300 mosmol/L, 4 mM sucralose), (iv) 500 ml normal saline (~ 300 mosmol/L), all labelled with 150 mg ^{13}C-acetate. Blood glucose increased only in response to sucrose ($P < 0.05$). GLP-1, GIP and insulin also increased after sucrose ($P = 0.0001$), but not after either load of sucralose, or saline. Gastric emptying of sucrose was slower than that of saline (T50: 87.4 ±

4.1 min vs. 74.7 ± 3.2 min, P < 0.005), whereas there were no differences in T50 between sucralose 0.4 mM (73.7 ± 3.1 min), or 4 mM (76.7 ± 3.1 min) and saline. We conclude that sucralose, delivered by intragastric infusion, does not stimulate insulin, GLP-1 or GIP release, or slow gastric emptying in healthy humans.

10.2 Introduction

The interaction of nutrient with the small intestine plays an important role in the regulation of appetite, energy intake and glucose homeostasis. For example, the suppression of energy intake induced by small intestinal fat infusion is much greater than that in response to an equivalent intravenous fat load (Welch et al. 1985). Exposure of the small intestine to nutrients is also associated with feedback inhibition to slow the rate of gastric emptying (Heddle et al. 1988a; Andrews et al. 1998). Both the slowing of gastric emptying and suppression of appetite are mediated by the secretion of gastrointestinal hormones, including GLP-1 (MacIntosh et al. 1999; Schirra et al. 2006), the release of which is strongly stimulated by carbohydrate (Lavin et al. 1998). GLP-1 is one of the two known „incretin" hormones that stimulate glucose-dependent insulin release (Deacon 2005). In healthy humans, GLP-1 and GIP account for at least 50 % of the postprandial insulin response (Shuster et al. 1988). GLP-1 in pharmacological doses also inhibits glucagon secretion, slows gastric emptying and suppresses

appetite, leading to weight loss in the long term (Nauck and Meier 2005). GLP-1, but not GIP, has preserved insulinotropic effects in patients with type 2 diabetes (Nauck et al. 1993c). Therefore, the GLP-1 analogues, such as exenatide and liraglutide, and enzyme dipeptidyl peptidase-IV (DPP-IV) inhibitors that enhance circulating concentrations of active GLP-1, such as sitagliptin, have been developed for therapeutic use (Ahren 2007; Drucker 2007).

The detection system for sweet taste on the tongue has been established for a decade. Sweet tastants are detected by G-protein-coupled receptors (GPCR) of the T1R family, of which T1R2 and T1R3 receptors heterodimerise to form broadly-tuned sweet taste receptors. T1R2+T1R3 couples to a G-protein, gustducin, and in turn, to the transient receptor potential ion channel TRPM5 (Ruiz-Avila et al. 2001). It has recently been established that the alpha subunit of gustducin, α-gustducin, is expressed in the mucosa of the murine gastrointestinal tract (Olsson et al. 2006; Sutherland et al. 2007b). Expression of α-gustducin is evident throughout the mouse small intestine, and amongst several cell types, appears to co-localize with GLP-1-secreting L-cells. The sweet taste receptor molecules T1R2, α-gustducin and TRPM5 have now been shown to also be expressed in the human small intestinal mucosa (Sutherland et al. 2007a).

Artificial sweeteners have been used to replace carbohydrate in the management

of diabetes and obesity (American Diabetes Association 2004). Sucralose is a noncaloric sweetener derived from sucrose, and is approximately 600 times sweeter. While sucralose (1-5 mM) has been shown to stimulate GLP-1 from human L-cells in vitro, in a concentration-dependent manner (Jang et al. 2007a), it is not known whether this effect occurs in vivo. A report that long-term (3-month) dietary supplementation with sucralose (667 mg daily) did not alter glycated haemoglobin in patients with type 2 diabetes (Grotz et al. 2003) argues against a significant effect. However, there is a lack of data about the effects of sucralose on gastric emptying or GLP-1 release, in either animals or humans.

The aims of our study were to evaluate the effects of sucralose, at a concentration chosen to match the sweetness of a sucrose load (0.4 mM), and at a much higher concentration (4 mM), in the range shown to stimulate GLP-1 release from a human enterendocrine cell line in vitro, on gastric emptying and GLP-1, GIP, insulin and blood glucose concentrations in healthy subjects.

10.3 Methods

10.3.1 Subjects

7 healthy subjects (age 24 ± 2 years; body mass index 21.6 ± 1.2 kg/m^2) were studied. None had a history of gastrointestinal disease, was taking

medications known to affect gastrointestinal motility or appetite, was a smoker, or habitually consumed more than 20 g of alcohol per day. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee, and each subject provided written, informed consent prior to taking part. The number of subjects was based on power calculations derived from our previous work (Gentilcore et al. 2005).

10.3.2 Protocol

Each subject attended the Discipline of Medicine at the Royal Adelaide Hospital at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids) on four occasions, each separated by 3-7 days. Women were studied in the follicular phrase of the menstrual cycle. On each study day, a catheter (external diameter ~ 3 mm) was introduced into the stomach via an anaesthetized nostril. Its intragastric position was verified by rapid injection of 10 ml air and auscultation over the upper abdomen. An intravenous cannula was inserted into a forearm vein to allow repeated blood sampling. All subjects received an intragastric infusion, over 3 min (t = -3 to 0 min), of either (i) 50 g sucrose dissolved in water to a total volume of 500 ml (~ 290 mosmol/L), (ii) 80 mg sucralose (Tate & Lyle Inc, Decatur, IL, USA) in 500 ml normal saline (~ 300 mosmol/L, 0.4 mM sucralose, equivalent sweetness to sucrose), (iii) 800 mg sucralose in 500 ml normal saline (~ 300 mosmol/L, 4 mM sucralose), or (iv)

500 ml normal saline (~ 300 mosmol/L), in randomized, single-blind fashion. All of the infusates were labeled with 150 mg ^{13}C-acetate, and breath samples were collected immediately before and every 5 min after intragastric infusion in the first hour, and every 15 min for a further 3 h (Chew et al. 2003). Blood was sampled immediately before the infusion (t = -3 min), and at t = 0, 5, 15, 30, 60, 90, 120, 150, 180 and 240 min for measurement of blood glucose, and plasma GLP-1, GIP and insulin. After 240 min, the nasogastric catheter and intravenous cannula were removed, the subject was offered lunch and then allowed to leave the laboratory.

10.3.3 Measurements

Gastric emptying

$^{13}CO_2$ enrichment in the breath samples was measured by mass spectroscopy (ABCA 20–20 mass spectrometer, Europa Scientific, Crewe, United Kingdom) to determine the percentage $^{13}CO_2$ recovery per hour and the cumulative percentage $^{13}CO_2$ recovery over 4 h (Chew et al. 2003). The gastric half-emptying time (T50) and gastric emptying coefficient (GEC) were calculated as measures of the gastric emptying rate (Maes et al. 1994) . Breath tests using the 13C acetate label have been validated against scintigraphy for the measurement of liquid gastric emptying (Braden et al. 1995; Chew et al. 2003).

Blood glucose, plasma GLP-1, GIP and insulin concentrations

Blood glucose concentrations were determined immediately using a portable glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). The remainder of each blood sample was placed in a prechilled EDTA tube containing 400 kIU aprotinin (Trasylol, Bayer Australia Ltd, Pymble Australia) per litre blood, and then centrifuged at 3200 rpm for 15 min (4 ^{o}C). Plasma was separated and samples were stored at -70 ^{o}C for subsequent analysis (MacIntosh et al. 1999). Total plasma GLP-1 was measured by radioimmunoassay (RIA) (GLPIT-36HK, Linco Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and the inter-assay coefficient of variation (CV) was 9.2 %. Total plasma GIP was measured by RIA (41). The sensitivity was 2 pmol/L, and both the intra- and inter-assay CVs were 15 %. Plasma insulin was measured by solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Sensitivity was 2 mU/L, intraassay CV was 3.9 % and interassay CV was 5.0 %.

10.3.4 Statistical analysis

Blood glucose and plasma hormone concentrations were analyzed by repeated measures analysis of variance (SuperANOVA, Abacus Concepts, Berkeley, CA, USA) with time and treatment as factors. T50 and GEC were also analyzed by ANOVA. Post hoc means comparisons were performed in the event of a

significant treatment $\times$ time interaction. Statistical significance was accepted at P < 0.05, and data are presented as mean values $\pm$ standard error.

10.4 Results

The study was well tolerated by all subjects. Fasting blood glucose concentrations and plasma GLP-1, GIP and insulin concentrations did not differ between the four study days.

10.4.1 Gastric emptying

There was a significant treatment effect for T50 between the 4 study days (P < 0.005). T50 was longer for sucrose than saline (87.4 $\pm$ 4.1 min vs. 74.7 $\pm$ 3.2 min; P < 0.005), whereas there were no differences in T50 between sucralose at 0.4 mM (73.7 $\pm$ 3.1 min) or sucralose at 4 mM (76.7 $\pm$ 3.1 min) and saline. Accordingly, GEC was less for sucrose when compared to saline (4.3 $\pm$ 0 vs. 4.7 $\pm$ 0.1; P < 0.0005), whereas it was not different between sucralose 0.4 mM (4.8 $\pm$ 0.1) or 4 mM (4.6 $\pm$ 0.1) and saline.

10.4.2 Blood glucose concentrations (Figure 1A)

There was a rise in blood glucose after sucrose administration (P = 0.0001),

which was evident from t = 5 min and decreased progressively after about 30 min, falling below baseline levels at 90 min. There was a significant treatment $\times$ time interaction (P = 0.0001), with the blood glucose concentration being greater after sucrose (P < 0.05) between 5 min and 60 min and lower between 90 min and 120 min (P < 0.05) compared with saline infusion. There were no significant differences in blood glucose between either load of sucralose and saline.

10.4.3 Plasma insulin concentrations (Figure 1B)

Plasma insulin rose promptly after sucrose administration (P = 0.0001), with a maximum response at 15 min, then fell to basal levels by t = 240 min. Insulin concentrations were markedly greater after sucrose infusion between 5 min and 120 min compared to those after saline infusion (P < 0.05). Neither sucralose load stimulated an insulin response.

10.4.4 Plasma GLP-1 concentrations (Figure 1C)

There was a marked increase in plasma GLP-1 after sucrose administration (P = 0.0001), which was maximal at 15 min followed by a subsequent decline to baseline levels by t = 60 min. The GLP-1 level was greater after sucrose between 5 and 15 min, and less at t = 90 min, compared to saline (P < 0.05). There was no difference between sucralose at 0.4 mM or 4 mM and saline.

10.4.5 Plasma GIP concentrations (Figure 1D)

There was a prompt rise in plasma GIP after sucrose infusion (P = 0.0001). After a peak between 5-15 min, GIP declined progressively to baseline values by 240 min. Plasma GIP was greater between 5 min and 150 min after sucrose, compared to saline infusion (P < 0.05). There was no difference between sucralose at 0.4 mM or 4 mM and saline.

10.5 Discussion

This study is the first to evaluate the incretin, insulin and glycaemic responses to sucralose administration, and to determine whether this artificial sweetener is capable of generating feedback in the small intestine that slows gastric emptying, in healthy humans. In contrast to administration of sucrose, sucralose given by intragastric infusion had no effect on GLP-1, GIP, or insulin secretion, or gastric emptying, both at a concentration chosen to match the sweetness of the sucrose load (0.4 mM), and at a much higher concentration (4 mM), in the range shown to stimulate GLP-1 release from a human enterendocrine cell line in vitro (Jang et al. 2007a). This is consistent with reports that sucralose failed to influence plasma glucose or serum C-peptide in patients with type 1 and 2 diabetes (Mezitis et al. 1996), and that the noncaloric sweetener, stevioside, failed to stimulate GLP-1 or GIP in patients with type 2 diabetes (Gregersen et al. 2004).

Similarly, there was no effect of sucralose on fasting blood glucose or glycated haemoglobin levels over three months in patients with type 2 diabetes (Grotz et al. 2003).

We administered sucralose by intragastric infusion in this study, rather than infusing directly into the small intestine. However, since sucralose is stable in acidic solution (less than 1 % hydrolysis at pH 3 after 1 year (Grice and Goldsmith 2000)), it is unlikely that the properties of sucralose would have been altered by exposure to gastric acid, prior to emptying into the small intestine. We do not believe that giving the test solution orally, rather than by intragastric infusion, would have altered the outcome, since there is no evidence for an effect of the cephalic phase of digestion on incretin hormone release (Ahren and Holst 2001). We selected a higher concentration of sucralose that was towards the upper end of the effective range in vitro (Jang et al. 2007a), and a lower concentration that approximated that used in the food industry, and observed no effect of sucralose at either concentration. It should be recognized that while we cannot be absolutely certain that these concentrations were too high or too low to stimulate a response (Jang et al. 2007a), it would not have been feasible to undertake additional study days in the same volunteers, particularly given the volume of blood sampled. We also did not measure the concentration of sucralose within the small intestinal lumen, but given that the emptying of both

sucralose solutions from the stomach was rapid, we would not anticipate a substantial difference from the concentrations administered intragastrically.

The presence of carbohydrate in the small intestine is a well established stimulus for GLP-1 and GIP secretion, leading to glucose-dependent insulin secretion from the β-cells, and feedback that regulates gastric emptying (Lin et al. 1989). Direct exposure of carbohydrate to the mucosa of the small intestine appears to be an essential requirement for GLP-1 and GIP secretion (Deacon 2005), and the magnitude of the former is dependent on the rate of delivery of glucose into the small intestine (Pilichiewicz et al. 2007a). Elements of the sweet taste receptor present in the tongue have recently been identified in both rodent (Olsson et al. 2006; Sutherland et al. 2007b) and human (Sutherland et al. 2007a) small intestine. The T1R2+3 heterodimer should, by analogy to the tongue, respond to various sweet-tasting molecules as diverse as sucrose, saccharin, acesulfame-K and sucralose (Nelson et al. 2001; Zhao et al. 2003). Furthermore, it has been demonstrated in two mouse enteroendocrine cell lines, GLUTag and STC-1, that sweet taste receptors are co-localized with GLP-1 and GIP (Kieffer et al. 1995; Reimann and Gribble 2002), and that sucralose stimulates secretion of GLP-1 and GIP from GLUTag cells (Margolskee et al. 2007). Mice that lack α-gustducin show markedly defective GLP-1 secretion in response to glucose (Jang et al. 2007a). Furthermore, the human L-cell line, NCI-H716, expresses α-gustducin

and several other taste signaling elements (Jang et al. 2007a), and GLP-1 release from NCI-H716 cells is stimulated by glucose, sucrose and sucralose, and blocked by the sweet receptor antagonist, lactisole, or siRNA for α-gustducin (Jang et al. 2007a). However, until this study, there has been no information available regarding effect of artificial sweeteners on GLP-1 release in humans in vivo. Moreover, even in mice, only a minority (8 %) of L-cells coexpress α-gustducin (Sutherland et al. 2007b); the potential stimulus for GLP-1 release from the remainder is unclear. It should also be noted that major differences between species are evident with regards to incretin hormone release. For example, fructose stimulates the release of GLP-1 in rats (Ritzel et al. 1997) and humans (Rayner et al. 2000b), but not in dogs (Shima et al. 1990).

Although sucrose, sucralose and other sweet tastants all bind to the T1R2+3 heterodimer, they do not act in identical fashion, at least on the tongue. For example, sucrose binds with a different affinity from sucralose (Nie et al. 2005), while absence of T1R3 receptors in knockout mice completely abolishes any taste preference for sucralose, but merely diminishes the preference for sucrose (Damak et al. 2003). Moreover, functional brain imaging in humans indicates differences in central activation between sucrose and identically sweet sucralose solutions (Frank et al. 2008).

Absorption of monosaccharide may be necessary for GLP-1 or GIP release; this seems to be true for GIP at least (Wachters-Hagedoorn et al. 2006), since rapidly and slowly digestible carbohydrates differ considerably in their ability to stimulate GIP secretion (Wachters-Hagedoorn et al. 2006), while phloridzin, an inhibitor of the SGLT1 glucose transporter, suppresses GIP release (Fushiki et al. 1992). Absorption of monosaccharide via SGLT1 has also been suggested as a trigger for GLP-1 secretion from L-cells (Gribble et al. 2003).

We have not examined whether sucralose has any effect on carbohydrate absorption in humans. Supplementation of the diet with sucralose increases SGLT1 mRNA and glucose absorption in wild type mice, but not in T1R3 or α-gustducin knockout mice (Margolskee et al. 2007). Furthermore, sucralose, acesulfame potassium and saccharin stimulate glucose absorption in rats by enhancing apical insertion of GLUT2 (Mace et al. 2007). This raises the question as to whether the combination of an artificial sweeter with carbohydrate could have a synergistic effect on incretin release, even if sucralose has no effect alone, and might account for the observation that chronic exposure of mice to oligofructose (a non-digestible sugar) enhances GLP-1 secretion in response to a high fat diet (Cani et al. 2006).

It should also be recognized that GPCR other than T1R2 have recently been

linked to incretin hormone release. For example, GPR40 and GPR119 are both GPCR that may stimulate incretin release in response to fatty acids, and agonists of these receptors could be therapeutically useful in diabetes (Chu et al. 2008). Furthermore, sweet taste sensors other than T1R2, such as SGLT3 (Diez-Sampedro et al. 2003), could be important in the detecting carbohydrate in the small intestine.

In conclusion, we have not been able to demonstrate that sucralose given by intragastric infusion stimulates GLP-1 or GIP release in humans, or elicits a feedback response to slow gastric emptying. This implies that artificial sweeteners may have no therapeutic benefit in the dietary management of diabetes, other than as a substitute for carbohydrate.
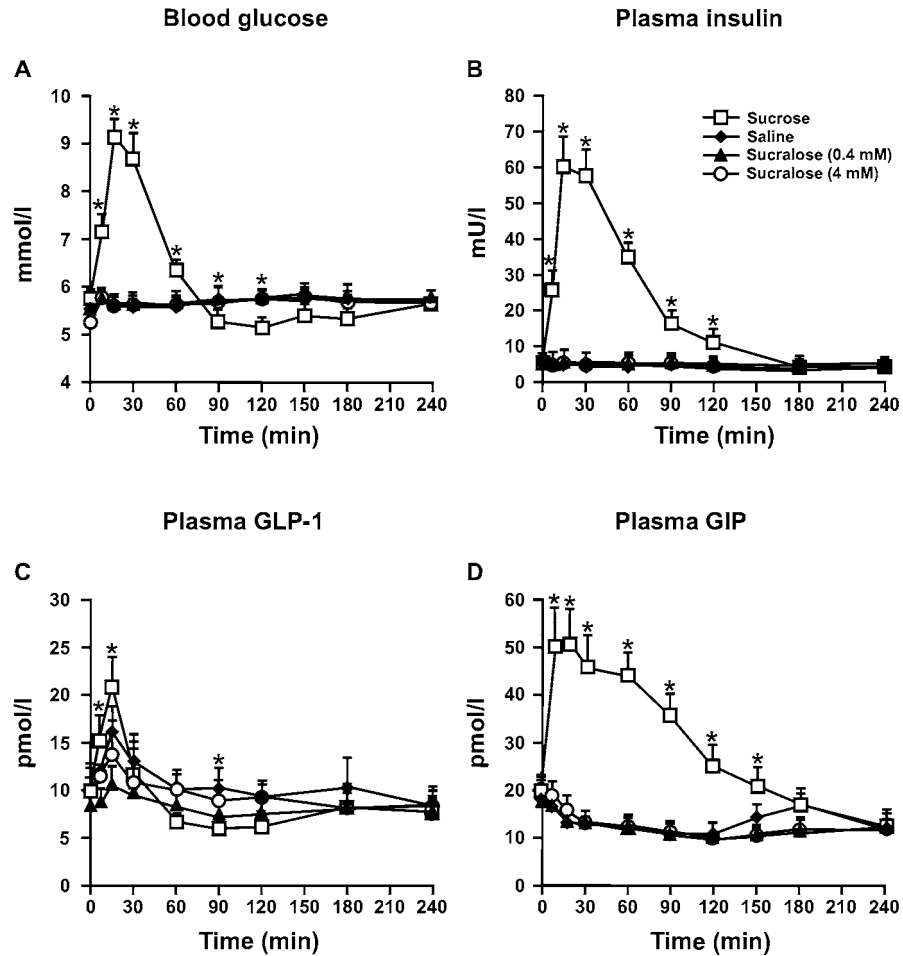
Figure 10.1: Concentrations of blood glucose (A), plasma insulin (B), plasma GLP-1 (C) and plasma GIP (D) in response to intragastric infusion of 500 ml solutions containing either (i) 50 g sucrose, (ii) normal saline, (iii) 80 mg sucralose (0.4 mM), or (iv) 800 mg sucralose (4 mM) in 7 healthy subjects. *sucrose vs. saline, $P < 0.05$. Data are mean values ± SEM. There were no significant differences in blood glucose, plasma insulin, plasma GLP-1 and GIP between either load of sucralose and saline.

CHAPTER 11: EFFECT OF THE ARTIFICIAL SWEETENER, SUCRALOSE, ON SMALL INTESTINAL GLUCOSE ABSORPTION IN HEALTHY HUMANS

11.1 Summary

It has been reported that the artificial sweetener, sucralose, stimulates glucose absorption in rodents by enhancing apical availability of the glucose transporter GLUT2. We evaluated whether exposure of the proximal small intestine to sucralose affects glucose absorption, and/or the glycaemic response to an intraduodenal (ID) glucose infusion, in healthy humans. 10 healthy subjects were studied on two separate occasions in single-blind, randomized order. Each subject received an ID infusion of sucralose (4 mM in 0.9 % saline), or control (0.9 % saline) at 4 mL/min for 150 min (t = -30 to 120 min). After 30 min (t = 0), glucose (25 %) and its non-metabolized analogue, 3-O-methylglucose (3-OMG; 2.5 %), were co-infused intraduodenally (T = 0 to 120 min; 1 kcal/min). Blood was sampled at frequent intervals. Blood glucose and serum 3-OMG concentrations increased during ID glucose/3-OMG infusion (P < 0.001 for both). However, there were no differences in either blood glucose or serum 3-OMG concentrations between sucralose and control infusions. In conclusion, sucralose does not appear to modify the rate of glucose absorption, nor the glycaemic

response to ID glucose, when given acutely in healthy humans.

11.2 Introduction

The mechanisms by which the gut senses nutrients are unclear, and the „receptor"
for detecting luminal carbohydrate has, until recently, been elusive. Recent
studies indicate the presence of G-protein coupled taste receptors, T1R2 and
T1R3, and their taste signal transduction partners, the G-protein gustducin and
the transient receptor potential ion channel TRPM5, in the mucosa of the mouse
and human gastrointestinal tract (Sutherland et al. 2007b; Young et al. 2009).
These receptors, analogous to sweet taste receptors on the tongue, broadly
respond to sugars and artificial sweeteners, and amongst several cell types,
appear to co-localize with glucagon-like peptide-1 (GLP-1)-secreting L-cells
(Jang et al. 2007b) .

It has been reported that the artificial sweetener, sucralose, stimulates the
secretion of both GLP-1 and glucose-dependent insulinotropic polypeptide (GIP)
from the mouse enteroendocrine cell line GLUTag (Margolskee et al. 2007), and
stimulates GLP-1 secretion from the human L-cell line NCI-H716 (Jang et al.
2007b), a response that is blocked by the sweet receptor antagonist, lactisole, and
siRNA for α-gustducin (Jang et al. 2007b). However, it was demonstrated in
Chapter 10 that sucralose, in two different loads, had no effect on GLP-1, GIP, or

insulin secretion, and did not elicit any feedback response on gastric emptying in healthy humans (Ma et al. 2009a). While this implies that artificial sweeteners may have no therapeutic benefit in the dietary management of diabetes, other than as a substitute for carbohydrate, it remains possible that sucralose affects small intestinal carbohydrate absorption, as a result of its interaction with the sweet taste receptor.

Glucose is absorbed from the small intestine through both the sodium-dependent glucose transporter 1 (SGLT1), and the facilitative transporter GLUT2 (Kellett et al. 2008). Supplementation of the diet with sucralose increases expression of SGLT1 in the enterocytes of wild type mice, but not in mice deficient in T1R3 or α-gustducin (Margolskee et al. 2007). The presence of sucralose enhances insertion of GLUT2 into the apical region of the enterocyte, and thus stimulates glucose absorption in the rat (Mace et al. 2007; Mace et al. 2009). For example, sucralose administration doubled the level of GLUT2 protein detected in apical membrane vesicles in response to low luminal glucose concentrations (20 mM) (Mace et al. 2007), and the maximum rate of glucose absorption was reached after 20 min of exposure to sucralose in vivo (Mace et al. 2009). This raises the question as to whether the combination of an artificial sweetener with carbohydrate could have a synergistic effect on glucose absorption in humans. The notion that consuming artificial sweeteners together with carbohydrates

could enhance glucose absorption, and therefore elevate postprandial blood glucose concentrations, is of fundamental clinical importance.

The aim of the current study was to evaluate whether exposure of the proximal small intestine to sucralose affects the subsequent response to glucose, in terms of the rate of glucose absorption and the glycaemic response.

11.3 Methods

11.3.1 Subjects

10 healthy subjects (8 male, 2 female; age 27 ± 2 yr; body mass index 23.4 ± 0.8 kg/m2) were studied twice in a randomized, single-blind, cross-over design. None of them had a history of gastrointestinal disease, upper or lower gastrointestinal symptoms, or significant previous surgery. Each subject provided written informed consent prior to participating, and the study was approved by the Royal Adelaide Hospital Research Ethics Committee.

11.3.2 Protocol

The study followed a randomized, single-blind, cross-over design. All participants attended the Discipline of Medicine at the Royal Adelaide Hospital at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids)

on two occasions, separated by at least 3 days. Women were studied in the follicular phase of the menstrual cycle, to avoid potential influences of the menstrual cycle on upper gastrointestinal function (Brennan et al. 2009).

On each study day, a multilumen silicone catheter (external diameter 4 mm) was introduced into the stomach through an anaesthetised nostril, and allowed to pass into the duodenum by peristalsis. This catheter incorporated a sidehole to deliver infusions into the proximal duodenum; its position was monitored continuously during the study by measurement of the transmucosal potential difference (TMPD), from two other saline-perfused side holes on either side of the pylorus, using established criteria (antral TMPD < -20 mV, duodenal TMPD > -15 mV, difference > 15 mV) (Heddle et al. 1988a). This required the insertion of a 20G saline-filled cannula subcutaneously in the forearm as a reference. When the catheter was positioned correctly, an intravenous cannula was inserted into a forearm vein for subsequent blood sampling.

After baseline blood samples were taken (t = -30), an intraduodenal infusion of sucralose (960 mg made up to 600 mL with 0.9 % saline (sucralose concentration 4 mM, total osmolality ~ 300 mosmol/L), or 600 mL 0.9 % saline alone as a control (~ 300 mosmol/L)), commenced and continued at a rate of 4 mL/min for 150 min (t = -30 to 120 min). At t = 0 min, an intraduodenal glucose solution (30

g glucose together with 3 g 3-O-methylglucose (3-OMG, Sigma-Aldrich, MO, USA), made up to a total volume of 120 mL with water, ~ 1390 mM glucose), also commenced via the same channel in the catheter and continued at a rate of 1 mL/min (= 1 kcal/min) for 120 min (t = 0 to 120 min).

Venous blood samples (~ 20 mL) were taken immediately before the intraduodenal infusion commenced, and at t = -15, 0, 5, 10, 20, 30, 40, 60, 90, and 120 min. Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). The remainder of each blood sample was placed in a serum tube, and centrifuged at 3200 rpm for 15 min. Serum was separated and samples stored at -70 $^{\circ}$C for subsequent analysis of 3-OMG concentrations by liquid chromatography/mass spectrometry. 3-OMG is an analogue of glucose that is absorbed from the gut in the same way as glucose but not metabolised; therefore serum concentrations represent an index of glucose absorption (Fordtran et al. 1962).

11.3.3 Statistical analysis

Repeated measures analysis of variance (SuperANOVA, Abacus Concepts, Berkeley, CA, USA) was used to analyze the blood glucose and serum 3-OMG concentrations with both treatment and time as factors. Statistical significance was accepted as $P < 0.05$, and data are presented as mean values $\pm$ standard

error.

11.4 Results

All subjects tolerated the study well.

11.4.1 Blood glucose concentrations (Figure 1A)

There was no difference in baseline glucose concentrations between the two study days. Blood glucose concentrations also did not differ over t = -30 min to 0 min, during infusions of sucralose or saline. There was a rise in blood glucose concentration following commencement of the ID glucose infusion, which was evident from t = 20 min (P < 0.001) on both days. There was no difference in blood glucose concentrations in subjects receiving sucralose or saline infusions.

11.4.2 Serum 3-OMG concentrations (Figure 1B)

Serum 3-OMG concentrations increased from 20 min following commencement of the ID glucose infusion (P < 0.001) on both study days. There was no difference in serum 3-OMG concentrations in subjects receiving sucralose or saline infusions.

11.5 Discussion

This study indicates that intraduodenal administration of sucralose has no effect on the rate of glucose absorption from the lumen of the small intestine, and does not elevate postprandial blood glucose concentrations, in healthy humans.

Sucralose is used as a non-caloric sweetener in the food industry and is widely consumed by individuals with obesity and diabetes. The recent identification of elements of the sweet taste receptor in the rodent and human small intestine, that are linked to peptide hormone release and modulation of glucose transport, suggests that artificial sweeteners could potentially be metabolically active. However, in vivo studies in mice (Fujita et al. 2009) and humans (Ma et al. 2009a) have failed to support any effect of sucralose on insulin, GLP-1 or GIP release. Recently, it was reported in rats that sucralose acted synergistically with glucose to activate the T1R2+T1R3 heterodimer and increase the rate of small intestinal glucose absorption by inserting GLUT2 into the apical membrane (Mace et al. 2007). Hence, consuming artificial sweeteners in conjunction with carbohydrate raises concerns about increasing the postprandial glycaemic response, particularly as there is already over-expression of SGLT1 and GLUT2 in the small intestine in animal models of diabetes (Burant et al. 1994; Dyer et al. 1997) and in diabetic humans (Dyer et al. 2002). Our study is the first to examine the potential interaction of intraluminal sucralose with glucose in relation to

small intestinal glucose absorption and blood glucose concentrations in healthy humans, and the negative outcome is consistent with studies that showed no effect of sucralose supplementation on glycaemic control in patients with diabetes (Mezitis et al. 1996; Grotz et al. 2003).

In the present study, we infused sucralose and glucose directly into duodenum, rather than administering them orally, in order to regulate precisely the exposure of the small intestine to these substances. In studies where sucralose stimulated the T1R2+T1R3 receptor, resulting in apical insertion of GLUT2, the concentration of sucralose was 1 mM (Mace et al. 2007). Therefore, 4 mM sucralose should have been a sufficient concentration to achieve a response. We used a high glucose concentration (1390 mM), which would itself be expected to induce apical GLUT2 insertion maximally (Mace et al. 2007), but this process would have taken about 20 min (Mace et al. 2009), so if prior exposure to sucralose did indeed modulate apical GLUT2, a difference in glucose absorption should have been evident early in the ID glucose infusion.

Species differences are likely to account for the lack of effect of sucralose in humans. It has been reported that there is much lower duodenal expression of GLUT2 in humans than in rats and mice, while expression of SGLT1 is much greater in humans (Kim et al. 2007). Therefore, SGLT1 is likely to play the

dominant role in glucose absorption in the human small intestine. Mutation of SGLT1 in humans results in glucose-galactose malabsorption, whereas absorption of these sugars is not disrupted by mutations of GLUT2 (Wright et al. 2003). Moreover, if apical GLUT2 insertion occurred in humans, one might expect this to ameliorate the effects of SGLT1 mutation and allow glucose to be tolerated in this disorder, but this appears not be the case (Dyer et al. 2002). Supplementation of a low-sugar chow with sucralose for two weeks in mice has been reported to increase SGLT1 protein and mRNA expression (Margolskee et al. 2007). Therefore, it is still possible that prolonged exposure to sucralose could increase small intestinal glucose absorption in humans, although this would need to be evaluated in a separate study.

In conclusion, this study has demonstrated that intraduodenal administration of sucralose does not acutely enhance the absorption of glucose from the small intestine, or increase blood glucose concentrations, in healthy humans.
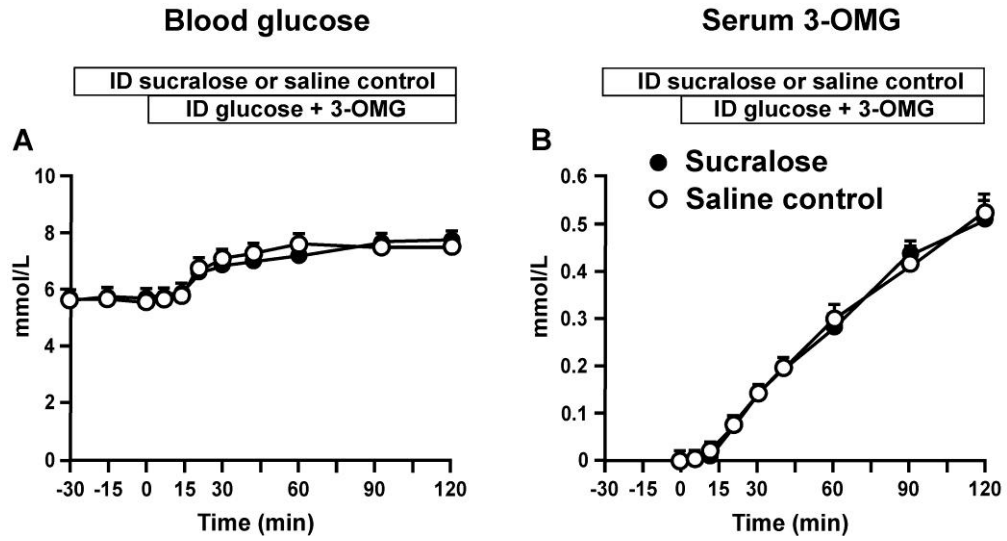
Figure 11.1: Concentrations of blood glucose (A) or serum 3-OMG (B) in 10 healthy subjects in response to an ID infusion of sucralose (4 mM in 0.9 % saline), or control (0.9 % saline) at 4 mL/min for 150 min (t = -30 to 120 min) with co-infusion of glucose (25 %) and 3-O-methylglucose (3-OMG; 2.5 %) between t = 0 and 120 min (1 kcal/min). Data are presented as mean values ± SEM. There are no significant differences in blood glucose or serum 3-OMG concentrations between saline or sucralose days.

CHAPTER 12: EFFECTS OF CEFACLOR ON GASTRIC EMPTYING AND CHOLECYSTOKININ RELEASE IN HEALTHY HUMANS

Adapted from Ma J et al, Regul Pept. 159(1-3): 156-9, 2010.

12.1 Summary

In rodents, cephalosporin antibiotics can mimic peptones and stimulate release of cholecystokinin (CCK), a hormone that slows gastric emptying. The rate of gastric emptying is a major determinant of postprandial blood glucose and insulin concentrations. The effect of orally administered cefaclor on plasma CCK and gastric emptying, as well as postprandial glycaemic and insulinaemic responses, was therefore evaluated in healthy humans. 8 healthy subjects were studied on two days each in double-blind, randomized order. On each day, subjects consumed 1000 mg cefaclor or placebo 30 min before a mashed potato meal labeled with ^{13}C octanoic acid. Blood and breath samples were collected for 4 hours after the meal. Blood glucose, serum insulin and plasma CCK increased in response to the carbohydrate meal on both study days, and cefaclor had no effect on these responses. Similarly, the gastric half-emptying time (measured by breath test) did not differ (placebo: 137.5 ± 6.0 min vs. cefaclor: 143.1 ± 8.0 min). Cefaclor, when given before a meal in the form of a capsule, does not stimulate CCK release or slow gastric emptying in healthy humans.

12.2 Introduction

Gastric emptying is regulated by the integrated activity of the proximal stomach, antrum, pylorus and duodenum. Exposure of the small intestine to nutrient induces feedback that slows gastric emptying by relaxing the fundus, suppressing antral pressures and augmenting pyloric contractions (Heddle et al. 1988a). This feedback inhibition is mediated by the secretion of gastrointestinal hormones, of which the best characterized is CCK (Schwizer et al. 1997). The CCK-1 receptor antagonist, loxiglumide, abolishes the inhibitory action of lipid on gastric emptying (Schwizer et al. 1997).

CCK is secreted from the I-cells of the proximal small intestine in response to nutrients (Liddle 1997). Protein, particularly protein hydrolysates (peptones), and fat, are strong stimuli for CCK release, whereas carbohydrate is less potent (Liddle 1997). In the intestinal cell line STC-1, peptones trigger CCK secretion and gene transcription directly (Cordier-Bussat et al. 1997). Various cephalosporin antibiotics, which have a tripeptide structure, and therefore mimic the molecular structure of peptones, have similar effects (Nemoz-Gaillard et al. 1998). In rats, intragastric administration of the cephalosporin antibiotic, cefaclor, between 15 and 500 mg/kg, results in a dose-dependent slowing of gastric emptying (Bozkurt et al. 2000), an effect mediated via a capsaicin-sensitive vagal afferent pathway involving CCK-1 receptors (Bozkurt et al. 2000; Darcel et al.

2005). Similarly, duodenal perfusion with cefaclor (500 mg/kg) stimulates the discharge of CCK-responsive vagal afferents in this species, an effect probably mediated via the peptone transporter PepT1 (Darcel et al. 2005). In mice, intraperitoneal administration of various cephalosporins accelerates gastric emptying over the range 2-200 mg/kg, and slows gastric emptying when given at higher doses (> 1000 mg/kg) (Kuo et al. 1998). Given the persuasive evidence that cephalosporins have the capacity to affect gastric emptying in rodents, it is surprising that little information is available in humans, although there is one report that intravenous cefazolin had no impact on gastric emptying in the critically ill (Chapman et al. 2003). However, parenteral administration of cephalosporins would not be expected to influence a luminal peptide sensing mechanism.

Control of postprandial glycaemia is increasingly recognized as fundamental to the management of diabetes. Gastric emptying accounts for about one third of the variance in postprandial blood glucose after an oral glucose load in healthy subjects (Horowitz et al. 1993) and in patients with type 2 diabetes (Jones et al. 1996), and minor variations in the initial rate of carbohydrate entry into the small intestine can have major effects on glycaemic and insulin responses (O'Donovan et al. 2004b). Accordingly, dietary or pharmacological interventions which affect gastric emptying influence the glycaemic response to carbohydrate meals. For

example, accelerating nutrient entry from the stomach to the small intestine with erythromycin increases the postprandial glycaemic response, whereas slowing gastric emptying with morphine attenuates postprandial blood glucose concentrations in type 2 patients (Gonlachanvit et al. 2003).

The aim of the current study was to evaluate the acute effects of orally administered cefaclor (about 15 mg/kg, a dose shown to be effective in rats and at the upper end of the clinical dosing range in humans) on CCK release, gastric emptying, and postprandial glycaemic and insulinaemic responses in healthy subjects.

12.3 Methods

12.3.1 Subjects

8 healthy subjects (4 male, 4 female; age: 28.3 ± 3.0 yr; BMI: 22.4 ± 0.7 kg/m^2) were studied twice in a randomized, double-blind, cross-over design. None was taking medication known to influence gastrointestinal function. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee, and each subject provided written informed consent prior to taking part.

12.3.2 Protocol

All participants attended the laboratory at approximately 0830 h after an overnight fast (14 h for solids, 12 h for liquids) on two occasions, each separated by at least 3 days. Women were studied in the follicular phrase of the menstrual cycle. On each day, an intravenous cannula was inserted into an antecubital vein for blood sampling and at t = -30 min, subjects consumed 2 capsules each containing 500 mg cefaclor (Link Pharmaceuticals Ltd., UK; encapsulated by the Pharmacy Department, Royal Adelaide Hospital) or identical placebo (containing starch), with 50 ml water, 30 minutes before a mashed potato meal (given at t = 0 min) in a double-blind and randomized design. The meal consisted of 65 g powdered potato (Deb, Epping, NSW, Australia) and 20 g glucose, reconstituted with 250 ml water and 1 egg yolk containing 100 μL ^{13}C octanoic acid. Subjects consumed the meal within 5 minutes. Breath samples were collected immediately before, and every 5 minutes after, meal ingestion for the first hour, and every 15 min for a further 3 hours. Venous blood samples (~ 18 ml) were taken immediately before administration of the capsules (ie. t = -30 min), and then at t = -15, 0, 15, 30, 60, 90, 120, 150, 180 and 240 min. Blood glucose concentrations were measured immediately using a glucometer (Medisense Precision QID, Abbott Laboratories, Bedford, MA, USA). The remainder of each blood sample was placed in both a prechilled-EDTA tube containing 400 kIU aprotinin (Trasylol, Bayer Australia Ltd, Pymble Australia) per litre blood for

CCK assay and an ice-chilled serum tube with clotting activator for insulin assay, both stored on ice, and centrifuged at 3200 rpm for 15 min. Plasma and serum were separated and samples were stored at -70 ºC for subsequent analysis.

12.3.3 Mesurements

<u>Gastric emptying</u>

Breath samples were measured for isotope enrichment by an isotope ratio mass spectrometer (ABCA 2020, Europa Scientific, Crewe, England) with an on-line gas chromatographic purification system. The half-emptying time (T50) and gastric emptying coefficient (GEC) were calculated using the formulae 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).

<u>Serum insulin and plasma CCK concentrations</u>

Serum insulin was measured by a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000 Insulin, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity was 2 mU/L, intraassay coefficient of variation (CV) was 3.9 % and interassay CV was 5.0 %. Plasma CCK-8 was measured by radioimmunoassay using an adaption of the method of Santangelo et al (Santangelo et al. 1998), (antibody C2581, Lot 105H4852, Sigma Chemical, St. Louis, MO, USA) with less than 2 % cross-reactivity for

gastrin. Intra-assay CV was 6.2 %, inter-assay CV was 14.2 % and sensitivity was 1 pmol/L.

12.3.4 Statistical analysis

Paired t-tests (Statview, SAS Institute Inc., Cary, NC, USA) were used to compare T50 and GEC, and baseline blood glucose, serum insulin and plasma CCK values, while repeated measures analysis of variance (SuperANOVA, Abacus Concepts, Berkeley, CA, USA) was used to compare blood glucose, serum insulin, and plasma CCK concentrations with time and treatment as factors. $P \leq 0.05$ was taken to indicate statistical significance, and data are presented as mean values ± standard error.

12.4 Results

All subjects tolerated the study well.

12.4.1 Blood glucose, serum insulin and plasma CCK concentrations (Figure 1A-C)

Baseline blood glucose, serum insulin and plasma CCK concentrations did not differ between the two study days. There was a rise in blood glucose and serum

insulin after the meal on both study days (P ≤ 0.001), with no difference between cefaclor and placebo. Between t = -30 to 0 min (ie. prior to the meal), there was no increase from baseline CCK after either cefaclor or placebo. After the meal, plasma CCK increased on both days (P ≤ 0.001), without any difference between them.

12.4.2 Gastric emptying

There was no significant difference in T50 between the 2 study days (cefaclor: 143.1 ± 8.0 min vs. placebo: 137.5 ± 6.0 min; P = 0.33). Similarly, the GEC did not differ between cefaclor (3.0 ± 0.1) and placebo (2.9 ± 0.1).

12.5 Discussion

In this study, it was not possible to demonstrate that oral cefaclor, at a dose of 1000 mg, stimulates any CCK release or slows gastric emptying, and, accordingly, it had no effect on blood glucose or insulin responses to a high carbohydrate meal in healthy subjects.

In humans and rats, protein and fat are potent stimuli for CCK release. Free fatty acids have recently been shown to stimulate CCK release via G-protein-coupled fatty acid receptors such as the GPR120 receptor, which mediates Ca^{2+} influx in

STC-1 cells in response to long chain fatty acids (Tanaka et al. 2008). While the gut mechanisms responsible for CCK secretion in response to protein are yet to be fully elucidated, it has been suggested that di- and tri-peptides, which are more potent stimuli for CCK release than polypeptides or monomeric amino acids (Cuber et al. 1990), are transported by the oligopeptide transporter PepT1, a process linked to CCK release in the murine enteroendocrine cell line STC-1 (Matsumura et al. 2005). More recently, another G protein-coupled receptor, GPR93, has been identified in rat enterocytes as a molecular sensor which is activated by peptones, and accompanied by an increase in CCK transcription (Choi et al. 2007b). Cefaclor, as a peptidomimetic, has the capacity to stimulate CCK secretion from STC-1 cells in vitro (Nemoz-Gaillard et al. 1998), and from rat small intestine in vivo (Darcel et al. 2005), an effect attributed to Pep T1 transport-induced activation of signal transduction pathways (Matsumura et al. 2005). However, cefaclor itself does not activate GPR93, but does act synergistically with protein hydrolysate to potentiate CCK release via this mechanism (Choi et al. 2007a), in a rat enterocyte line (hBRIE 380i) that does not express PepT1 (Choi et al. 2007a).

The reason that cefaclor failed to stimulate CCK release in humans remains uncertain. However, the dose given (about 15 mg/kg) is comparable to that shown to be effective in rats (3 ml of a 3 mM solution) (Bozkurt et al. 2000) and

was four times thse standard therapeutic dose. Accordingly, any substantial dose increase would have caused ethical concerns. Cefaclor was administrated 30 min before the meal, in the expectation that the presence of cefaclor in the small intestine would both induce the release of CCK, and slow gastric emptying in advance of meal ingestion, an approach we have used previously with other substances that stimulate gut peptide secretion (Ma et al. 2009c). It was anticipated that this time interval would coincide with maximal exposure of the small intestine to cefaclor, given that the peak plasma concentration occurs between 30 min and 60 min after an oral dose of 1000 mg (Lode et al. 1979). Using the mean values and standard deviations derived from the results, the current study had a 90 % power to detect a 15 % change in half emptying time, and a 90 % power to detect a 75 % increase from fasting in plasma CCK at 30 minutes after study drug administration, at the alpha 0.05 level. Accordingly, this study appears to exclude a clinically significant effect of cefaclor on either gastric emptying or CCK secretion. Although the study design did not incorporate a positive control, our group has previously demonstrated clear effects of small protein loads on gastric emptying using breath tests (Karamanlis et al. 2007) and scintigraphy (Ma et al. 2009c), and on gut peptide secretion, in a similar number of subjects.

Rather than an issue of dose, it may be more likely that species differences

between rodents and humans account for the discrepant observations on the stimuli that induce CCK release; in humans the presence of nutrient might be a necessary condition for the secretion of CCK (Liddle 2000).

The current observations are important because they highlight the caution needed in extending in vitro or rodent data to humans. Furthermore, they appear to exclude an acute effect of cephalosporin antibiotics in the clinical dose range on CCK release, gastric emptying or blood glucose metabolism.
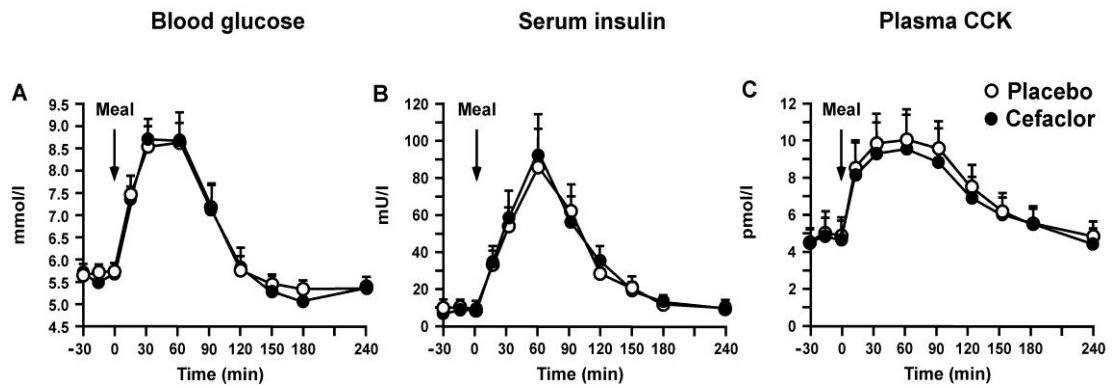
Figure 12.1: Concentrations of blood glucose (A), serum insulin (B) and plasma CCK (C) in 8 healthy subjects in response to oral cefaclor (1000 mg) or placebo administered 30 minutes prior to a potato meal. On one study day, subjects consumed 2 capsules each containing 500 mg cefaclor (total dose 1000 mg) with 50 ml water, 30 minutes before a mashed potato meal consisting of 65 g powered potato and 20 g glucose, reconstituted with 250 ml water and 1 egg yolk containing 100 μL ^{13}C octanoic acid. On the other study day, subjects consumed 1000 mg placebo prior to the mashed potato meal. Data are presented as mean values ± SEM. There were no significant differences in blood glucose, serum insulin or plasma CCK levels between placebo and cefaclor days.

CHAPTER 13: CONCLUSION

The studies reported in this thesis provide support for the fundamental importance of gastrointestinal function in the regulation of glycaemia and appetite in healthy individuals and patients with type 2 diabetes.

Gut functions involved in glycaemic regulation include gastric emptying and incretin hormone release, which are modified by the interaction between nutrients and the small intestine. Among the macro-nutrients, fat is most potent inhibitor of gastric emptying. A previous study had established that giving a small amount of fat (oil) 30 min before a carbohydrate meal, delayed the postprandial increase in blood glucose, plasma insulin and GIP, and stimulated the release of GLP-1, in patients with diet-controlled type 2 diabetes (Gentilcore et al. 2006b). When compared to giving the oil as a „preload", ingestion of oil with the meal had modest effects on gastric emptying, glycaemia and incretin hormone responses. The putative underlying mechanism is that when oil was given before the meal, both digested and non-digested fat would be present in the small intestine to slow gastric emptying and stimulate incretin hormones, in advance of meal consumption. In this fat preload study, there was only a trend for reduction of peak blood glucose levels, and measurements had not been continued for sufficiently long to determine whether the overall glycaemic

response was less.

Protein is a stronger stimulus for insulin release, and has a lower energy density, than fat. In the study presented in Chapter 5, ingestion of a whey protein preload (55 g) 30 min before, or with, a high carbohydrate meal, markedly slowed gastric emptying, and was associated with stimulation of GLP-1, as with the fat preload in patients with diet-controlled type 2 diabetes. In contrast to the fat study, protein stimulated GIP and insulin secretion - the latter substantially. Glycaemic responses after protein were markedly decreased (peak blood glucose by about 3 mmol/L), which is comparable to pharmacological therapy. However, it should be noted that this dose of whey protein, which was chosen on the basis of previous data that indicated it would be sufficient to stimulate gut hormones (Bowen et al. 2006c), does impose a caloric load of 220 kcal. Therefore, it would be important to confirm whether the observed effects would be maintained with a smaller load of protein, in order to minimize the additional energy load. It would also be interesting to determine whether the protein preload included suppressed appetite and energy intake, and whether the acute effects were maintained with continued use over the long term.

The next study, therefore, aimed to determine whether a whey preload at a smaller dose (25 g) remained effective in lowering postprandial glycaemia, and

also whether whey suppressed appetite and energy intake at a subsequent ad libitum meal in patients with type 2 diabetes (Chapter 6). There was a dose-dependent increase in fullness and an increase in plasma CCK and GIP after both protein preloads (25 g and 55 g) as well as a reduction in postprandial blood glucose concentrations in patients with diet-controlled type 2 diabetes; GLP-1 and insulin data were not available at the time of writing. Despite the increase in fullness, there were no significant differences in energy intake between either protein preload and placebo. However, the number of subjects was relatively small, and the ability of the preload to suppress energy intake might be critically dependent on the timing of the subsequent meal.

The study reported in Chapter 7 represents the first evaluation of the chronic effects of a protein preload on gastric emptying and glycaemic control in type 2 diabetes. Patients with diet-controlled type 2 diabetes were exposed to 4 week periods of either a whey or placebo preload before each main meal, in a randomized crossover design. The dose of whey (25 g) was the lower of the doses shown to be effective in reducing glycaemia acutely (Chapter 6). This study showed that the effects on gastric emptying and postprandial glycaemia were sustained over 4 weeks.

The site, and length of exposure, of the small intestine to nutrient is likely to be

important in determining the release of gut peptides, including GLP-1. In the study reported in Chapter 8, the capacity of an enteric coated pellet formulation of lauric acid (CTM#3) to release GLP-1 and improve the postprandial glycaemic response in type 2 diabetes was evaluated. CTM#3 was designed to release a small amount of fatty acid over a long length of terminal ileum. A glucose-lowering effect of CTM#3 was observed after both breakfast (containing the pellets) and lunch, associated with prolonged stimulation of GLP-1 release. Neither serum insulin, nor the insulin to glucose ratio, were increased significantly by CTM#3, suggesting that paracrine and portal effects of GLP-1 may be at least as important as its endocrine effects. Other potential actions of GLP-1 that may be relevant include the suppression of glucagon and possibly, slowing of gastric emptying.

In healthy subjects, the incretin hormones account up to 70 % of the postprandial insulin response. In patients with type 2 diabetes, the incretin response is reported to be attenuated. The secretion of GIP in type 2 diabetes appears to be normal, whereas the insulinotropic effect of GIP is reduced, while the insulin response to GLP-1 appears intact, but its postprandial secretion is said to be impaired. However, no studies had previously taken into account the potential confounding influence of different rates of gastric emptying in determining the incretin response. In the study reported in Chapter 9, the effects of intraduodenal

infusion of glucose at 1, 2, and 4 kcal/min, which spans the physiological range of gastric emptying, on blood glucose, serum insulin and plasma incretin hormones were evaluated, and were compared with data from healthy subjects. In both groups, incremental increases in serum insulin and plasma GLP-1 with increasing glucose loads were not linear, and were much greater in response to 4 kcal/min than 1 or 2 kcal/min, while GIP increased more directly in proportion to the glucose load. However, with intraduodenal glucose delivery at a known rate, the incremental increases in GLP-1 and GIP were qualitatively similar in patients with type 2 diabetes and healthy subjects, implying that the incretin responses to glucose are not grossly impaired in well-controlled type 2 diabetes. Furthermore, the results suggested that if glucose entry to the small intestine is at the low end of the physiological range (about 1 kcal/min), the incretin response is relatively less important, adding to the rationale for interventions that slow gastric emptying in the treatment of type 2 diabetes. Finally, it was observed that defective insulin secretion was evident even in these well controlled type 2 patients, but was less marked in response to lower than higher rates of duodenal glucose delivery.

Direct exposure of the small intestine to nutrients, including carbohydrate, is important to regulate gastric emptying and gut hormone release. It has recently been established that elements of the sweet taste receptor, identified in the tongue,

are also found in the small intestine. Among several cell types, sweet taste receptors appear to colocalize with GLP-1-secreting L-cells, and sucralose was recently reported to stimulate GLP-1 release in vitro. In the study reported in Chapter 10, the effects of sucralose at a concentration chosen to match the sweetness of a sucrose load (0.4 mM), and at a much higher concentration (4 mM), in the range shown to stimulate GLP-1 release in vitro, were examined. In contrast to sucrose, neither load of sucralose, given by intragastric infusion, had any effect on GLP-1, GIP or insulin secretion. It has been reported in rats that sucralose also acts synergistically with glucose, by stimulating the sweet taste receptor and, thereby, inducing the insertion of GLUT2 into the apical membrane to enhance the rate of glucose absorption (Mace et al. 2007). In the study reported in Chapter 11, the potential interaction of intraluminal sucralose with glucose, in relation to small intestinal glucose absorption and blood glucose concentrations, was examined in healthy humans. Sucralose did not increase the rate of absorption of the glucose analogue, 3-OMG, nor the glycaemic response to intraduodenal glucose, when given acutely. The outcome of the studies in Chapters 10 and 11 indicates that artificial sweeteners have no therapeutic role in the management of diabetes, other than as a substitute for carbohydrate.

Protein, particularly protein hydrolysates (peptones), is a known stimulus for CCK release (Liddle 1997). In rats, intragastric administration of the

cephalosporin antibiotic, cefaclor, which shares a tripeptide structure with peptone, results in a dose-dependent slowing of gastric emptying by a CCK-dependent mechanism (Bozkurt et al. 2000). In Chapter 12, it was reported that oral cefaclor, at a dose of 1000 mg in healthy humans, had no effect on CCK release, gastric emptying, or postprandial glycaemic and insulinaemic responses to a carbohydrate meal. The reason that cefaclor failed to stimulate CCK release in humans remains unclear, but as for sucralose, species differences between rodents and humans may well account for the discrepant observations.

In summary, the studies presented in this thesis indicate that a small preload of protein, given in advance of a meal, can stimulate gut hormone release and insulin secretion, slow gastric emptying, and dramatically improve the glycaemic response to a meal. Furthermore, a relatively small protein load is well tolerated over several weeks, and without apparent adaptation to its effects on glycaemia. Similar effects appear to be achievable by administrating small macronutrient loads in enteric coated formulations, so as to target release over a long length of terminal ileum. The fact that GLP-1 secretion appears intact, at least in well controlled type 2 diabetes, provides a rationale for strategies to stimulate endogenous GLP-1 release. Unfortunately, neither of the non-nutrient stimuli tested in these studies (sucralose and cefaclor) had any effects on GLP-1 or CCK secretion in humans. Nevertheless, dietary strategies to stimulate small intestinal

feedback appear extremely promising and should be developed further for the

management of patients with type 2 diabetes.

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