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THE ROLE OF INCRETIN PEPTIDES AND GHRELIN IN UPPER GUT MOTILITY AND METABOLIC CONTROL

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To Mom and Dad

A cheerful heart is good medicine
Proverbs 17:22

CONTENTS

ABSTRACT

Metabolic control is essential for the individual and is strictly regulated via a wide range of factors. One important mechanism in maintaining an adequate blood sugar level is the regulation of insulin release and gastric emptying rate by gastrointestinal (GI) hormones.

The aims of this study was to (1) investigate the effects of the two incretin hormones GIP and GLP-1 on insulin secretion and small bowel motility in a rat model of diabetes type 2 and (2) to investigate the effect of GIP and GLP-1 on gastric emptying, metabolic control and appetite after intake of a mixed meal in man. In addition, I (3) studied the effects of ghrelin on smooth muscle contractility in rat and (4) its effect on gastric emptying in humans after intake of a mixed meal.

The effect of GIP and GLP-1 on insulin secretion was studied in isolated perfused pancreas in a rat model of diabetes type 2. Migrating motor complex (MMC) is a marker for small bowel motility recorded by electromyography. The effect of GIP and GLP-1 was studied in both diabetic and non-diabetic rats. In man, gastric emptying during GIP or GLP-1 infusion was investigated scintigraphically after intake of a ^{99}Tc-labelled omelette. Simultaneously, appetite ratings were measured using visual analogue score (VAS) and blood samples collected for later analysis of GI hormones, e.g. insulin, glucagon, PYY, GLP-1, GIP and ghrelin by radioimmunoassay (RIA).

GIP and GLP-1 stimulated insulin secretion in normal rats. The basal insulin level was higher in diabetic rats and insulin response to glucose stimulation was severely impaired. The potentiatory effect of both GIP and GLP-1 on glucose-induced insulin secretion was preserved in diabetic animals, being even more pronounced than in controls. In diabetic and non-diabetic animals GIP and GLP-1 showed a similar inhibition of small bowel motility. GIP induced a small acceleration of gastric emptying in man, whereas GLP-1 potently inhibited gastric emptying. Both peptides showed a trend towards increased insulinogenic index. GIP did not affect appetite, while GLP-1 decreased hunger and increased satiety.

The effect of ghrelin was studied on isotonic contractions of smooth muscle strips of the gastric fundus and jejunum in an organ bath. MMC was studied during intravenous infusion of ghrelin in normal, atropinised and vagotomised rats. The effects of ghrelin on gastric emptying, VAS and GI hormones were studied in the same way as for GIP and GLP-1.

Ghrelin caused dose-dependent contraction of the fundus and jejunum. Pre-treatment with atropine abolished the response. Ghrelin also dose-dependently shortened the MMC. In man, ghrelin accelerated gastric emptying followed by increased hunger and deceased satiety. The effect was independent of growth hormone (GH) secretion.

In conclusion, there is an insulinotropic effect of GIP and GLP-1 in both normal and diabetic rat and in man. Novel finding is that GIP accelerates gastric emptying, whereas GLP-1 inhibits it. Ghrelin stimulates motility *in vitro* and *in vivo* in rat and accelerates gastric emptying in man. Moreover, both GLP-1 and ghrelin seems to have independent effects on appetite, which make them interesting tools for the study of diabetes type 2, diabetes type 1 as well as obesity.

LIST OF PUBLICATIONS

I **Edholm T**, Cejvan C, Efendic S, Holst JJ, Schmidt PT, Hellström PM. *The incretin hormones GIP and GLP-1 in diabetic rats: Effects on motility and insulin secretion.* (manuscript)

II **Edholm T**, Grybäck P, Lundberg S, Schmidt PT, Hellström PM. *The incretin hormones GIP and GLP-1 in healthy volunteers: Effects on gastric emptying rate and insulin response.* (manuscript)

III **Edholm T**, Levin F, Hellstrom PM, Schmidt PT. *Ghrelin stimulates motility in the small intestine of rats through intrinsic cholinergic neurons.* Regul Pept. 2004, 121:25-30.

IV Levin F, **Edholm T**, Schmidt PT, Grybäck P, Jacobsson H, Degerblad M, Höybye C, Holst JJ, Rehfeld JF, Hellström PM, Näslund E. *Ghrelin stimulates gastric emptying and hunger in normal weight humans.* J Clin Endocrinol Metab. 2006, 91:3296-3302.

LIST OF ABBREVIATIONS

Ach	acethylcholine
AUC	area under the curve
CCK	cholecystokinin
DD	diabetic diarrhoea
DFZ	diabetic fatty zucker rat
DPP IV	dipeptidyl-peptidase IV
ENS	enteric nervous system
GH	growth hormone
GHD	growth hormone-deficient
GHS-R	growth hormone secretagogue receptor
GI	gastrointestinal
GIP	glucose-dependent insulinotropic polypeptide
GK	goto-kakizaki rat
GLP-1	glucagon-like peptide-1
GPCR	G-protein coupled receptor
i.p.	intraperitoneal
i.v.	intravenous
MMC	migrating myoelectric complex
PI 3-K	phosphoinositide-3-kinas
PKA	protein kinas A
PKB	protein kinas B
PP	pancreatic polypeptide
PYY	peptide YY
RIA	radioimmunoassay
SEM	standard error of the mean
VIP	vasoactive intestinal polypeptide

INTRODUCTION

THE INCRETIN EFFECT

Oral intake of glucose induces a greater insulin response than intravenous (i.v.) glucose, though resulting in the same blood glucose elevation [1-3] (fig 1). Early estimates assumed that 50% of the insulin response after oral glucose intake was released by gastrointestinal (GI) factors [4]. Today, three criteria are used to determine whether a hormone should to be classified as an incretin [5, 6]:

(1) The hormone must be released from gut endocrine cells after ingestion of nutrients, especially of glucose.
(2) The circulating hormone must stimulate insulin secretion at a concentration which is achieved after ingestion of a nutrient.
(3) The hormone releases insulin only at elevated glucose levels (known as glucose-dependence).
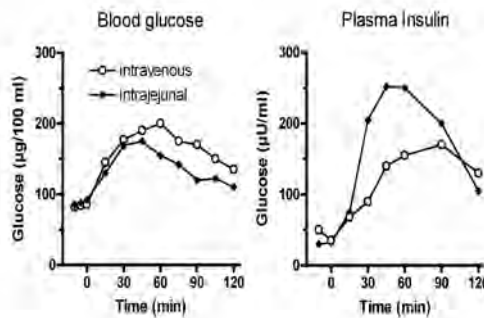
Fig 1. Schematic demonstration of the incretin effect. Blood glucose and insulin responses after either intravenous or intrajejunal glucose infusion in normal subjects. Plasma glucose levels after intravenous glucose infusion are similar to intrajejunal glucose infusion, but the latter generates a larger insulin response.

Development of concept

Already in 1906 it was discovered that the duodenum yielded a chemical excitant for internal pancreas secretion [7], but it took almost 25 years until the importance of the gut in the regulation of insulin secretion was called to attention. It was then demonstrated that extract from the duodenal mucosa could lower blood glucose [8]. Interest in the incretin concept was raised again 30 years later, when two independent groups, using radioimmunoassay (RIA) to show evidence that glucose per oral stimulated insulin secretion far more potently than the same amount of glucose i.v. [5, 6]. The first incretin hormone was isolated and sequenced 1970 [9] and it was named gastric inhibitory polypeptide (GIP), due to its inhibitory effect on gastric acid secretion in dogs. After insulinotropic action of GIP in man had been demonstrated [10] the name was changed to "glucose-dependent insulinotropic polypeptide". Fifteen years later, a strong insulinotropic effect was seen with glucagon-like peptide (GLP-1) a new intestinal peptide identified by cloning the pre-proglucagon. The discovery of GLP-1 has been the start of a new possibility to treat diabetes, based on the incretin concept.

GLP-1 analogues or inhibitors of degradation of GLP-1 have now been approved for the US market and are commercially available for treatment of type 2 diabetes since 2005 [11].

Quantification of the incretin effect

The incretin effect is quantified by comparing the insulin response to oral and intravenous glucose loads. In healthy subjects, the contribution of the incretin effect on insulin response ranges between 25 and 73 % [12-15], depending on the amount of glucose given. In patients with diabetes type 2.

Interaction between the gut and the endocrine pancreas

The interaction between the gut and the endocrine pancreas is complicated and not fully understood. Both GIP and GLP-1 are secreted from the gut lumen after intake of glucose, carbohydrate, fat and protein [16-18]. These peptides have different actions on the small intestine; at least GLP-1 acts as a "brake" on gastric emptying and gut motility [19-22]. GIP and GLP-1 are both glucose-dependent in their action on the β-cell and insulin secretion. The blood glucose threshold for the peptides is 4.5 mmol/L [23]. Incretin hormones do not only induce insulin secretion, but also interact with pancreatic islets via neurocrine action [24]. In parasympathetic neurons, the transmitter substances acetylcholine (Ach), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating polypeptide and gastrin-releasing peptide are localized. In sympathetic neurons, on the other hand, we can find norepinephrine, galanin and neuropeptide Y. All these neurotransmitters stimulate or inhibit insulin release and glucagon secretion [25].

GIP AND ITS RECEPTORS

GIP is a 42 amino acid peptide secreted from the K-cells [26-28] in response to nutrient ingestion [29, 30]. The main stimuli of GIP secretion are carbohydrates and a lipid-rich meal [18, 31-33]. K-cells are found in the proximal gut. The K-cells express glucokinase and are believed to have a glucose-sensing system, similar to the mechanism seen in the pancreatic β-cell [34]. GIP secretion reaches peak concentrations already 15-30 min after intake of a meal, long before the nutrients are present in the gut [18, 31-33]. Therefore it is highly likely that the vagus nerve is involved in the stimulation of secretion [35]. Shortly after GIP is released into the circulation the full-size peptide (1-42 amide) is cleaved by the enzyme dipeptidyl-peptidase IV (DPP IV) [36, 37] at the NH_2-terminal part resulting in the truncated GIP (3-42 amide) [38-41]. The biological activity is lost for the truncated GIP (3-42 amide) and the metabolite can even act as an antagonist to the GIP receptor [38, 42, 43]. The half-life of the intact GIP (1-42) is approximately 7 min.

There are two isoforms of the human GIP receptor, 466 and 493 amino acids. The receptors are expressed in islet β-cells, adipose tissue, heart, and brain. GIP glucose-dependently stimulates insulin secretion via activation of specific G protein-coupled receptors (GPCR) expressed directly on islet β-cells [44]. GPCR activation is followed by adenylyl cyclase activation, an increase in intracellular Ca^{2+} and arachidonic acid efflux. Activation of the GIP receptor also stimulates cyclic AMP formation and protein kinase A activation.

GLP-1 AND ITS RECEPTOR

GLP-1 is a product of the pro-glucagon gene and is mainly expressed in mucosal L-cells located in the distal intestine (ileum and colon) [45, 46]. GLP-1 is expressed in pancreatic alpha cells, as well as in neurons from several brain areas (hypothalamus, pituitary, nucleus of the tractus solitarius, reticular nucleus) [23]. GLP-1 secretion is stimulated by intake of carbohydrates and fat [47, 48]. The secretion is glucose-dependent [49, 50]. GLP-1 exists in two bioactive forms, GLP-1(7-37) and the most common GLP-1(7-36 amide) [44, 51]. The peptides are equipotent, with an equal plasma half-life and identical biological activity through activation of the same receptor. Plasma levels of GLP-1, similar to GIP, elevate rapidly after food intake. GLP-1 concentration peaks within 30 min after a meal [52]. The rapid response is probably caused by vagus nerve activation [35]. The peptide has a half-life of a few min [53], due to effective cleavage by the enzyme DPP IV.

The GLP-1 receptor (GLP-1R) is a 463 amino acid receptor and has been found in pancreatic islets, kidney, lung, heart, and multiple regions of the peripheral and central nervous system. GLP-1 acts on the β-cell by binding to the specific, seven-transmembrane receptor [54]. Activation of this G-coupled receptor causes an increase in intracellular cAMP concentration [55] and activation of protein kinase A (PKA). GLP-1 acts directly through the cAMP/PKA pathway to enhance and sensitize β-cells resulting in glucose-stimulated insulin secretion (fig 2).

Fig 2. Model of the proposed ion channels and signal transduction pathways in a pancreatic ß-cell involved in the mechanisms of insulin secretion in response to glucose and GLP-1. The key elements of the model are the requirement of dual inputs of the glucose-glycolysis signaling pathway resulting in the generation of ATP and an increase in the ATP:ADP ratio, and the GLP-1 receptor (GLP-1R)-mediated cAMP PKA pathways to effect closure of ATP-sensitive potassium channels (K-ATP) consisting of the inward rectifier Kir6.2 and the sulfonylurea receptor SUR1. The closure of these channels results in a rise in the resting potential (depolarization) of the ß-cell, leading to opening of voltage-sensitive calcium channels (L-type VDCC). A major component of the depolarizing current is carried by NSCCs that import Na+ (and Ca^{2+}). In response to activation of NSCC and influx of Na$^+$ there is import of Ca^{2+} by the Na$^+$/Ca^{2+} exchanger (Na:Ca Exch). Release of intracellular membrane stores of calcium (Ca^{2+} stores) is induced by intracellular free Ca^{2+}, so called calcium-induced calcium release. The influx of Ca^{2+} through the open-end L-type VDCC triggers vesicular insulin secretion by the process of exocytosis. Phosphorylation of vesicular (granule) proteins by PKA may also trigger insulin secretion. Repolarization of the ß-cell is achieved by opening of calcium-sensitive potassium channels (Ca-K). It is believed that the GLP-1 receptor is coupled to a stimulatory G-protein (Gs) and a calcium-calmodulin-sensitive adenylate cyclase (AC).

INSULIN SECRETION AND BLOOD GLUCOSE UPTAKE

Insulin is a 51 amino acid hormone that was isolated for the first time in 1922 by Banting and Best [56]. It is synthesised as an 86 amino acid proinsulin molecule in the pancreatic β-cell [57]. After glucose stimulation, proinsulin is cleaved enzymatically and insulin and the cleaved-off chain, the C-peptide, are co-released into the circulation. Insulin secretion is strictly glucose-dependent, but several other mechanisms also stimulate or inhibit the release (table 1). No secretion is seen at blood glucose levels below (4.4-5.6 mmol/L) [57]. The secretion is bi-phasic with the first peak seen within min of a meal intake. Binding of insulin to the insulin receptor activates phospho-inositide-3-kinase (PI 3-K), via insulin receptor substrate. Downstream of PI 3-K phosphoinositide-dependent protein kinase mediates activation of protein kinase B (PKB). Activated PKB regulates glucose uptake by recruiting the glucose transporter GLUT-4 to the plasma membrane [58].

Table 1. Regulation of insulin release in humans

Stimulants	Amplifiers of glucose-induced insulin release	Inhibitors
Glucose	**1. Enteric hormones:**	**1. Neural:**
Mannose	GLP-1	alpha-adrenergic effect
Leucine	GIP	of catecholamines
Vagal stimulation	CCK	**2. Humoral:** somatostatin
Sulfonylureas	Secretin	**3**. **Drugs:**
	Gastrin	diazoxide
	2. Neural amplifiers: beta-adrenergic	phenytoin
	stimulation	vinblastine
	3. Amino acids: arginine	colchicine

INCRETINS AS THERAPY FOR DIABETES TYPE 2

In patients with diabetes type 2 there seems to be a reduced or absent incretin effect [12, 59-61]. The insulinotropic effect of GLP-1 is preserved in much greater extent than that of GIP [50]. Infusion of GLP-1 to type 2 diabetes patients can normalize fasting [62-65] and postprandial [63, 66] blood glucose. Short-term studies with repeated daily injections [67] or continuous subcutaneous administration of GLP-1 to type 2 diabetes patients has shown consistent blood glucose lowering, but the rapid degradation of GLP-1 prevent the natural peptide being used as therapy. Instead there has been centered focus on finding a degradation resistant GLP-1 receptor agonist or increasing endogenous GLP-1 by inhibition of DPP-IV.

One interesting synthetic GLP-1R agonist is exenatide (exendin-4). Exendin-4 was originally isolated from the venom of a lizard, *Heloderma suspectum* [68]. It is encoded by a distinct gene not present in the human genome [69]. Exendin-4 exhibits 53% amino acid identity relative to the GLP-1 sequence, and is resistant to DPP-IV cleavage. Moreover, there is liraglutide, an acylated human GLP-1 analogue that has completed phase 2 clinical trials. Liraglutide binds to albumin. It can be administered once daily, and exhibits a prolonged pharmacokinetic profile [70] relative to native GLP-1 or exenatide [71, 72]. A third interesting substance is vildagliptin, an orally administered DPP-IV inhibitor [73].

GHRELIN AND ITS RECEPTORS

Ghrelin is a 28 amino acid gut peptide mainly produced by X/A-like cells in the mucosa of the gastric fundus [74-77], but ghrelin cells are found throughout the whole GI tract, most abundantly in the stomach and then gradually decreasing aborally. Ghrelin producing cells are also present in the pancreas [75-78]. The peptide was identified 1999 and found to be a growth hormone (GH) releasing peptide [79]. The named origins from "ghre", which is the Indo-European root of the word "to grow" followed by "relin" a suffix used for releasing substances. Independently of this finding, another group isolated the same peptide and published the sequence under the name motilin-related peptide [80]. The peptide exists in two forms, acylated and des-acylated. The latter is less common and believed to be biologically inactive, but recent data suggests a role in energy homeostasis and GI motility [81-84].

Ghrelin binds to a specific GPCR, with seven transmembrane domains that exist in two isoforms, growth hormone secretagogue receptor (GHS-R) 1a and 1b. The GHS-R 1a is active, whereas type 1b is inactive [85]. GHS-R exists in both the nervous system, including the arcuate and ventromedial nuclei, and in regions of the hypothalamus [85, 86] and in peripheral organs such as gut, pituitary, myocardium, spleen, pancreas and adrenals [86-89]. Ghrelin, as well as synthetic GHS, possesses strong and dose-related GH-releasing effects, but has been found to have a wide range of biological activities, including stimulation of somatostatin, promotion slow-wave sleep [90] in humans, stimulating appetite and food intake [91, 77], stimulation of adipogenesis and protection against cardiovascular damage [93]. Ghrelin has also been reported to stimulate gastric motility [93, 94] and acid secretion [95]. This effect is blocked by pre-treatment with atropine or bilateral cervical vagotomy [93, 96] indicating that the motor effect of ghrelin is mediated via vagal mechanisms. On the other hand, ghrelin has recently been shown to accelerate gastric emptying in patients suffering from diabetic gastroparesis [97, 98], a complication due to autonomic neuropathy. These findings point towards non-vagal pathways of action. This is further supported by identification of both ghrelin and ghrelin receptors in the myenteric plexus of the enteric nervous system (ENS) [99].

GASTROINTESTINAL MOTILITY

Anatomy of the stomach and the small intestine

The human stomach is divided into two functionally different regions, the proximal (fundus) and distal part (antrum), divided by a "midgastric transverse band" [100, 101]. The distal part of the stomach and the proximal part of the small intestine are separated by the pyloric sphincter. The small intestine is approximately 4-6 m long and divided in three segments, duodenum, jejunum and ileum.

The GI tract consists of two muscle layers, the longitudinal and the circular, which are built up by smooth muscle cells with the myenteric plexus in between. The circular layer is mainly mediates contractility, while the longitudinal layer controls transit. Efferent neurons innervating smooth muscle cells in the gut wall constitutes the ENS and controls the motor and secretory actions of the digestive channel [99].

Slow-waves

The basal myoelectrical activity, or slow waves, of the GI tract is mediated by pacemaker cells, i.g. Cajal-cells, distributed throughout the whole intestine [102]. One gastric pacemaker zone is located in the corpus of the stomach and generates slow-waves (3 waves/min) in man [103, 104] (Fig 3). The slow-waves propagate in aboral direction and diminish throughout the GI tract.
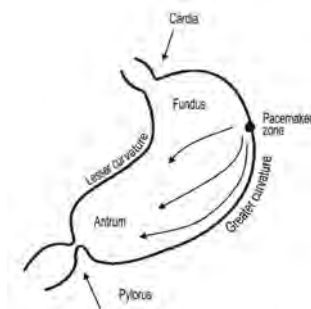

Fig 3. One gastric pacemaker zone is located at the upper part of the great curvature in the corpus of the stomach and generates slow-waves with a frequency of three waves per minute. This is the maximum frequency of gastric contractions . The frequency of the slow-waves is gradually reduced throughout the whole GI tract.

In the small intestine there is a pacemaker area located distally of the pylorus and it generates slow-waves at a frequency of 10-12 waves/min [105]. The start of a muscle contraction is initiated by an action potential caused by a slow-wave [104] and can be modulated by neuro-hormonal input (Table 2) and locally by the ENS. Contractions of the stomach and the small intestine follow two distinct patterns, the interdigestive (fasting) and the digestive (fed) motility.

Table 2. Gut hormones controlling gastric emptying and small bowel motility. Some of these peptides are also regulatory factors of insulin secretion, appetite and food intake [22, 94, 106-117].

Peptide	Gastric emptying	Peptide	Gastric emptying
Amylin	- - -	Neurotensin	++
CCK	- - -	Orexin A	+
Gastrin	- - -	Oxyntomodulin	- -
GIP	-	PP	-
GLP-1	- - -	PYY	- -
GLP-2	-	Secretin	- - -
Glucagon	- - -	Somatostatin	- - -
Ghrelin	+++	Tachykinins	+ +
Glicentin	- -	VIP	- - -
Motilin	+++		

Fasting and fed motility

At the end of food intake a cyclic pattern of motor activity occurs, which migrates from the distal part of the stomach towards the ileum. This pattern, the so-called migrating motor complex (MMC), was first observed in dogs and later in other species, including man [118-122]. The MMC consists of a re-cycling pattern that is divided into three parts; phase I, a period of quiescence when no spikes are seen, phase II with sporadic spiking and phase III (the "activity front") when the intestine contracts at its maximal frequency [118, 121, 123]. Eating disrupts the MMC and the pattern is changed to random contractions, similar to phase II (fig 4)

Fig 4. The migrating motor complex (MMC). During fed state the small intestine contracts randomly, which is interrupted by food intake, and the cyclic pattern of the interdigestive phase occurs. The interdigestive phase is characterised by three distinct patterns; phase I, a period of quiescence when no spikes are seen, phase II with sporadic spikings and phase III, which is the "front" or MMC when the intestine contracts at its maximal frequency.

GASTRIC EMPTYING

The human stomach has three major functions. First, during food intake the tension of the stomach increases, and as a response to this the fundus and proximal corpus relax to retain food. The relaxation keeps the intraluminal pressure constant during the meal, despite the increased volume [124, 125]. The proximal adaptation to the stomach content is then followed by a tonic contraction, pushing liquids and solids to the distal part of the stomach. Second, the gastric contents are degraded in the stomach by a mixing chyme with gastric secretions. The digestion is partly carried out by digestive enzymes and partly by mechanical contraction starting in the middle of the corpus, propagating distally, and ending with contraction of the antrum [103]. The third function is to empty the gastric contents into the duodenum at a controlled rate. The rate is dependent on the resistance of the pylorus and the contraction strength of the muscle in the stomach. Gastric emptying is also controlled by neurons and hormones [108, 126]. Liquids enter the duodenum more quickly than solids. Liquids are emptied from the stomach at first order kinetics [127], with a half-emptying time of 15-20 min in man. Emptying of solids follows a bi-phasic pattern. During the first phase, the *lag-phase*, the stomach content is redistributed from the fundus. When the food is broken down to smaller particles (1-2 mm), the stomach starts to empty it in a linear fashion, the *linear emptying phase* [128, 129] (fig 5).
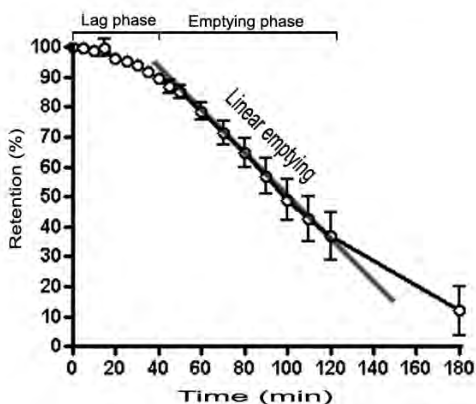

Fig 5. Emptying of solids follows a bi-phasic pattern. The first phase, which is the time it takes for the stomach to empty it-self 10 %, is the *lag-phase*. After the first phase the gastric starts to empty the contents in a linear way, called the *linear emptying phase* and continues until 90 % of the content of the stomach has been released in to the intestine.

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FOOD INTAKE AND OBESITY

GI peptides are secreted the after intake of a meal, to control gastric emptying and small bowel motility, but many of them are also potent regulators of hunger, satiety and appetite, all in concert to control the caloric intake and energy balance. The most important peptides known today to alter food intake and body weight are CCK, ghrelin, GLP-1, oxyntomodulin, PP and PYY [106]. These peptides regulate food intake both locally on the GI tract, but also centrally by signalling to the nucleus arcuatus and the nucleus tractus solitarius in the appetite regulating area in the hypothalamus [108].

AIMS

The overall aim of this study was to investigate the effect of GI hormones on smooth muscle contractility in rat and in man and to investigate in an interdisciplinary way the role and effect of gut peptides on the endocrine systems. More specifically, the aims were:

- To investigate the effects of GIP and GLP-1 on insulin secretion and small bowel motility in a rat model of diabetes type 2.

- Investigate the effect of GIP and GLP-1 on gastric emptying after intake of a mixed meal, and monitor hunger and satiety feelings, as well as blood glucose levels and insulin secretion in humans.

- Investigate the effects of ghrelin on (i) the contractility of smooth muscle strips *in vitro* as well as effects on (ii) the migrating myoelctrical complex (MMC) *in vivo*.

- To study the effect of ghrelin on gastric emptying in normal humans after intake of a mixed meal and to monitor the effects of ghrelin on hunger, satiety, and on gut hormones CCK, GLP-1, PYY, and motilin.

- To study gastric emptying in GH-deficient (GHD) patients before and after GH.

- To localize the distribution of GHS-R in different regions of the human stomach.

MATERIALS

ANIMALS

The regional animal ethics Committee in northern Stockholm approved the experiments.

Paper I
Twenty diabetic male GK-rats, bred at Karolinska University Hospital, and sixteen male Wistar rats obtained from B&K Universal (Sollentuna, Sweden) were used for perfusion of isolated pancreas. Sixteen male Wistar (B&K) and 16 male GK (Karolinska University Hospital Solna) rats were used for the MMC experiments. All animals had a similar weight, approx. 300 g, and were fed *ad libitum* with free access to water. The rats were kept under standardized conditions (temperature 22°C, humidity 60% and regulated lighting in 12-h cycles).

Paper III
A total of 37 male Sprague–Dawley rats (B&K Universal), weighing 200–300 g, were used for the two different experimental methods. The animals were kept under standard conditions as above.

HUMAN STUDIES

Subjects
All study protocols were approved by the ethics Committee of the Karolinska Institutet North, and all subjects gave written informed consent.

Paper II and *Paper IV*
Solid scintigraphic gastric emptying and plasma concentrations of the gut peptides were measured. In *paper II*, infusion of GIP (NeoMPS, Strasbourg, France) were studied in eight healthy volunteers, (5 men, 3 women, 29.3 ± 3.4 yr), with a mean body mass index (BMI) of $22.3 \pm 0.5 \text{kg/m}^2$, and infusion of GLP-1 (NeoMPS) were studied in nine different healthy volunteers, (8 men, 1 woman, 30.1 ± 3.0 yr), with a BMI of $23.6 \pm 0.8 \text{ kg/m}^2$.

In *paper IV*, ghrelin were studied in eight healthy, non-smoking volunteers (5 men, 3 women, 26.5 ± 1.6 yr), with a mean BMI of $24.0 \pm 1.1 \text{ kg/m}^2$ and in six GH deficient (GHD) patients (3 men, 3 women, 58.3 ± 3.0 yr) before and after 6 months of GH substitution therapy. All patients had GH of adult onset.

METHODS

ISOLATED PANCREAS PERFUSION

Each animal was anesthetised with an intraperitoneal injection (i.p.) of sodium thiopental (100 mg/kg, Apoteksbolaget AB, Stockholm, Sweden). The pancreas was dissected free from adjacent tissues, as previously described [130]. A cannula was inserted in the abdominal aorta to enable administration of perfusion medium, which consisted of Krebs-Ringer buffer [131]. After a 10-min basal period with glucose 3.3 mmol/L a 30-min stimulation period with glucose 16.7 mmol/L was initiated. Finally, the pancreas was perfused with glucose of 3.3 mmol/L for the last 10 min. Samples were collected and stored at -20°C for subsequent radioimmunoassay (RIA) of insulin.

ORGAN BATH

Rats were sedated by intraperitoneal (i.p.) injection of sodium–pentobarbital (50 mg/kg, Apoteksbolaget AB). The abdomen was opened and the proximal jejunum was quickly removed and placed in cold modified Krebs–Ringer buffer [93]. Segments of jejunum, 1.5 cm long, were mounted in 5-ml organ bath chambers containing continuously oxygenated (5% CO_2 : 95% O_2) modified Krebs–Ringer buffer at 37°C. Isometric tension was continuously recorded with a PowerLab recording unit and further analysed using Chart 4.1™ software (ADInstruments, Oxfordshire, UK).

In the beginning of each experiment, acetylcholine chloride (Ach 10^{-5} M) was applied to achieve a maximal contraction. All experiments were ended with a single dose of Ach (10^{-5} M) to verify the tissue vitality, and as positive control. Test substances were applied every 2 min to establish a cumulative dose–response curve followed by washing and recovery for minimum 20 min. Results were expressed as percent of maximal contraction induced by Ach.

GASTROINTESTINAL MOTILITY IN VIVO IN RATS

Small bowel motility

The animals were anaesthetised by i.p. pentobarbital (50 mg/kg, Apoteksbolaget AB) and the abdomen was opened via a midline incision. All rats were supplied with three bipolar insulated stainless steel electrodes (SS-5T, Clark Electromedical Instr., Reading, UK) implanted into the muscular wall of the small intestine, 5 (D), 10 (J1) and 15 (J2) cm distal from the pylorus. All animals were supplied with a catheter in the jugular vein for administration of test substances. Electrodes and catheter were tunnelled subcutaneously to exit at the back of the animal's neck. The animals recovered for 7 days before experiments were performed. Experiments were then carried out in conscious animals after an overnight fasting period in wire-bottomed cages with free access to water. The electrodes were connected to EEG pre-amplifiers (7P5B) operating a Grass Polygraph 7 B (Grass Instr., Quincy, MA, USA), with time constant set at 0.015 s, and the low and high cut-off frequencies at 10 and 35 Hz, respectively, and paper-speed 10 mm/min. For more details and analyses of small bowel motility see *paper I* and *III*.

In *paper I*, GIP (20, 100 or 200 pmol/kg/min or GLP-1 (1, 2, 5 or 20 pmol kg/min) were administrated and in *paper III* ghrelin (100, 400 or 1000 pmol/kg/min) was administrated. In *paper III* I also pre-treated animals with a bolus of atropine 1 mg/kg. Futher more, the effect of ghrelin infusion at 1000 pmol/kg/min was studied in vagotomised animals.

GASTRIC EMPTYING

After an overnight fast an indwelling catheter was placed in each antecubital vein in each test person for administration of substances and plasma sampling. Concomitantly with the intake of a 310 kcal omelette labelled with 12-15 MBq ^{99}mTc-macroaggregated albumin (Pulmonate plc; Amersham International, Little Chalfont, UK) and a glass of fruit punch, either saline or test substance (dissolved in 0.9% saline containing 0.1% albumin (Albumin Kabi, 200 g/L, Kabi, Stockholm, Sweden), subjected to sterile filtration, and stored at -70°C until use) was started in one of the i.v. catheters and continued for 180 min.

Anterior and posterior 1-min acquisitions were performed with the subject in standing position. Acquisitions were then obtained every 5 min during the first 50 min and thereafter every 10 min during 70 min and finally one acquisition at 180 min. Image data were collected using a gamma camera (General Electric Maxicamera 400 T, General Electric, Milwaukee, WI, USA). The following parameters were calculated: lag phase, defined as the time period from termination of the meal until 90% radioactivity remained in the stomach; gastric emptying rate, defined as percentage of radioactivity decreasing per min during the linear slope after termination of the lag phase; and half-emptying time (T_{50}), defined as the time after termination of the meal when 50% of the gastric radioactivity had been emptied. Time 0 was defined as the time of the first acquisition (10 min after beginning the meal and the infusion of peptides. For more details see Grybäck et al. [132].

VISUAL ANALOGUE SCORES

Measurements of hunger, desire to eat, fullness and prospective food consumption were assessed with 100 mm visual analogue scores (VAS) [133] at -20, 10, 30, 60, 120 and 180 min in the healthy volunteers.

BLOOD SAMPLE COLLECTION

Blood samples were collected in pre-chilled EDTA tubes every 10 min from –20 until 60 min and at 90, 120, and 180 min for measurements of plasma concentrations of gut hormones and glucose levels. Samples were centrifuged at 4°C for 10 min at 3000 rpm. Plasma was collected and stored at -20°C until analysis. Plasma glucose concentrations were measured during the experiments using a glucose oxidase method and a Glucose Analyzer (Yellow Springs Instrument Model: YSI 2300 STAT plus analyzer, Ohio, USA). Individuals displaying fasting glucose in the range of diabetes or clearly outspoken glucose intolerance were excluded from further analyses.

INSULINOGENIC INDEX

An insulinogenic index was obtained based on the results from paper II and calculated as AUC for insulin release divided by AUC for glucose levels after infusion of GIP or GLP-1 and intake of a mixed meal [134].

RIA

Insulin was analysed with an enzyme immunoassay (DAKO Insulin Kit K6219, Copenhagen, Denmark). Plasma glucose concentrations were measured during the experiments using a glucose oxidase method and a Glucose Analyzer (Yellow Springs Instrument Model: YSI 2300 STAT plus analyzer, Ohio, USA). Individuals displaying fasting glucose in the range of diabetes or clearly outspoken glucose intolerance were excluded. Total GIP was measured using the C-terminally directed antiserum R65 [135, 136]. Intact, biologically active GIP was measured using an assay as described in [41] and [61]. The plasma concentrations of GLP-1 were measured against standards of synthetic GLP-1 (7–36) amide using antiserum which is specific for the amidated C-terminus of GLP-1 [51]. Ghrelin (total) was measured with a commercially available RIA kit (Linco Research, St. Charles, MO) [94]. RIAs of PYY in plasma were performed using antiserum code no. 8412–2II [137], which reacts equally with PYY1–36 and PYY3–36. Synthetic human PYY 1–36 (Peninsula Laboratories, St. Helens, UK) was used for standards. ^{125}I-PYY1–36 (code no. IM259) was from Amersham Biosciences (Buckinghamshire, UK) [138]. CCK was assayed using an antibody (92128) raised in rabbits against an *O*-sulfated human CCK-12 analogue [139]. Plasma concentrations of motilin were determined on EDTA plasma extracted with ethanol against standards of human motilin as previously described [140].

PCR AND RNA PREPARATION

Tissue collection

Human tissue for RNA preparation was collected during surgery, were segments from corpus and antrum were quickly removed and placed in RNAlater. Pancreatic islets were obtained from Wistar and GK-rats euthanised with CO_2. A 10 ml collagenase mixture (Sigma-Aldrich, Munich, Germany) was injected carefully into the bile duct. The expanded pancreas was cut out and put into Hanks buffer. The pancreatic tissue was incubated and washed. Small bowel tissue was obtained from animals anesthetised with i.p. pentobarbital (50 mg/kg, Apoteket AB). The tissue was surgically removed and quickly placed in RNAlater (Qiagen, Hilden, Germany).

RNA extraction

About 50 mg of each tissue was homogenised and total RNA purified using RNeasy mini-kit and RNase-free DNase set (Qiagen). The quality and concentration of the RNA was controlled by 1% agarose-gel electrophoresis and spectrophotometry (λ260:λ280).

PCR

Total RNA was reverse transcribed to cDNA using random primers and a thermal cycler (Eppendorf, Hamburg, Germany). The thermal profile and primers were selected with the software Primer-3 (Whitehead Institute, Massachusetts Institute of

Technology Centre for Genome Research, Boston, MA). Protocols, primer sequences, annealing temperature, and size of PCR products are given in *paper III* and *IV.* Detection of the PCR amplification products was carried out by size fractionation on 2% agarose gel electrophoresis.

STATISTICS

Values are expressed as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

Paper I
Mann-Whitney U-test was used for analyses of perfusion of isolated rat pancreata and non-linear regression for analysis of myoelectric activity. In order to achieve dose-response curves for calculation of pD_2 values.

Paper II and *paper IV*
Wilcoxon signed rank test for matched pairs and ANOVA for repeated measurements was used to analyse the data with time (12 time-points) and treatment (saline or active substance) as dependent factors (peptide data and gastric emptying plot). With regards to the VAS differences were evaluated as delta-values between the control and peptide infusion group.

Paper III
ANOVA followed by Tukey's multiple comparison test and the Student's t-test were used.

RESULTS

PERFUSION OF ISOLATED RAT PANCREAS

In non-diabetic Wistar rats, a glucose level of 16.7 mmol/L elicited a bi-phasic insulin response, that was potently enhanced by 10 nmol/L GIP and 10 nmol/L GLP-1 (10-fold each, $P < 0.05$) (Fig 6A and C).
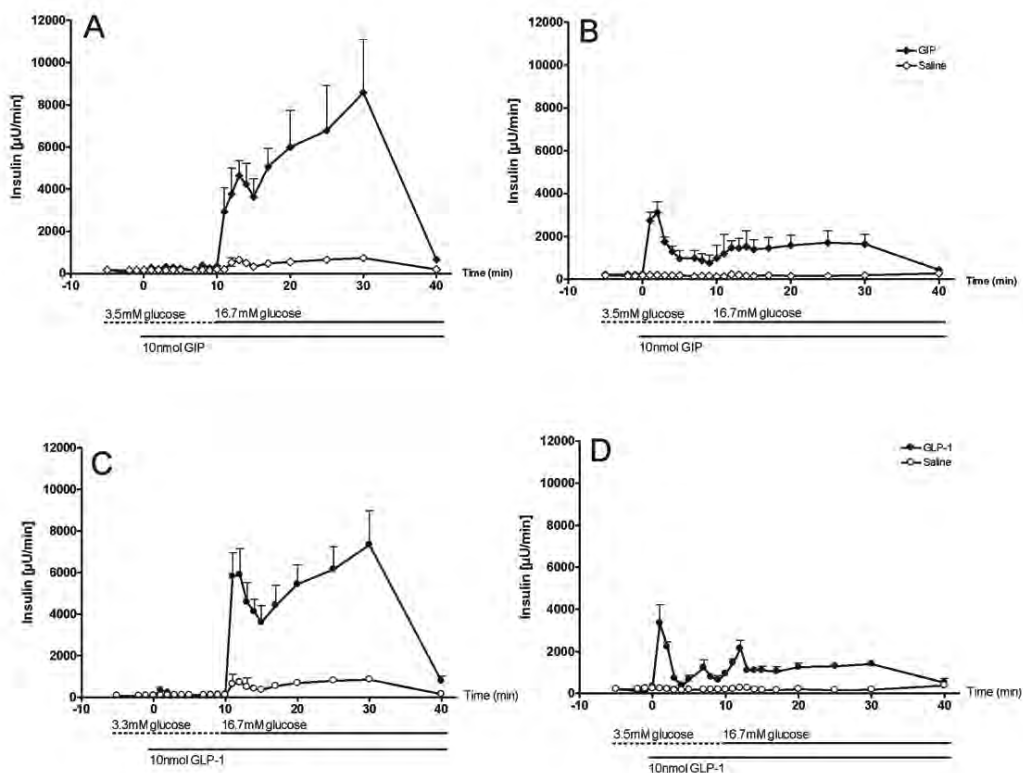
Fig 6. Effects of GIP (10 nmol/L) and GLP-1 (10 nmol/L) on insulin response to 16.7 mmol/L glucose in isolated perfused pancreas of control Wistar rat (A and C) and in diabetic GK rat (B and D). Glucose concentration was 3.3 mmol/L except where stated otherwise. Results are expressed as mean ± SE of 4-6 experiments.

Furthermore, at non-stimulatory glucose concentration (3.3 mmol/L) GIP and GLP-1 elicited peaks of insulin secretion of maximum 291 ± 100μU/min and 284 ± 49 μU/min, respectively, compared to the basal level of around 13 μU/min. In GK rats both peptides elicited marked insulin release at 3.3 mmol/L glucose with maximum peaks of 3262 ± 349 for GIP and 2080 ± 312 μU/min for GLP-1. Basal insulin level was higher in GK rats (around 100 μU/min) and insulin response to 16.7 mmol/L glucose was severely impaired (Fig 6B and D). Interestingly, the potentiatory effect of both GIP and GLP-1 on glucose-induced insulin secretion was preserved in GK rat pancreas, being even more pronounced than in control animals (131- and 50- fold AUC, $P < 0.05$ and $P < 0.01$ for GIP and GLP-1, respectively).

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ORGAN BATH

Ghrelin caused concentration-dependent contractions of jejunum segments, with a pD_2 of 7.97 ± 0.47. At 10^{-6} M, ghrelin contracted the muscle strips to $97 \pm 7\%$ of the maximal contraction amplitude with Ach (fig 7). Atropine (10^{-6} M) blocked the effect of ghrelin. The ghrelin receptor antagonist (10^{-6} M) blocked the effect of ghrelin at 10^{-8} M, but had no effect on the higher ghrelin concentrations. The contractile response to ghrelin at 10^{-8} was $40.6 \pm 9.0\%$ of maximal Ach response, while after additional administration of the antagonist the contractile response was $12.1 \pm 2.8\%$, $P < 0.05$.
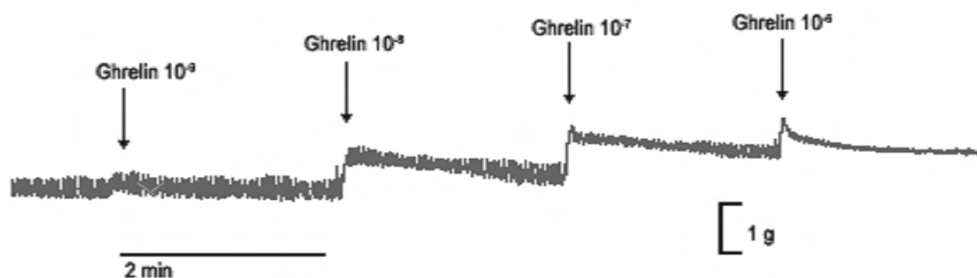
Fig 7. Recordings from the organ bath showing the effect of ghrelin at 10^{-9} to 10^{-6} M on isometric contractions of a segment of rat jejunum.

GASTROINTESTINAL MOTILITY IN VIVO

GIP and GLP-1

In normal Wistar rats, the administration of GIP decreased the time occupied by phase III of MMC in proportion to the dose, at all three recording points. GLP-1 had a similarly inhibiting effect on the MMC but was more potent. No differences were seen between GK and Wistar rats. In diabetic GK rats, the administration of GIP decreased the time occupied by phase III of MMC in proportion to the dose, at all three recording points. GLP-1 had similar inhibitory effect on the MMC as GIP, but was more potent. No differences were seen between GK and Wistar rats.

Ghrelin

Ghrelin shortened the MMC cycle length at all three recording points in proportion to the dose. The cycle length in the duodenum was shortened from 17.2 ± 2.0 to 9.9 ± 0.8 min during infusion of ghrelin ($P < 0.01$). At the two jejunal sites, the MMC cycle length was shortened from 17.5 ± 2.2 to 10.5 ± 0.8 min ($P < 0.01$) and from 18.4 ± 2.5 to 10.4 ± 0.9 ($P < 0.05$).

A small, but significant, reduction of the interval was seen at all three recording sites after administration of atropine at 10^{-6}. The effect of ghrelin at 1000 pmol/kg/min was, however, blocked by atropine, and vagotomy also abolished the effect of ghrelin on the phase III interval (fig 8).
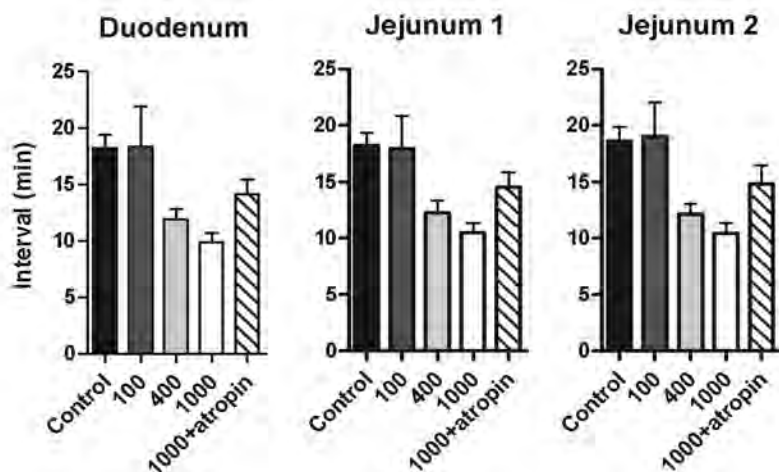
18

Fig 8. The effects of ghrelin without and with pre-treatment with atropine on the interval of phase III of the MMC. The black bar represents control (no ghrelin infusion), the dark gray bar ghrelin infusion (100 pmol kg/min), the light gray bar ghrelin infusion (400 pmol kg/min), the white bar ghrelin infusion (1000 pmol kg/min), and the striated bar represents ghrelin infusion (1000 pmol kg/min) after atropine administration (1 mg/kg). Mean ± S.E.M., n=8.

GASTRIC EMPTYING

GIP

GIP had a small but significant accelerating effect on gastric emptying ($P < 0.05$). There were no differences of the lag phase or linear emptying rate among the three treatments, but GIP at both doses decreased the half-emptying time and the meal retention after 120 min. There was an increased emptying rate of the proximal stomach with the low dose GIP whereas no effect was observed with the high dose. In the distal part of the stomach the emptying rate was decreased during infusion of the low dose of GIP, whereas the high dose increased the distal gastric emptying compared to saline.

GLP-1

GLP-1 slowed gastric emptying compared to saline ($P < 0.001$). GLP-1 markedly prolonged the lag-phase, half-emptying time and food retention at 120 min. When separating the gastric emptying into fundamental events, emptying of the proximal part was delayed, whereas emptying of the distal part of the stomach was almost blocked by GLP-1.

Ghrelin

Infusion of ghrelin resulted in a marked increase in the emptying rate compared with saline ($P < 0.001$). Both the lag phase and the T_{50} of solid gastric emptying were shorter during ghrelin infusion compared to saline (fig 9).

VISUAL ANALOGUE SCORES

GIP and GLP-1

During GIP infusion no effect on hunger, desire to eat, satiety or prospective consumption was observed compared to saline. GLP-1 infusion decreased hunger, desire to eat and prospective consumption at t = 180 min compared to saline ($P < 0.05$) with a corresponding trend towards increased satiety.

Ghrelin

Hunger and desire to eat were significantly increased and fullness ratings lower during ghrelin infusion compared with saline ($P < 0.05$, for all). Prospective food consumption tended to increase ($P = 0.06$).

Correlation between gastric emptying and appetite

A linear correlation between gastric emptying and satiety and hunger was observed in all studies of gastric emptying after saline infusion ($r = 0.13 \pm 0.02$ and -0.25 ± 0.04, respectively)(fig 10). After infusion of ghrelin there was also a linear correlation between gastric emptying and satiety and hunger ($r = 0.18 \pm 0.04$ and -0.21 ± 0.04, respectively). After infusion of GLP-1 there was still a linear correlation between gastric emptying and satiety ($r = 0.36 \pm 0.05$), but not between gastric emptying and hunger, indicating a greater importance of gastric fullness for perception of satiety than for hunger.
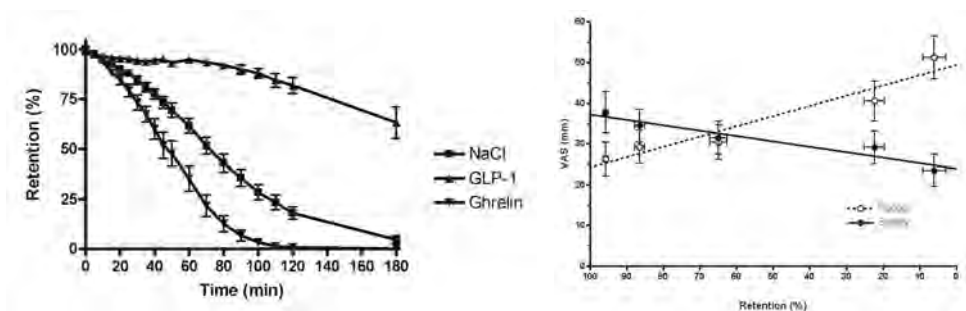
Fig 9. THe gastric emptying profile as stimulated by ghrelin (10 pmol/kg/min intravenuously, with a mean of 49 min, compared to to controls (NaCl), with a half-emptying time of 76 min, and GLP-1 (1.2 pmol/kg/min), displaying a half-emptying time of 217 min.

Fig 10. Scoring of hunger and satiety measured by a visual analogue score (VAS) plotted against gastric emptying rate in normal subjects given i.v. infusion of saline. Dotted line represents hunger and solid line represents satiety.

BLOOD GLUCOSE AND INSULIN RESPONSES

Blood glucose

GIP infusion at 2 pmol/kg/min did not change glucose plasma levels compared to saline after meal taking. 5 pmol/kg/min on the other hand, inhibited the plasma glucose rise after the meal ($P < 0.001$).

GLP-1 infusion blunted the plasma glucose rise 30 min after meal intake and stayed low throughout the study.

Ghrelin increased gastric emptying followed by slightly elevated glucose and insulin levels for the first 60 min. After 60 min, the glucose level rose and stayed elevated throughout the ghrelin infusion.

Insulin
GIP infusion at 2 pmol/kg/min did not alter insulin plasma levels compared to saline. During 5 pmol/kg/min, on the other hand, insulin secretion was smaller compared to saline ($P < 0.05$), but when comparing the insulinogenic index, GIP 5 pmol/kg/min displayed a greater release (0.16 ± 0.06 mmol x min/L) than both GIP 2 pmol/kg/min (0.10 ± 0.01 mmol x min/L) and saline (0.08 ± 0.01 mmol x min/L).

In the GLP-1 group, subjects displayed a markedly reduced insulin release as compared to control conditions. The insulinogenic index tended to be greater after infusion of GLP-1 (0.28 ± 0.12 mmol x min/L), compared to saline (0.08 ± 0.02 mmol x min/L) but not significant ($P = 0.08$).
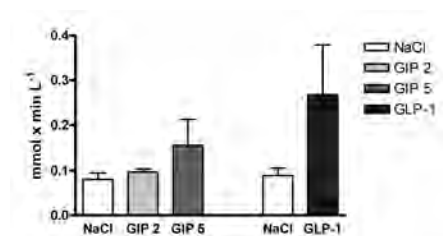
Fig 11. Insulinogenic index. Both GIP and GLP-1 showed a trend towards increased insulinogenic index after intake of a mixed meal.

Hormone levels
Infusions of GIP at low and high doses increased the total GIP plasma levels of the subjects, whereas plasma concentrations of GLP-1 and glucagon showed no differences.

Infusion of GLP-1 increased GLP-1 plasma levels of the subjects, lowered plasma glucagon and C-peptide, but had no effect on PYY or ghrelin.

Infusion of ghrelin to subjucts increased plasma concentrations 5-fold from a preinfusion concentration of 300-1500 pmol/liter. Plasma concentrations of GLP-1 and CCK increased more rapidly after intake of the solid meal, and there was a greater total amount of each peptide secreted in the postprandial period during ghrelin infusion. In contrast, motilin were not changed significantly by ghrelin infusion.

PCR
Expression of GIP and GLP-1 receptor in pancreas and small bowel in rat
RNA expression of the GIP receptor gene, as well as the GLP-1 receptor gene, was found in duodenum, gastric fundus, jejunum and in pancreatic islets in both the GK and the Wistar rat.

Expression of ghrelin and GHS receptor in human gut
Expression of the ghrelin gene, GHS-1a and GHS-1b was found in both the antrum and corpus.

GENERAL DISCUSSION

GIP AND GLP-1: FROM RAT TO MAN

I investigated the role of gut peptides on GI motility and the endocrine regulation in rat and in man.

In isolated perfused pancreata from euglycemic Wistar rats an increase of glucose concentration in the perfusate from euglycemia to hyperglycemia induced a marked biphasic insulin response. In agreement with a study by Abdel-Halim et al [131] basal insulin secretion was increased while insulin response to glucose was severely impaired in the GK rat. GIP and GLP-1 enhanced insulin in response to hyperglycemia as much as 10-fold in Wistar rat, which reflects the established incretin function of these hormones. The novel finding is that both GIP and GLP-1 exerted a pronounced potentiation of glucose-induced insulin release in isolated pancreata of GK rats. Thus, in the GK rat, the insulinotropic effect of GIP and GLP-1 was preserved.

When investigating the small bowel motility *in vivo*, both GIP and GLP-1 inhibited the MMC in proportion to the dose administered. GLP-1 was almost 10 times more potent than GIP. The effect was similar in diabetic and non-diabetic animals. The different potency of the two incretins finds support in previous observations in man, where GIP at 2 pmol/kg/min showed negligible effect on gastric emptying, whereas GLP-1 inhibited gastric emptying at 0.3 pmol/kg/min [141, 142].

After investigation *in vitro* and *in vivo* I continued to study the effect of GIP and GLP-1 on motility and metabolic control in man. In the rat, the two incretin hormones had similar effects on perfused isolated pancreata and on small bowel motility, even thought their potency differed, but the results in rat did not mirror in man.

Most studies fail to show insulinotropic effects of GIP in man [142], suggesting that GIP is not a true incretin. My study confirms these observations, as I found that a low dose of GIP did not change insulin secretion, but lowered plasma glucose, whereas a high dose of GIP blunted the postprandial plasma insulin and glucose responses. Unlike previous studies, GIP showed a dose-dependent, increasing trend for the insulinogenic index [134], reflecting an insulinotropic effect of GIP in healthy volunteers.

In my experiments, GIP had an inhibitory effect on small bowel motility in rat, whereas in man GIP increased gastric emptying. At first the stimulated gastric emptying was surprising considering the results *in vivo*. One previous study on gastric emptying in man after a mixed meal however show similar results [142]. This finding could be detected because previous studies of GIP have been performed with an oral glucose load [10] whereas only a few studies have been done with GIP and a mixed meal.

The different effects between rat and man can partly be explained by different experimental design. In the rat the standard procedure is to infuse GIP after overnight fasting, whereas in man the infusion started at initiation of a meal. Thus, comparing interdigestive motility response to digestive. GIP release is stimulated by

carbohydrates and proteins [16-18], indicating that the experimental protocol used in man is more physiological than in rat. GIP did not affect appetite, even thought it slightly increased the gastric emptying.

Infusion of GLP-1 in man blunted the plasma glucose rise after meal intake and kept it low throughout the study period. But in spite of the low blood glucose, a small peak of insulin release after meal was still seen. When comparing the insulinogenic index, GLP-1 infusion elicited a higher index compared to saline, indicating an insulinotropic effect induced by the incretin, even in the absence of any postprandial glucose rise.

Since GLP-1 have an determined inhibitory effect on gastric emptying the true incretin effect of GLP-1 has lately been questioned, and a new concept, "physiological incretin effect" where the inhibitory action on gastric emptying is excluded, has been suggested [143]. Even though the insulin response after the intake of a meal was low in my experiments, the insulinogenic index was elevated, indicating an insulinotropic effect of GLP-1 in man, independent of the strong inhibition of gastric emptying. The existence of an independent insulinotropic effect of GLP-1 after a mixed meal has further been verified by a recent study [144]. In that study, healthy volunteers were given erythromycin in order to compensate for the inhibited gastric emptying.

The gastric emptying rate is thought to be correlated to hunger and satiety. In placebo experiments I was able to show correlation between gastric emptying and both satiety and hunger. The relationship was further verified by the fact that GLP-1 via decreased gastric emptying also decreased hunger, desire to eat and prospective consumption and to some extent increased satiety. It is difficult to distinguish if the effect of GLP-1 on appetite is a central effect or caused by the inhibition of gastric emptying. If there is an independent central or peripheral role for GLP-1 on appetite control, this will of course make GLP-1 an interesting tool, not only for diabetes type 2 and diabetes type 1, but also as a therapy for obesity.

GHRELIN: FROM RAT TO MAN

In this study I investigated the effect of ghrelin on rat smooth muscle contractility and on gut motility in both rat and in man. I also looked into the effects of ghrelin on appetite and endocrine regulation.

In *in vitro* experiments on rat, ghrelin dose-dependently contracted smooth muscle strips from both the fundus and the small intestine. The effect could be blocked by pre-treatment with Ach. *In vivo*, ghrelin increased small intestine motility by decreasing the intervals between MMC. The *in vivo* effect was practically abolished by pre-treatment with atropine. Ghrelin had no effect in vagotomised animals. These findings together indicate the that effect of ghrelin is centrally regulated by the vagus nerve, but regulated locally by cholinergic neurons.

In man, all subjects exhibited a clear increase in gastric emptying rate when given ghrelin i.v. Similar findings have been made in experimental animals [77, 145, 146]. Ghrelin increased gastric emptying followed by slightly elevated glucose and insulin levels for the first 60 min. After 60 min, the glucose level rose and stayed elevated throughout the ghrelin infusion.

It is well documented, that ghrelin is a GH secretagogue [74, 79, 147, 148]. Thus, one possible mechanism by which ghrelin could influence gastric emptying is by altering GH concentrations. To test this hypothesis, I studied gastric emptying in GHD patients before or after GH substitution therapy. No significant effect was seen on any parameter of gastric emptying before and after substitution or when compared with healthy volunteers, indicating that the ghrelin effect on gastric emptying is most likely not mediated by GH.

The increased gastric emptying rate in human was associated with increased hunger and desire to eat and decreased satiety after ghrelin infusion. Prospective food consumption also tended to increase.

Taken together, increased gastric emptying induced by ghrelin in man is most likely direct and does not seem to be mediated via GH or motilin. CCK and GLP-1 is elevated postprandially, but is possibly a consequence of the enhanced gastric emptying rate, and not induced by ghrelin *per se*.

CLINICAL IMPORTANCE

Gastric emptying is a complicated process where the emptying rate is carefully regulated by blood sugar levels as well as enteric nervous signalling and gastrointestinal peptide hormones. Of the different hormones involved in regulation of gastric emptying, ghrelin seems to be one of the most important peptides signalling hunger and stimulating gastric motor activity. GLP-1, on the other hand, is released after food intake to promote satiety and inhibit gastric emptying rate.

GLP-1 has for a long time been a hot candidate for treatment of diabetes type 2 , and the GLP-1R agonist exenatide is now commercially available. Treatment with exenatide has been associated with a reduction in hemoglobin A1c levels and weight loss but also with nausea and vomiting [149]. The side effects are most likely caused by the inhibition of gastric emptying that occurs already at 0.3 pmol/kg/min [141]. GIP has limited or no effect on gastric emptying. The insulinotropic response in diabetes type 2 patients to GLP-1 is preserved to much greater extent than that of GIP [50], but most previous studies have used GIP at doses approximately at 2 pmol/kg/min. The findings in my studies indicate that GIP has a preserved insulinotropic effect in the diabetic GK rat and in man at high dose, 5 pmol/kg/min, but is not associated with inhibited gastric emptying as seen with GLP-1 treatment. This makes GIP analogues or GIP receptor agonists interesting candidates for treatment of diabetes type 2.

Another clinical aspect of GI hormones is treatment of motility disorders and for decreased appetite. Ghrelin is known to stimulate appetite in cancer patients with anorexia [150] and it accelerates gastric emptying in patients suffering from diabetic gastroparesis [97, 98]. Gastroparesis, a disorder where the stomach takes too long to empty its contents, is seen in at least 20 % of diabetes type 1 patients, and can also occur in patients with diabetes type 2 [151]. Symptoms of gastroparesis include early fullness, nausea, vomiting, and weight loss. Gastroparesis makes gastric emptying unpredictable, which can cause erratic blood glucose levels that are difficult to control [152]. When food that has been delayed in the stomach finally enters the small intestine and is absorbed, blood glucose levels suddenly rise, unbalanced by insulin.

A different motility disorder associated with diabetic disease is diabetic diarrhoea (DD) [153]. Typically it occurs in patients with a long history (> 8 yr) of diabetes and insulin treatment [154]. Peripheral neuropathy is present in most patients, and autonomic neuropathy is common [155]. No studies have been done to study the effects on GLP-1 on both insulinotropic effect in diabetic patients and improvement of DD or decreased abdominal pain and discomfort. This group of patients may benefit from either GLP-1 or GLP-1 analogue therapy.

SUMMARY AND CONCLUSIONS

In this thesis I have showed that GIP and GLP-1 stimulated insulin secretion in normal rats. The basal insulin level was higher in diabetic rats and insulin response to hyperglycemic glucose stimulation was severely impaired, but the potentiatory effect of both GIP and GLP-1 on glucose-induced insulin secretion was preserved in diabetic animals, being even more pronounced than in control animals.

Diabetic GK rats and normal controls had a similar small bowel inhibitory response to infusion of GIP and GLP-1, indicating a preserved incretin-mediated inhibitory effect in the gastrointestinal tract in the GK rat. This finding suggests the presence of a functional GIP receptor in the GK rat, in contrast to what is seen in Diabetic Fatty Zucker rats.

GIP had a small accelerating effect of gastric emptying in man, whereas GLP-1 potently inhibited gastric emptying. The low dose of GIP infusion did not affect insulin release after a mixed meal, but slightly lowered the plasma glucose. A higher dose of GIP, clearly reduced the insulin release and abolished the plasma glucose response. GLP-1 infusion reduced insulin release and lowered the blood glucose to levels below baseline. In spite of this, both peptides showed a trend towards increased insulinogenic index. GIP did not affect appetite, while GLP-1 decreased hunger and increased satiety.

Ghrelin caused dose-dependent contractions of rat fundus and jejunum. Pre-treatment with atropine abolished the response. Ghrelin also dose-dependently shortened the MMC at all recording sights. In man, i.v. administration of ghrelin stimulated gastric emptying. This effect is likely direct and not mediated by GH or motilin. The postprandial peak in plasma concentrations of CCK and GLP-1 was increased by ghrelin, possibly as a consequence of an enhanced gastric emptying rate. Ghrelin receptor agonists may achieve a role as prokinetic agents.

I have shown that gastric emptying rate is correlated to hunger and satiety. When the gastric emptying is accelerated the correlation is maintained, but deceleration and high retention of the gastric content is only related to satiety, indicating that gastric fullness is of less importance for perception of hunger than satiety.

In conclusion, there is an insulinotropic effect of GIP and GLP-1 both in normal and diabetic rats and in man. GIP accelerates gastric emptying in man and at the same time GLP-1 inhibits emptying rate. GIP and GLP-1 display insulinotropic properties, independent of gastric emptying rate. Ghrelin stimulates motility *in vitro* and *in vivo* and accelerates gastric emptying in man. The effect is not mediated by GH. Both GLP-1 and ghrelin seem to have effects on appetite, independent of gastric emptying, which makes them interesting tools for further studies of diabetes type 2 and diabetes type 1, as well as for obesity and diabetic diarrhoea.

SUMMARY IN SWEDISH

Att upprätthålla kontroll över sin metabolism är livsavgörande för den enskilda individen och sker via strikt reglering på flera olika nivåer. En viktig mekanism för att bibehålla stabila blodsocker nivåer över dygnet är genom att gastrointestinala (GI) peptidhormon påverkar frisättningen av insulin och kontrollerar magsäckstömningshastigheten. Målet med denna avhandling var först att undersöka vilken effekt de två inkretina hormonerna GIP (glucose-dependent insulinotropic polypeptide) och GLP-1 (glucagon-like peptide-1) har på insulinfrisättning och på tunntarms-motilitet i en djurmodell för diabetes typ 2. Därefter att undersöka vad dessa peptider har för effekt på människors magsäckstömningshastigheten, parametrar för metabolismen, samt på aptit. Utöver detta ville jag studera effekten av ghrelin på glatt muskel från råttor och på magsäckstömning efter måltid hos människor.

Effekten av GIP och GLP-1 på insulinfrisättning undersöktes i isolerade pankreas från friska och diabetiska GK-råttor. MMC (migrerande motorkomplex) är ett mått på tunntarms-motilitet och aktiviteten registrerades via elektroder inopererade i övre tunntarmens vägg. På råttor GIP och GLP-1 gavs intravenöst (i.v.). Djuren var vakna under hela försöket. Effekten av GIP och GLP-1 på magsäckstömning hos friska frivilliga försökspersoner mättes scintigrafiskt efter intag av en radioaktiv ^{99}Tc-märkt omelett. I samband med den scintigrafiska undersökningen graderades hunger och mättnad, samt togs blodprov för vidare analys av GI hormoner, som insulin, glukagon, PYY, GIP, GLP-1 och ghrelin. Även blodsocker mättes.

Vi har kunnat visa att både GIP och GLP-1 stimulerar frisättning av insulin i normala råttor. Basala insulin-nivåer är högre i GK-råttor och insulin utsöndringen vid hyperglykemi var kraftigt försämrad, men förmågan att potentiera insulinsekretionen vid glukosstegring var bevarad i GK-råttor och till och med högre än i kontrollgruppen. I både GK-råttan och kontrollråttan minskade tarm-motiliteten efter infusion av GIP eller GLP-1. I människor accelererade GIP magsäckstömningshastigheten något, medan GLP-1 bromsade upp den kraftigt. Båda peptiderna visade en trend mot ökad insulinogeniskt index, oberoende av magsäckstömningshastigheten. GIP hade ingen påverkan på aptiten medan GLP-1 minskade hungern och ökade mättnaden.

I organbad registrerades isotonisk kontraktion av segment från tunntarmen och magsäcken i närvaro av ghrelin. MMC studerades i friska djur efter i.v. infusion av ghrelin. I människor studerades effekten av ghrelin på magsäckstömning och aptit på samma sätt som för GIP och GLP-1. Ghrelin visade en dos-beroende kontraktion av segment från tunntarmen och magsäcken. Förbehandling med atropin förhindrade kontraktionen helt. Ghrelin ökade även hastigheten för MMC i tunntarmen i råttor. I människor ökade ghrelin magsäckstömningshastigheten och det fick till följd att försökspersonerna kände en ökad hunger och en minskad mättnad. Ghrelins effekt på magsäckstömning var oberoende av utsöndring av tillväxthormon.

Slutsatsen man kan dra av dessa studier är att GIP och GLP-1 har en ökad inneboende förmåga att öka insulinfrisättningen hos både normala och diabetiska råttor. En ny

upptäckt är även att GIP har en accelererande effekt på magsäckstömningen. Dessutom påverkar både GLP-1 och ghrelin aptiten. Detta gör dessa peptider till intressanta redskap, inte bara för att vidare studera diabetes typ 1 och typ 2, utan även för att behandla fetma, gastropares samt diabetes orsakad diarré.

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