Università degli Studi di Palermo

DIPARTIMENTO DI SCIENZE E TECNOLOGIE MOLECOLARI E BIOMOLECOLARI

DOTTORATO DI RICERCA IN

"ALIMENTAZIONE E NUTRIZIONE UMANA"

XXIII CICLO - SSD: BIO/09

GLUCAGON-LIKE PEPTIDE-1: GASTROINTESTINAL MOTILITY AND FOOD INTAKE

Dottoranda

Dott. Alessandra Rotondo

Tutor

Prof. Flavia Mulè

Coordinatore del Dottorato

Prof. Caterina Mammina

Triennio 2009/2011

INDEX

PRELIMINARY CONSIDERATIONS

Many peptides involved in the eating behaviour are found in the brain, in the enteric nervous system (ENS) and enteroendocrine cells of the gastrointestinal (GI) tract. The gut senses the presence of nutrients and signals to the brain, via neural and endocrine mechanisms, to regulate short-term appetite and satiety. After food intake, GI tract releases anorexigenic peptides, to mediate satiety; on the contrary, during fasting it releases appetite stimulating orexigenic factors. The mechanisms by which gut hormones modify feeding are object of ongoing investigations. Peptides can act as circulating hormones with direct effects in the brain or with indirect effects through afferent and efferent vagal fibres. In parallel, most of them can also affect GI motility, considered an additional component in the regulation of the feeding behavior (Konturek et al., 2005).

In fact, the successful carryout of the digestion and absorption of nutrients depends on the presence of well-coordinated GI motility, which regulates the rates at which nutrients are being processed. Gastric distension and emptying play a crucial role in the regulation of satiety and an enhanced gastric emptying has been related to overeating and obesity (Wright et al., 1983) Moreover, acceleration in intestinal transit may reduce the absorption and lead to a weight loss, whereas a delay in intestinal transit may increase the absorption and lead to a weight gain.

Glucagon-like peptide-1 (GLP-1), that is the object of this Ph.D. thesis, is included among the satiety peptides. GLP-1 is an intestinal peptide hormone, released in response to nutrient ingestion, which is receiving notable attention for its ability of increasing insulin release, reducing appetite and food intake and influencing gastric and intestinal motility. This potential makes GLP-1 an attractive tool in the fight against obesity, and several companies are developing weightlowering drugs based on GLP-1.

The present research has been addressed mainly to explore the presence and the importance of peripheral action sites in mediating the motor effects of GLP-1 in the different regions of gastrointestinal tract (stomach, small and large intestine), using an animal model (mouse). Subsequently, in the attempt to correlate gastric motor responses with the regulation of food intake, the effects of stable GLP-1 analogue, liraglutide, were examined on gastric accommodation and satiation in humans.

The results are presented in relation to the original published papers or submitted for publication, therefore methods are described within each chapter.

Specifically, the first article concerns the analysis of the effects of exogenous GLP-1 on spontaneous and evoked mechanical activity of mouse duodenal and colonic circular and longitudinal muscle. Intestinal segments were mounted in a horizontal organ bath in order to record both the intraluminal pressure (index of circular muscle mechanical activity) and the isometric tension (index of longitudinal muscle mechanical activity). Functional data were coupled with results from immunohistochemical analysis performed in collaboration with prof. Vannucchi research group (University of Florence, Italy).

The second article concerns the study of GLP-1 effects on mouse gastric tone. Endoluminal pressure from the whole stomach was recorded *in vitro* in order to study the muscle function under conditions where the influence of external factors is removed, but the muscle performs in a manner analogous to its *in vivo* capacity. Biomolecular techniques were also used to analyze the expression of GLP-1 receptor in the different gastric region (antrum and fundus).

The third article concerns the investigation about liraglutide effects on the satiation feeling and gastric accommodation in human. This study was performed at the Translational Research Centre for Gastrointestinal Disorders, Katholieke Universiteit Leuven, Belgium, under the supervision of prof. J. Tack. A high resolution manometry probe positioned in the proximal stomach was used to measure the intragastric pressure changes during a liquid meal (nutrient drink) infusion. Barostat technique was utilized to verify changes in the compliance of the proximal stomach.

Within the Introduction, it is my intention to provide an overview about the gastrointestinal signals that influence food intake and to provide a state of art about GLP-1 physiology.

PUBLICATIONS

The following publications have arisen due to work performed during my PhD candidature and are the basis of this thesis:

Journal Articles

AMATO A., CINCI L., ROTONDO A., SERIO R., FAUSSONE-PELLEGRINI MS., VANNUCCHI MG., MULÈ F. (2010). Pheriperal motor action of glucagon like peptide-1 through enteric neuronal receptors. *Neurogastroenterology & Motility*. **22**: 664-672.

ROTONDO A., AMATO A.,LENTINI L., BALDASSANO S., MULÈ F. (2011). Glucagon-like peptide-1 relaxes gastric antrum through nitric oxide in mice. *Peptides* **32**:60-64

ROTONDO A., JANSSEN P., MULÈ F., TACK J. Effect of the GLP-1 analogue liraglutide on satiation and gastric sensorimotor function during nutrient drink ingestion. *Submitted for publication* 2011.

Abstracts

MULÈ F., AMATO A., ROTONDO A., SERIO R. (2009). Effects of glucagon-like peptide 1 on intestinal mechanical activity in mouse. *Acta Physiologica, 197 (suppl 672), 86.*

ROTONDO A., AMATO A., BALDASSANO S., MULÈ F. (2009). Glucagon-like peptide-1 modulates excitatory cholinergic neurotransmission of mouse duodenum and colon. VI° National Meeting of Cellular and Developmental Biology, Palermo University.

ROTONDO A., AMATO A., BALDASSANO S., MULÈ F. (2010) Evidence for region-specific effects of glucagon-like peptide-1 in mouse stomach. *Acta Physiologica, 200 (suppl 681), 168.*

ROTONDO A, JANSSEN P, MULÈ F, TACK J. (2011). Effect of the glucagon-like peptide-1 analogue liraglutide on gastric sensorimotor function. Oral presentation "Annual Meeting of Physiology Young Researchers"

ROTONDO A., PAPATHANASOPOULOS A., VOS R., JANSSEN P., TACK J. (2011). Effect of the GLP-1 analogue liraglutide on intragastric pressure and satiation during nutrient drink infusion in healthy volunteers. Oral presentation XXIII Belgian Week of Gastroenterology.

ROTONDO A., JANSSEN P, TACK J. (2011). Effect of liraglutide on intragastric pressure and satiation during intragastric nutrient drink infusion in healthy volunteers. Appetite, 57 Suppl 1, s37.

INTRODUCTION

1. Control of food intake

Despite substantial fluctuations in daily food intake, animals maintain a remarkably stable body weight, because overall caloric ingestion and expenditure are exquisitely matched over long periods of time, through the process named energy homeostasis. Energy homeostasis is a complex physiological phenomenon that encompasses diverse processes integrated by the brain to maintain energy-relevant parameters within optimal levels given environmental conditions, thus includes the regulation of nutrient levels in key storage organs (fat in adipose tissue and glycogen in the liver and elsewhere) as well as in the blood (blood glucose) (Woods, 2005). The two key variables involved in the maintenance of body weight are energy expenditure and food intake (Fig.1).

Energy expenditure is the amount of energy to maintain a level of physical activity sufficient to actively participate in social and economic life and it is compatible with body size and composition of a good state of health in the long-term. The energy expenditure is divided into different components, the basal metabolic rate, the diet induced thermogenesis and the physical activity.

Food intake relies on a balance between hunger, satiation (the disappearance of hunger during a meal) (Blundell & Halford, 1994) and satiety (the sensation of satisfaction after a meal that gradually disappears to make way for hunger) (Strubbe & Woods, 2004). However, food intake in humans is influenced by emotional factors, social cues, and learned behaviour. These influences often can overlay highly conserved systems within the brain that sense and integrate signals to maintain energy balance (Strubbe & Woods, 2004). Choices of high fat or low fat foods, energy density of foods consumed, variety of foods accepted, palatability of the diet and variability in day-to-day intake can play a role in encouraging energy intake to exceed energy expenditure thereby creating a positive energy balance. If this persists then it will lead to weight gain.

Then, feeding behaviour is a complex process and it depends on a series of interactions between central nervous system (CNS) circuitries and peripheral signals.

Among the brain regions involved in the control of energy homeostasis, the hypothalamus is regarded as the "feeding control center" (Schwartz et al., 2000). In fact, the hypothalamus receives and integrates inputs from other areas of the brain or from peripheral organs (gastrointestinal tract, pancreas, liver and adipose tissue) and exerts homeostatic control over food intake and energy expenditure (Badman & Flier, 2005). In particular, the arcuate nucleus plays a central role in both long-term regulation of energy balance and body mass and short term adjustment of the food intake. In fact, its location, in a region that is without blood-brain barrier, allows that chemical messengers circulating affect neurons in an extremely efficient manner (Funahashi et al., 2000).

Satiety is also regulated by the hindbrain. The nucleus of solitary tract (NTS) and the area postrema, component of the dorsal vagal complex, receive inputs from vagal afferents and circulating factors, and are reciprocally connected with the hypothalamic nuclei controlling energy balance (Berthoud, 2008).

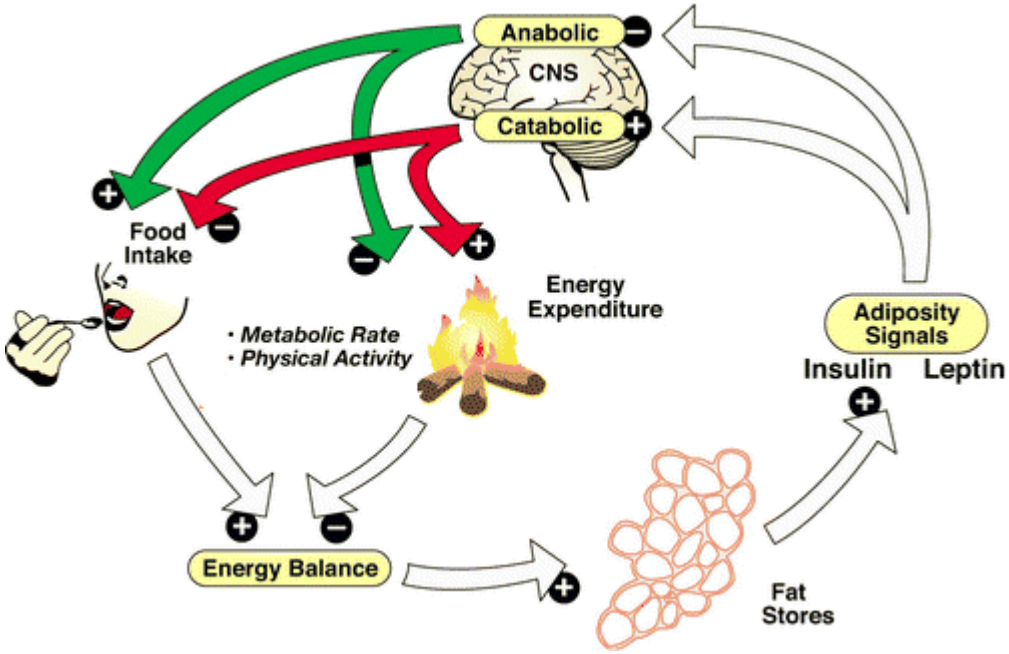


Fig. 1 *Maintenance of body weight: energy expenditure, food intake and adiposity signals*

The peripheral signals that influence food intake can be separated into: hunger signals, satiety signals or short term signals and adiposity signals or long term signals (Woods et al., 1998).

Hunger signals. Besides the gustatory system which acts in a positive feed-forward fashion, ghrelin is the first gut hormone that increases appetite. Ghrelin is an acylated peptide secreted primarily by the gastric mucosa with circulating levels at their peak just before a meal is taken and rapidly decreasing when nutrients are emptied into the duodenum (Cummings et al., 2005). It has been suggested the ghrelin is a meal initiator (Cummings et al., 2001). Beyond its proposed role in short-term feeding control, ghrelin also contributes to long-term body-weight regulation, influencing neuronal activity through its receptor in several areas of the brain governing long-term energy homeostasis, including the hypothalamus and brainstem (Cummings et al., 2005). Like many of the peptides involved in the regulation of food intake ghrelin also influences GI motility. In rats, intravenous ghrelin increases fasting motility and increases the rate of gastric emptying. There are ghrelin receptors in the gut, which most likely mediate these effects of ghrelin on gut function. These data, in addition to those demonstrating inhibitory effects of many gut peptides involved in satiety responses demonstrate a link between motor function in the gut and appetite regulation (Naslund & Hellstrom, 2007).

Satiety signals arise from the gastrointestinal tract and related organ during a meal. They influence the sensations of satiation and satiety by activating neurons in NTS in the hindbrain. Most satiety signals interact with specific receptors on peripheral nerves passing from the gastrointestinal tract to the hindbrain, especially the vagus nerves, or else circulate to the hindbrain via the blood and interact with local receptors there. Short-acting gastrointestinal signals are typified by gut hormones such as CCK and mechanical factors, such as gastric distension, which characteristically relay a sense of “fullness” resulting in postprandial satiation and meal termination (Wren & Bloom, 2007).

Adiposity signals in contrast, are hormones whose secretion is proportional to the amount of fat in the body, and include insulin from the pancreatic islets; leptin and other adipokines from adipose tissue (Fig.1). These hormones are transported from the circulation into the brain through the blood-brain barrier, and once inside the brain they interact with specific receptors on nerve cells. These long-term signals exert a tonic pressure on the expression of appetite in depending upon the nutritional status reflecting

the level of energy stores, regulating body weight and the amount of energy stored as fat over the long term (Berthoud, 2008).

1.1 Satiation and satiety signals

In addition to the obvious role of the gastrointestinal tract in the digestion and absorption of nutrients, the gut and associated visceral organs (pancreas, liver and visceral adipose tissue) have important sensing and signaling roles in the regulation of energy homeostasis. To accomplish this role, the gut uses neural and endocrine pathways to communicate with the controllers of energy balance in the hypothalamus and hindbrain.

During food intake and shortly thereafter, gastric distension and accommodation are major determinants in the regulation of satiation.

Sensations of satiation are mainly mediated by gastric mechanosensitive receptors that relay their information via vagal nerves to the nucleus of the solitary tract in the hindbrain. After food intake, when the stomach gradually empties, the role of gastric distension in the determination of appetite decreases and the regulation of satiety is shifted to gastric emptying and intestinal exposure of the nutrients. In humans, Goetze and collaborators, (2007) found that postprandial hunger and satiety were correlated to postprandial gastric volumes, without significant effects of the nutrient composition of the meal. Therefore, gastric satiety is volume-dependent. Although the stomach is able to sense some aspects of the nutrient content, this does not seem to play a role in gastric satiety (Maljaars et al., 2007).

Sensations of satiety are mainly mediated by enteroendocrine cells in the mucosa of the small intestine that sense intestinal contents and release a variety of peptides and small molecules that can act locally (and activate vagal nerves that signal to the NTS) or enter the blood stream as hormones and signal to the arcuate nucleus in the hypothalamus. Most of these hormones are anorexigenic peptides which, directly or indirectly in the hypothalamic centres inhibits the expression and release of factors stimulating the food intake (such as NPY) and then inducing satiety (Woods, 2004; Naslund & Hellstrom, 2007). Many of these gut peptides delay gastric emptying, thereby contributing to prolonged distension of the stomach, satiety and subsequently to

meal termination (Fig.2). Regional differences exist in the release if gut peptides: in the duodenum and jejunum, exposure of the gut wall to fat and protein results in the release of cholecystokinin (CCK), while in the distal small intestine, the presence of nutrients induces the release of PYY and proglucagon-derived peptides.

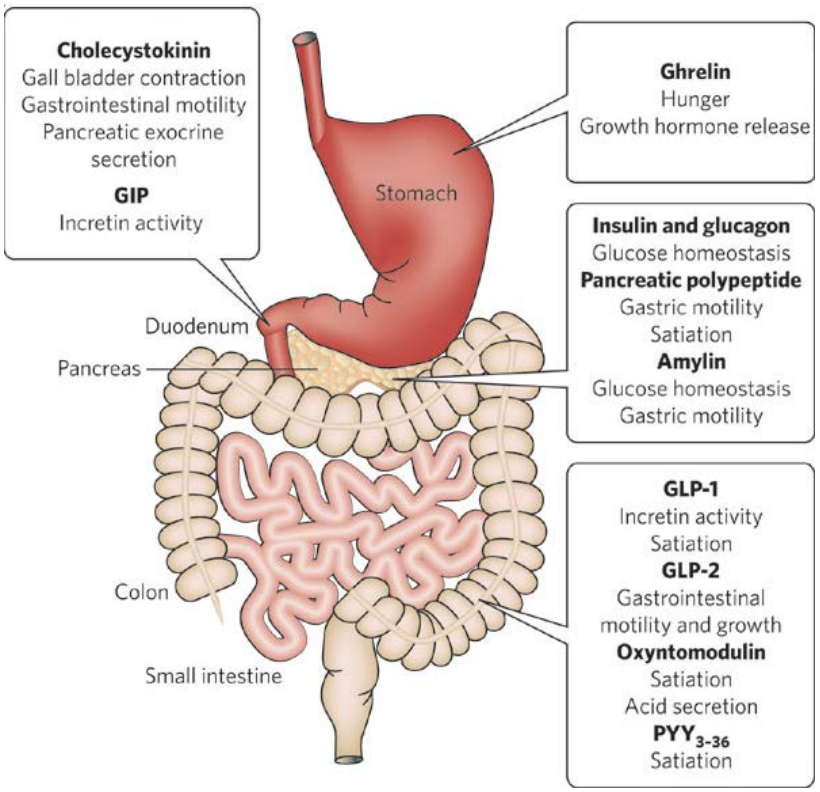


Fig. 2 *Main gastrointestinal peptides involved in control of food intake and regulation of digestive function*

CCK is the archetypal intestinal satiation peptide produced by I cells in the duodenal and jejunal mucosa, as well as in the brain and enteric nervous system. The CCK prepropeptide is processed by endoproteolytic cleavage into at least six peptides, ranging from 8 to 83 amino acids in length (Rehfeld, 2004), which interact with two receptors expressed in the gut and brain, CCK receptor 1 (CCK1R, formerly known as CCK-A, for “alimentary”) predominates in the GI system, whereas CCK2R (formerly known as CCK-B, for “brain”) predominates in the brain (Reeve et al., 2003). Through endocrine and/or neural mechanisms, CCK regulates many GI functions, including satiation. The most important role for CCK in body-weight regulation might be its synergistic interaction with long-term adiposity signals, such as leptin (Schwartz et al., 2000; Morton et al., 2006). CCK mediates the control of food delivery to the small

intestine through inhibition of gastric emptying and inhibition of food intake. The two responses can be considered together because both are likely to involve activation of a vagal afferent pathway by CCK, and because there is evidence for functional links. The gastric emptying of test meals is reduced by administration of CCK-1 receptor antagonist, suggesting this is a physiologic effect of the hormone (Fried et al., 1991). In addition there is considerable evidence that CCK activates a vago-vagal reflex leading to relaxation of the proximal stomach (Raybould & Tache 1988). Inhibition of gastric emptying and food intake may well be linked because gastric retention due to inhibition of emptying by endogenous CCK enhances sensations of fullness or satiety in healthy subjects (Lal et al., 2004). In addition reduction of food intake in humans is increased by the combination of a low dose of exogenous CCK and moderate gastric distension, suggesting synergic interactions between CCK and gastric mechanoreceptor stimulation (Kissileff et al., 2003).

PYY is produced mainly by distal intestinal L cells, most of which co-express glucagon like peptide-1 (GLP-1). It is secreted postprandially in proportion to caloric load, with a macronutrient potency of lipids being greater than that of carbohydrates, which is greater than that of proteins. The postprandial secretion is biphasic, initially stimulated by atropine-sensitive neural projections from the gut, followed by direct nutrient stimulation in the gut (Lin and Taylor, 2004). PYY1–36 is rapidly proteolyzed by dipeptidyl peptidase-4 (DPP-IV) in the bioactive form, PYY3–36. A role for PYY3–36 in satiation was asserted in a recent set of studies heralding this peptide as a promising antiobesity therapeutic. Indeed peripheral PYY3–36 administration, at doses generating physiologic postprandial blood excursions, reduces food intake and body weight in rats (Batterham et al., 2003).

Proglucagon-derived peptides include **oxyntomodulin** (OXM), glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2), which are secreted from intestinal L cells following nutrient ingestion. Exogenous administration of OXM decreases food intake and body weight in rodents and humans (Dakin et al., 2001; Dakin et al., 2005). Although the mechanisms mediating these effects are controversy, GLP-1 receptor (GLP-1R) is probably involved, since OXM does not alter feeding in GLP-1R-deficient mice (Baggio et al., 2004), and the GLP-1R antagonist exendin(9–39) blocks oxyntomodulin-induced anorexia (Dakin et al., 2001).

GLP-1 and GLP-2 exhibit an increasing number of biologically important actions (Hellstrom, 2011). GLP-1 will be treated more in detail in the section 2.

GLP-2 is considered an anorexigenic peptide because intracerebroventricular injections of GLP-2 reduce the food intake in rodents (Lovshin et al., 2001) as well as peripheral administrations of the stable analogue of GLP-2, [Gly^2]GLP-2 (Baldassano and Mulè, 2011). GLP-2 affects multiple facets of gastrointestinal physiology, including growth, absorption and motility (Wallis et al., 2007). In particular, GLP-2 has been shown to be an important intestinotrophic factor that stimulates intestinal epithelial cell proliferation and inhibits apoptosis, increases crypts and villi and enhances intestinal digestive and absorptive capacity (Wallis et al., 2007). GLP-2 inhibits the GI motility, thus providing another mechanism to increase digestion and absorption of nutrient. Specifically, GLP-2 reduces the vagally-induced antral motility in pigs (Wøjdemann et al., 1998) and it decreases the mouse gastric fundic tone leading to an increase of the stomach capacity (Amato et al., 2009). Although it is not clearly established if the GLP-2 effect on mouse gastric stomach is physiological or pharmacological, the GLP-2 action on gastric fundus seems particularly interesting, because could represent a satiety signaling which well fits with the finding that GLP-2 is a chemical mediator inhibiting rodent feeding behaviour (Tang- Christensen et al., 2001). In mouse GLP-2 inhibits the intestinal transit in vivo (McDonagh et al., 2007), and it reduces spontaneous or electrically-evoked cholinergic contractions of the small and large intestine in vitro (Amato et al., 2010b; Cinci et al., 2011). The peptide modulation on the gastrointestinal motility may be due to central nervous mechanisms (Wøjdemann et al., 1998), but involvement of the enteric nervous system has been also clearly shown through *in vitro* studies (Amato et al., 2009; Amato et al., 2010b; Cinci et al., 2011). Because GLP-2R is expressed in the subepithelial myofibroblasts (Oskov et al., 2005) and enteric nervous system as well as human enteroendocrine cells (Yusta et al., 2000), it has been proposed that the peptide exerts its actions indirectly via downstream mediators deriving from GLP-2R-expressing cells (Yusta et al., 2000). Indeed, neural VIP, nitric oxide and reduction of the acetylcholine release from enteric nerves have been reported to be involved in the inhibitory motor effects induced by GLP-2 in different regions of the mouse GI tract (Amato et al., 2009; Amato et al., 2010; Cinci et al., 2011).

1.2 Gastrointestinal motility and control of food intake

The gastrointestinal tract exerts a fundamental role in the regulation of food intake being the system in which the nutrients enter and can be absorbed to provide energy. Indeed, GI tract is the site of origin for a wide range of signals that control hunger, food intake and satiety (Cummings & Overduin, 2007). Its importance in appetite regulation has been long demonstrated because the removal of ingesta from the gut during the ingestion of a meal increases food intake (Chaudhri et al., 2006). Also the control of the gut motor function, upon eating, may play a key role in the context of regulation of satiation, satiety and feeding state (Janssen et al., 2011). In fact, gastrointestinal motility is closely linked to the rate at which nutrients become systematically available. Alterations in gastrointestinal motility have been observed in obese patients and these alterations could be contributing factors to the development and maintenance of obesity and changed eating behaviour (Xing & Chen, 2004 - Park & Camilleri, 2005) Usually, obese subjects have a larger than normal gastric capacity (Näslund & Hellström, 2007). Up to date, however there is limited evidence (Janssen et al., 2011), although the role of gastrointestinal motility and sensation becomes evident in patients. Some functional dyspeptic patients have early satiation and weight loss and that can be due to impaired gastric accommodation and hypersensitivity to distension (Tack et al., 2001). In addition, so far the attention has been focused on the potential role of gastric distension and emptying as physiological functions associated with appetite regulation (Janssen et al., 2011), while the relationship between intestinal motor function and appetite is less clear.

The GI motor activity is allowed because of the gut wall composition and properties (Fig.3). In general, the gut wall comprises two layers of smooth muscle, **circular and longitudinal layers**, between them there is a ganglionated nervous plexus, the *myenteric (or Auerbach's) plexus*. On the circular muscle layer luminal side a connective tissue with glands and a second ganglionated nerve plexus, the *submucous (or Meissner's) plexus,* represent the submucosa, under which there is the mucosa layer.

There is a remarkable variety of motor patterns, uniquely suited to each gut organ, including segmentation contractions, to mix the food, and peristaltic movements, to propulse the food in an anterograde (from mouth to anus) or retrograde (vomiting, regurgitation) direction. The motor pattern takes place through cooperation of sensory and motor nerves, smooth muscle cells, interstitial cells of Cajal (ICC), and endocrine

or paracrine factors associated with secretory glands, secretory mucosal cells, and the gut flora. Consequently, the expression of various motor patterns is generally not the consequence of independent actions of different control systems but rather, depending on specific stimuli, of varying domination of one or more of the control activities (Huizinga & Lammers, 2009).

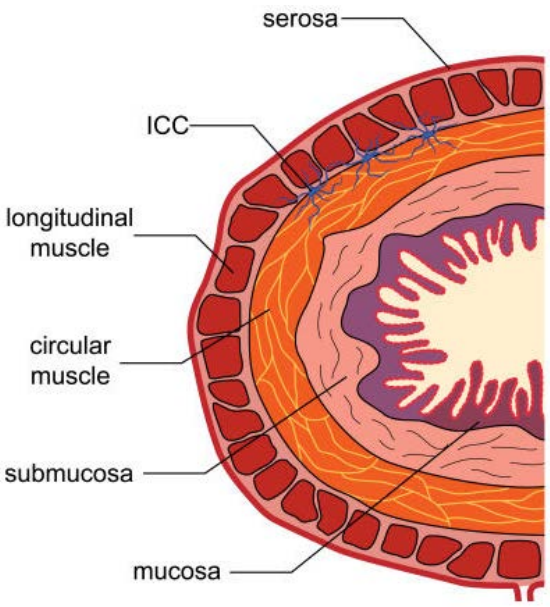


Fig. 3 *Gut wall layers.*

In the GI smooth muscular cells, cyclic changes in the membrane potential due to activation and inactivation of different ion channels or pumps represent electrical slow waves (Sanders & Ward, 1996). These rhythmic oscillations are initiated in the ICCs (Sanders & Ward, 1996), some mesenchimal cells, spindle shaped or with several processes, typically situated between muscle cells within the muscle layers or between neuronal plexuses and the muscular layers. ICCs act as pacemakers in the gut wall, by developing spontaneous slow waves, which spread passively to the smooth muscle cells (Sanders and Ward, 1996). If the amplitude of the slow wave exceeds the threshold of excitability of the cell, it generates one or more potential of action. However smooth muscle can contract even in absence of a significant variation in membrane potential, this occurs when chemical ligands, such as hormone and drugs, bind membrane receptors and induce contractions.

Beyond the myogenic intrinsic activity, the regulation of GI motility depends on neurocrine, endocrine and paracrine mechanisms (Kunze & Furness, 1999). The neural

regulation of GI motility involved both intrinsic and extrinsic neurons belonging to the autonomic nervous system (ANS). The extrinsic mechanism by which ANS controls the GI functions consists of sympathetic and parasympathetic components, while the intrinsic component is due to the enteric nervous system (ENS). The sympathetic innervation is represented by adrenergic postganglionic fibres and takes contact with intramural plexus neurons. The parasympathetic innervation is represented by the vagus nerve and pelvic nerves and are articulated with the post-synaptic neuron in intramural plexus (Olsson & Holmgren, 2001). The ENS is organized in the two major ganglionated networks, the myenteric and the submucous plexus, and also in several aganglionated plexus within the mucous and muscular layer, and underneath the serosal layer. The number, morphology, and neurochemical characteristics of these networks differ depending on localization along the gastrointestinal axis, as well as from species to species (Timmermans et al., 1997). It participates in the coordination of motor and secretory activities of the entire GI tract. The modulation of GI motility is made possible by the release from the ENS neurons of neurotransmitters and neuromodulators that may assume an excitatory or inhibitory action. Among the excitatory neurotransmitters tachykinin and acetylcholine are included, instead, inhibitor neurotransmitters are the NO, the adenosine triphosphate (ATP) and vasoactive intestinal peptide (VIP) (Bornstein et al., 2004).

Gastrointestinal motility is also regulated by the cells of the endocrine system, whose secretions are able to inhibit, stimulate or modulate, directly or indirectly, the activity of smooth muscle cells. The endocrine regulation is expressed through the production of paracrine substances and hormones (Olsson & Holmgren, 2001).

Gut motility is different in considering the nutritional state; traditionally the responses to ingestion of food are complex and have been considered in three phases highly coordinated: cephalic, gastric, and intestinal phase The *cephalic phase* (pre-ingestion phase) includes the auditory, cognitive, visual and olfactory stimuli induced by the meal which the parasympathetic outflow to trigger secretor and motor events in both the proximal and distal gastrointestinal tract. Secretory events include secretion of salivary, acid, pepsinogen, intrinsic factor, gastric and pancreatic enzymes (Cummings & Overduin, 2007). Motor events include relaxation of the sphincter of Oddi, gallbladder contraction and, most importantly, adaptive relaxation of the gastric fundus, which serves to prepare the stomach to receive food (Cuomo & Sarnelli, 2004). The

gastric phase includes the accommodation of the stomach to a meal and is represented by activation of inhibitory neurones of the gastric wall in order to relax the proximal stomach (Camilleri, 2006). Gastric accommodation is followed by gastric emptying that is different for liquid and solid meals, indeed the emptying of liquid food is driven mainly by the tone of the gastric fundus and spend approximately 20 min; emptying of solid food requires grinding or trituration through antral contractions and it is completely over in 3–4 h (Camilleri, 2006). The *intestinal phase* (post-ingestion phase) is mainly characterised by duodenal and colonic motor pattern (Xing & Chen, 2004).

In the interprandial period, intestinal motility pattern is represented in by the migrating motor complex (MMC), which comprises three phases. Phase I is a period of motor quiescence, phase II is characterised by intermittent and irregular contractions; and phase III is a period of intense, rhythmic contractions that propagate from the proximal to the distal intestine. After a meal, the fasting pattern of motility is switched to the fed pattern, which is characterised by intermittent phasic contractions of irregular amplitude similar to those of phase II of MCC (Xing & Chen, 2004; Camilleri, 2006).

In general, the stomach play an important role in the regulation of food intake, even if the mechanisms involved are only partly understood yet.

Anatomically, the stomach is divided into a fundus, corpus and antrum, but when it comes to motor function two parts can be distinguished: the proximal stomach, consisting of the fundus and the proximal part of the corpus, and the distal stomach consisting of the fundus and the proximal part of the corpus and antrum (Fig. 4). With regard to motility the proximal stomach is characterised by tonic contractions but not slow waves activity, whereas the distal stomach is characterised by slow waves activity and peristaltic contractions. During the interdigestive phase, the proximal stomach muscle tone is high, whereas the distal region is engaged in migrating motor complex. Upon food intake the motor pattern if the stomach changes: the proximal region relaxes to function initially as a reservoir. After food intake, a tonic contraction of the proximal stomach pushes the food distally, whereas the distal stomach mixes and grinds the food by powerful and regular peristaltic contractions. Therefore the stomach has three main motor functions: i) it acts as a reservoir that enables a large amount of food without a significant increase in pressure; ii) it mix and grinds food to smaller particles for further intestinal processing; iii) it generates controlled flow to the duodenum through tonic and

propulsive contractions. Each of these gastric motor functions could potentially influence appetite and food intake.

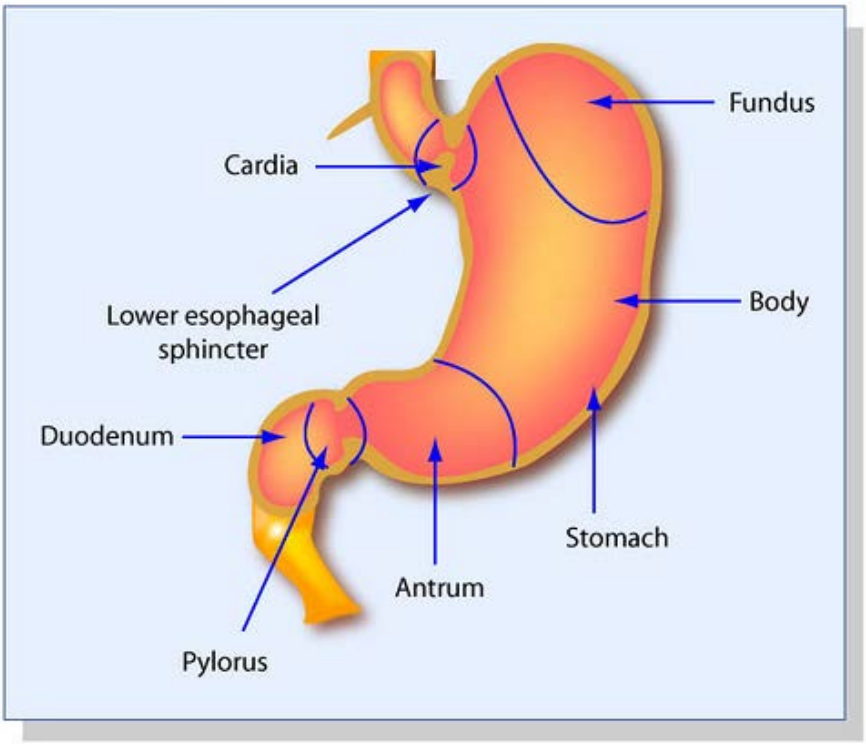


Fig.4 *Gastric regions*

Gastric distension induces a satiating effect (Janssen et al., 2011) (Lee et al., 2004), then gastric mechanosensation is a key factor in the regulation of satiation during food intake (Naslund & Hellstrom, 2007; Janssen et al., 2011). Inflated intragastric balloon has been used in the treatment for obesity and induces early satiation during the meal (Vanden Berghe et al., 2009). Gastric distension, particularly in the proximal stomach, trigger tension and stretch mechanosensitive receptors, which are relayed to the brain by vagal and spinal sensory nerves, through a complex array of neurotransmitters and neuromodulators (Park & Camilleri, 2005; Cummings & Overduin, 2007). Several evidence have shown that gastric distension-induced satiation can be also regulated by gut hormones. In fact, the satiating effect of gastric distension has been shown to be enhanced by CCK (Kissileff et al., 2003). Also central release of GLP-1 from neurons of STN, which reduces food intake in humans (Gutzwiller et al., 1999; Hayes et al., 2009) has been suggested to be involved in gastric distension-induced appetite. After food intake, when the stomach gradually empties, the role of

gastric distension in the determination of appetite decreases and the regulation of satiety is shifted to gastric emptying and intestinal exposure to nutrients.

Therefore, in addition to distension, gastric emptying is an important determinant of ingestion behaviour. Indeed, gastric emptying has been correlated to ingestion behaviour in many studies (Sturm et al., 2004). However, some studies do not find such a relationship (Carney et al., 1995; Lavin et al., 2002) either because gastric distension is more important than gastric emptying, the intestinal effects are more important, or because not the meal itself but one of its meal components is a more important determinant of gastric emptying or satiety (Janssen et al., 2011). A meal might consist of many components that behave and might be handled differently in the stomach, and consequently leave the stomach at different rates (selective emptying). In any case, gastric emptying affects not only the magnitude and duration of gastric distension, but also the rate of nutrient delivery to the intestine in which they become systemically available. Rapid gastric emptying would reduce the negative feedback satiety signal produced by the presence of nutrients inside the intestinal lumen and thus precipitate a feeling of hunger and shorten the interval between consecutive meals. On the other hand, in obese subjects a delay in gastric emptying is associated with by an increased gastric capacity which slows negative feedback produced by satiety signals (Xing & Cheng, 2004).

Differently from the gastric mechanisms, the relationship between the intestinal functional and appetite is less clear. The control of feeding behaviour by intestinal signals derive largely from the chemical effects of foods which induce release of gut peptides and neurotransmitters that induce a reduction in hunger levels and food intake (Cummings, 2007). The peptides diffuse through interstitial fluids to activate nearby extrinsic sensory fibres (vagal and spinal afferents) and⁄or enter the bloodstream to function as hormones (Park & Camilleri, 2005; Cummings, 2007). In addition, they may influence the gastric motor activity and intestinal transit rate with consequences in the absorption of nutrients which in turn influences food intake. Acceleration in intestinal transit might reduce the absorption and lead to a weight loss, whereas a delay in intestinal transit might increase the absorption and lead to a weight gain (Xing & Cheng, 2004).

Upon entry of nutrients into the small intestine, motility changes from the propagative contractions of peristalsis of the fasted state to the non-propagative pattern

that slows intestinal transit in the fed state and vice versa when fed changes to fasted state. Manometry has been used to examine various motility parameters that may be associated with ingestion behaviour (Brennan et al., 2005) but the relationships between the various aspects of gastrointestinal motility and appetite appeared variable and unclear.

The intestine is surely involved in the regulation of food intake through the "brake mechanism". The exposure of gut mucosa of the distal intestine activates a negative feedback mechanism that potently inhibits the functions of more proximal parts of the gastrointestinal tract. In particular, animal and human studies have shown that activation of the intestinal brake by local perfusion with nutrients delays gastric emptying and small intestinal transit; inhibits exocrine pancreatic secretion resulting in reduction of food intake and inhibition of hunger (Lin & Taylor, 2004; Goetze et al., 2007). Which neural or hormonal mechanisms mediate the intestinal brake is not exactly known. As the effects of an ileal infusion on pancreatic enzyme secretion could be abolished by infusion of a specific GLP-1 receptor antagonist, GLP-1 is thought to play an important role in the ileal brake (Schirra et al., 2006).

2. Glucagon-like peptide-1

GLP-1 is a gastrointestinal regulatory peptide secreted from small intestine in response to ingested nutrients (Schirra et al., 1996) and it circulates in two equally potent forms, GLP-1(7-36)amide and GLP-1(7-37), although the amidated form is more abundant after eating (Dhanvantari et al., 1996; Brubacker, 2006).

GLP-1 is co-encoded within the proglucagon gene, which, in mammals, gives rise to a single mRNA transcript that is expressed in the α-cells of the endocrine pancreas, in the enteroendocrine L-cells of the intestine, in the hypothalamus and brainstem in the CNS (Drucker & Brubacker, 1989). The proglucagon mRNA is translated into a single 160 aminoacid precursor protein that undergoes tissue-specific post-translational processing to produce several biologically active proglucagon-derived peptides (PGDPs). In particular in pancreatic α-cells, proglucagon is cleaved by prohormone convertase (PC)-2 to form glucagon, the major glucagon fragment and intervening peptide (IP)-1. In the gastrointestinal tract and in the brain processing of proglucagon, which is operated by PC1/3, results in GLP-1, GLP-2, IP2, oxynthomodulin and glicentin (Holst, 2007) (Fig.5).

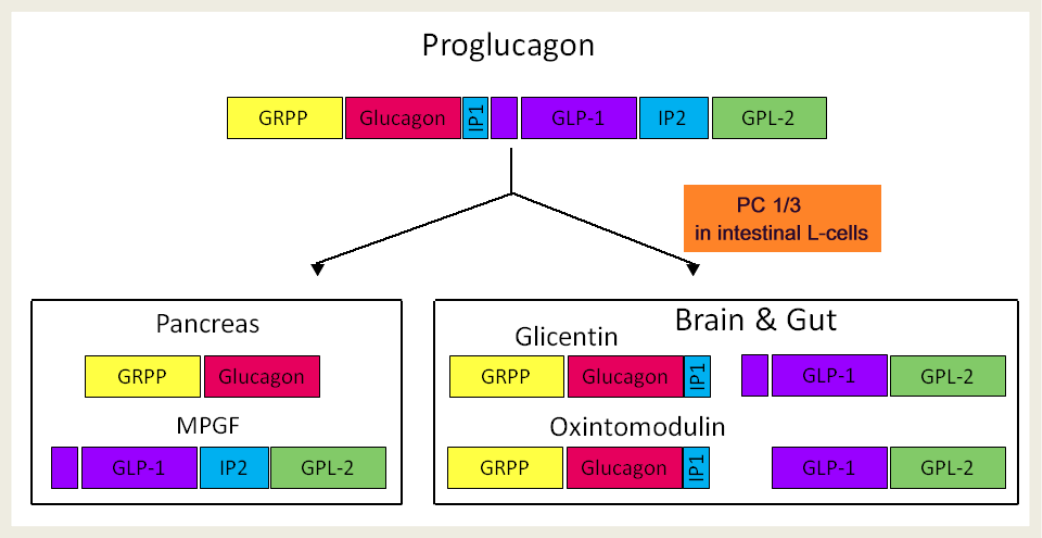


Fig. 5 *Proglucagon and proglucagon-derived peptides*

2.1 Secretion

GLP-1 is secreted by enteroendocrine L cells, most of which are located in the distal ileum and colon (Baggio & Drucker, 2007). Multiple forms of GLP-1 are secreted in vivo, including GLP-1(1-37) and GLP-1(1-36)NH_2, which are inactive, and GLP-1(7-37) and GLP-1(7-36)NH_2, which are biologically active (Dhanvantari et al., 1996).

The chief stimulus for intestinal secretion of GLP-1 is the ingestion of nutrients (Brubaker, 2006). The peptide is secreted in a biphasic pattern, with an early peak followed by a longer second phase after ingestion of nutrients. The early phase initiates within minutes of eating and may last for 30–60 min. The second phase is more prolonged, lasting 1–3 h after a meal (Hermann et al., 1995).

It is likely that the early phase of GLP-1 secretion is due to the stimulation by some neural and endocrine factors, in contrast the second or late phase is caused by direct stimulation of intestinal L-cells by digested nutrients (Baggio & Drucker, 2007). After ingestion of nutrients, plasma levels of GLP-1 increase 2- to 5-fold, depending upon the size and nutrient composition of the meal (Roberge & Brubaker, 1991). It has been supposed that the peptide diffuse across the subepithelial lamina propria to activate afferent nerves and/or to enter the circulation, thus it may act also as paracrine agents besides as endocrine hormones (Cummings & Overduin, 2007).

The mechanisms by which nutrients induce release of the peptide from the enteroendocrine cells have not been definitively clarified yet. It is known that GLP-1 release can be stimulated by mixed meals or individual nutrients including glucose and other sugars, fatty acids, essential amino acids, and dietary fiber, however intravenous glucose administration does not stimulate GLP-1 secretion in humans (Hermann et al., 1995). Because the majority of GLP-1–secreting L-cells are located in the distal small intestine, it is unlikely that the early phase of GLP-1 secretion can be mediated by direct nutrient contact with the L-cell. However, several studies have shown the autonomic nervous system involvement through stimulation by neurotransmettitor and other peptides. It has been shown that bilateral subdiaphragmatic vagotomy completely blocks fat-induced GLP-1 secretion, whereas direct electrical stimulation of the celiac branches of the vagus, that innervate the jejunum, ileum, and colon, increases GLP-1 secretion (Rocca & Brubaker, 1999). In humans, administration of atropine, a nonspecific muscarinic- receptor antagonist, diminishes oral glucose-stimulated first-phase GLP-1 secretion independently of gastric emptying (Balks et al., 1997).

GLP-1 secretion is stimulated by activation of a number of intracellular signals including protein kinase A (PKA), protein kinase C (PKC), calcium, and mitogen-activated protein kinases (MAPK). Studies using a mouse intestinal L-cell line suggest that glucose stimulates GLP-1 secretion via glucose metabolism and the adenosine triphosphate (ATP)-sensitive potassium channel closure, whereas non metabolizable sugars promote GLP-1 release via a sodium-glucose cotransporter–dependent mechanism (Gribble et al., 2003). Unsaturated long-chain free fatty acids stimulate GLP-1 secretion *via* GPR120, a G-protein coupled receptor expressed in the intestine. On the other hand, few studies have examined the factors responsible for inhibition of GLP-1 release and it has been hypothesized that insulin, somatostatin, and the neuropeptide galanin can inhibit GLP-1 secretion from intestinal L-cells *in vitro* and *in vivo* (Saïfia et al., 1998; Chisholm & Greenberg, 2002; Velasquez-Mieyer et al., 2004).

2.2 Metabolism

After release, GLP-1 is quickly degraded through cleavage of N-terminal histidine and alanine by the ubiquitously expressed DPP-IV. DPP-IV, also known as CD26, is a serine protease that is found in multiple tissues and cell types including the kidney, lung, adrenal gland, liver, intestine, spleen, testis, pancreas, and CNS. Notably, DPP-IV also is found on the surface of endothelial cells, including those lining blood vessels that drain the intestinal mucosa, which are positioned directly adjacent to the sites of GLP-1 secretion (Jiang & Zhang, 2003).

DPP-IV specifically cleaves dipeptides from the amino terminus of oligopeptides or proteins that contain an alanine or proline residue in position 2, thereby modifying or inhibiting their activity. GLP-1, which contains a penultimate alanine residue and thus is a substrate for DPP-IV, is metabolized rapidly to GLP-1 (9-37) or GLP-1 (9-36)NH_2 (Holst, 2010) (Fig.6). Neutral endopeptidase 24.11 (NEP-24.11), a membrane-bound zinc metallopeptidase, has been shown to have endoproteolytic activity on GLP-1 in vitro and up to 50% of GLP-1 entering the circulation may undergo C-terminal cleavage by NEP-24.11 (Kendall et al., 2005).

GLP-1 is extremely susceptible to the catalytic activity of the DPP-IV and NEP and it seems that the large part of the GLP-1 that leaves the gut is already degraded to the inactive metabolite and only about 25% of newly secreted GLP-1 leaves the gut in

an intact active form (Hansen et al., 2000). Then the DPP-IV activity results in an apparent half-life for intact GLP-1 in plasma of 1–2 min.

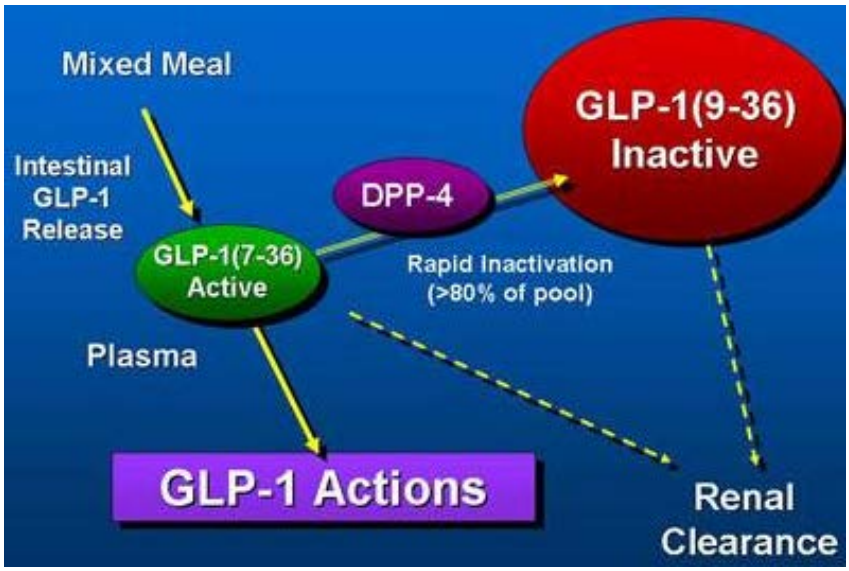


Fig. 6 *GLP-1 cleavage by DPP-4*

The kidney provides the major route of clearance for GLP-1 and patients with chronic renal insufficiency have elevated levels of circulating GLP-1 compared to healthy control individuals (Meier et al. 2004).

2.3 Biological actions of GLP-1

One of the most important functions of GLP-1 is to act as an incretin hormome, but further important effects include inhibition of gastrointestinal secretion and motility an reduction of food intake (Holst, 2007) (Fig.7).

The biological actions of the peptide are mediated by a specific receptor, the GLP-1 receptor (GLP-1R).

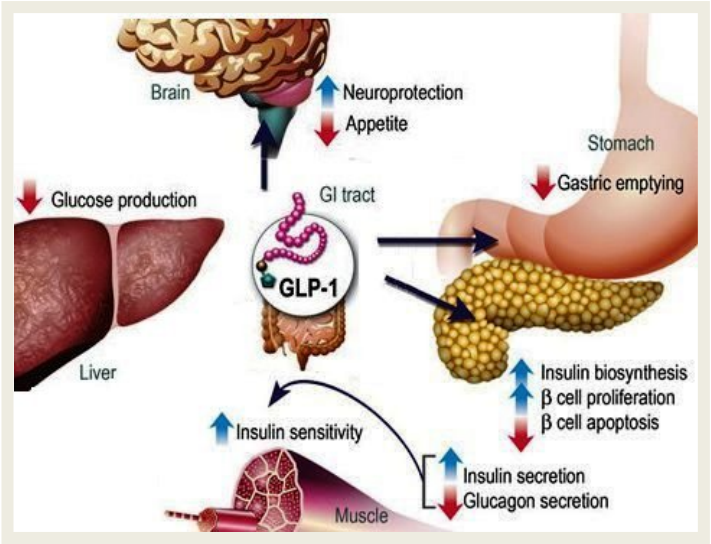


Fig.7 *GLP-1 biological effects*

GLP-1 receptor (GLP-1R)

The GLP-1 receptor (GLP-1R) belongs to the class B family of 7-transmembrane–spanning heterotrimeric G-protein–coupled receptors, which also includes receptors for glucagon, GLP-2, and GIP (Mayo et al., 2003) (Fig.8). The receptors for glucagon and for GLP-1 are highly homologous in their amino acid sequences, however they are highly specific and have no cross reactivity (Munroe et al.,1999; Osaka et al., 2005).

Rat and human GLP-1R cDNAs were cloned and sequenced in the early 1990s from their respective pancreatic islet cDNA libraries. The human GLP-1R gene spans 40 kb, consists of at least 7 exons, and has been mapped to chromosome 6, band p21.1 (Baggio & Drucker, 2007).

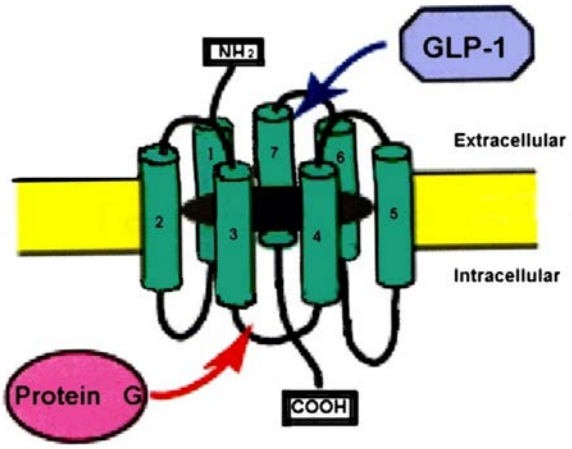


Fig.8 *GLP-1 receptor*

Although GLP-1R tipically couples via a stimulatory G protein to adenylate cyclase and activation of PKA (Holst, 2007), involvement of Gq, Gi, and Go proteins, increases in intracellular Ca^{2+}, activation of phospolipase C, PKC, phospatidylinositol-3 kinase (PI-3K), Epac2, and MAPK have been reported (Baggio & Drucker, 2007).

In rodents and humans GLP-1R is expressed in pancreatic islets, brain, heart, kidney, small and large intestine and in the stomach (Campos et al., 1994; Bullock et al., 1996; Nakagawa et al., 2004; Dunphy et al., 2008; Kumar et al., 2008; Tornehave et al., 2008). GLP-1R has been identified in several regions of central nervous system that control feeding behavior (Goke et al.,1995), as brainstem and hypothalamus and in the nodose ganglion of the vagus nerve (Bullock et al, 1996; Nakagawa et al., 2004). Although the distribution of the GLP-1R in the central nervous system and in the nodose ganglion has suggested that GLP-1 effects are exerted through the interaction with centres in the brain or afferent neural pathways (Imeryuz et al., 1997; Tolessa et al., 1998; Schirra et al., 2000; Baggio & Drucker, 2007; Kumar et al., 2008; Bucinskate et al., 2009), the mechanisms by which the GLP-1 exerts its action in gut remain poorly understood. One major question regarding the actions of the GLP-1 in the gastrointestinal tract is the relative importance of the local enteric nervous system.

GLP-1R agonists and antagonists initially contributed to clarify and to define the different biological actions of the peptide and today some of them are employed in therapy. GLP-1 mimetics, as liraglutide (Victoza, Novo Nordisk) and exendin-4, (Exenatide, Byetta) are resistant to inactivation by DPP-IV, thus prolong and enhance the effect of the hormone (Fig.9).

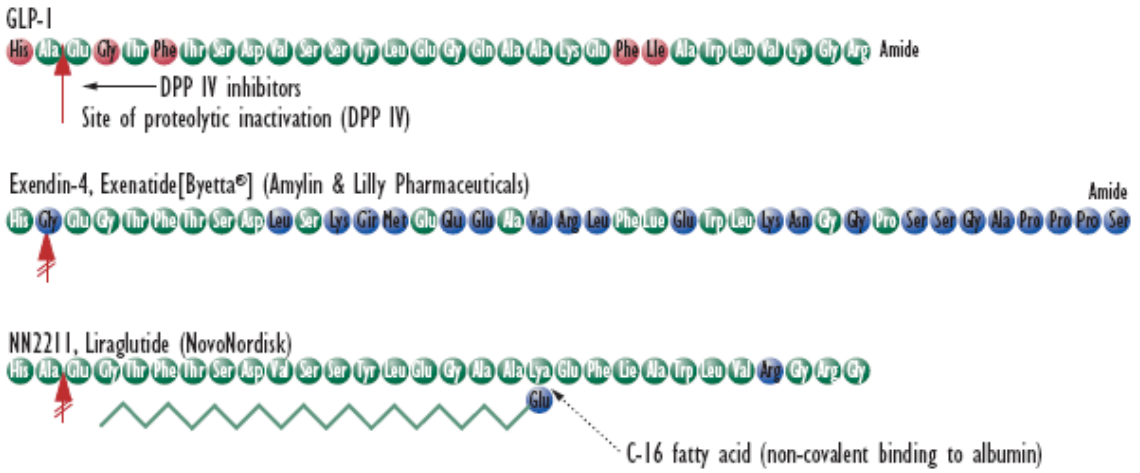


Fig. 9 *GLP-1 and GLP-1 receptor agonist, exenatide and liraglutide*

Liraglutide is a GLP-1 analogue that shares 97% sequence identity to native GLP-1. The addition of a 16-carbon fatty acid and a small amino acid based spacer confers reversible binding of the agonist to albumin and increases resistance to DPP-IV activity, providing liraglutide with a half-life of approximately 13 hours (Knudsen et al., 2000). Liraglutide was initially developed for the treatment of type 2 diabetes mellitus and has shown benefits for glycaemic control (Vilsboll et al., 2007; Garber et al., 2009; Madsbad 2009; Nauck et al., 2009). Although *in vitro* studies suggest that liraglutide binds GLP-1 receptors with similar potency as native GLP-1 (Knudsen et al., 2000), clinical data indicate that the spectrum and magnitude of actions of GLP-1 and liraglutide are not identical. These differences may be attributed to differences in concentrations but potentially also to different binding profiles (Mikhail, 2010). Several studies have suggested that daily administration of liraglutide (1.8 mg/day) is associated with weight loss (Astrup et al., 2009; Garber et al., 2009; Nauck et al., 2009; Zinman et al., 2009). However the mechanism underlying weight loss with liraglutide treatment remains to be elucidated, probably delay in gastric emptying, sensations of early satiation and a decreased sense of appetite may be implicated.

Exenatide is the synthetic version of exendin-4, a 39-amino-acid peptide found in the saliva of the lizard *Gila monster* which shares 53% sequence homology to GLP-1 (7-36) amide (Heine et al., 2005). Exenatide is an insulin secretagogue with glucoregulatory effects. Indeed, exenatide augments pancreas responses increasing insulin secretion in response to eating meals, suppresses pancreatic release of glucagon in response to eating to stop the liver from overproducing sugar when it is unneeded,

then it prevents hyperglycemia (Bunck et al., 2009). Moreover exenatide helps slow down gastric emptying and thus decreases the rate at which meal-derived glucose appears in the bloodstream and has a subtle yet prolonged effect to reduce appetite promoting satiety via hypothalamic receptors (Folli & Guardado Mendoza, 2011). Most people using exenatide slowly lose weight, and generally the greatest weight loss is achieved by people who are the most overweight at the beginning of exenatide therapy (Vergès et al., 2011). The main side effects of exenatide use are gastrointestinal in nature, including acid or sour stomach, belching, diarrhoea, heartburn, indigestion, nausea, and vomiting; exenatide is therefore not meant for people with severe gastrointestinal disease (Buse et al., 2011).

Exendin (9-39) is a derivative of the nonmammalian peptide exendin-4, exendin(9-39)amide (ex(9-39)NH_2), has been found to act as a specific and competitive antagonist of GLP-1R (Baggio & Drucker, 2007). Ex(9-39)NH_2 has been applied in animal experiments. The effect of its intracerebroventricular injection in rats has implicated that GLP-1 acts as a central regulator of satiety as well as of water and salt homeostasis (Tang-Christensen et al., 1996; Turton, et al., 1996). Intravenous application of ex(9-39)NH_2 in rats has been used to demonstrate that GLP-1 is an important enhancer of postprandial insulin release and, therefore, it functions as a true incretin hormone in this species (Kolligs et al., 1995; Wang et al., 1995). So far, ex(9-39)NH_2 was established in vitro or in animal models as a GLP-1R competitive antagonist (Holst, 2007).

Pancreatic effects

The mainly effect of GLP-1 on pancreas cells is to induce insulin exocytosis and gene transcription. The process of glucose-dependent insulin secretion begin with the binding of GLP-1 to its specific receptor on pancreatic β–cells leads to activation of adenylate cyclase and production of cAMP. Subsequently, GLP-1 stimulates insulin secretion via mechanisms that include the following:

(1) direct inhibition of ATP-sensitive potassium (KATP) channels, which leads to β-cell membrane depolarization; (2) increases in intracellular Ca^{2+} levels resulting from GLP-1–dependent influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+}channels, activation of nonselective cation channels, and mobilization of intracellular Ca^{2+} stores;

(3) increases in mitochondrial ATP synthesis, which lead to further membrane depolarization;

(4) closure of voltage-dependent K^+ (Kv) channels and consequent reductions in Kv currents, thereby preventing β-cell repolarization;

(5) direct effects on β–cell insulin storage granule exocytosis that occur distal to increases in ATP and intracellular Ca^{2+} (Baggio & Drucker, 2007)

Synergistically with glucose, GLP-1 also promotes insulin gene transcription, mRNA stability, and biosynthesis, and thus has the potential to replenish β-cell insulin stores and prevent exhaustion of β-cell reserves (Baggio & Drucker, 2007) through cAMP/PKA-dependent and -independent signaling pathways, and increasing in intracellular Ca^{2+} levels (Fehmann & Habaner, 1992).

Moreover GLP-1 acts improving the capacity of β-cells to sense and respond to glucose, thus switching from glucose-resistant β-cells to glucose sensitivity cells because of an up-regulation of the expression of glucose transporters and glucokinases (Holz et al., 1993).

GLP-1 is also able to interact with other pancreatic cells stimulating somatostatin release by direct interaction with GLP-1R on somatostatin-secreting pancreatic δ-cells (Fehmann & Habaner, 1991) and inhibiting glucagon secretion through direct binding to pancreatic α-cell GLP-1R (Heller et al., 1997) or indirectly via stimulation of insulin and/or somatostatin secretion.

The cellular mechanisms that mediate the inhibitory actions of GLP-1 on glucagon secretion are less well characterized but are believed to involve

(1) cAMP/PKAdependent closure of α-cell KATP channels, which leads to membrane depolarization and subsequent inactivation of T-type Ca^{2+} and Na^+ channels;

(2) inhibition of A-type K^+ channels, which prevents α-cell membrane repolarization;

(3) reductions in intracellular Ca^{2+} levels.

GLP-1 is also able to modulate pancreatic β cell proliferation. There is evidence that GLP-1 increases pancreatic β cell mass. This effect may occur by enhancing proliferation and inhibiting apoptosis of β cells as well as by stimulating differentiation of stem cells in the ductal epithelium (Farilla et al., 2003).

GLP-1 augments cellular integrity and overall cell survival following exposure to a range of pro-apoptotic agents as peroxides, cytokines and fatty acids both in rodents and in humans (Aaboe et al., 2008). Additionally, β-cell damage induced by exposure to reactive oxygen species, such as peroxynitrite, is reduced in the presence of GLP-1 (Tews et al., 2009). In addition GLP-1 appears to increase expression of anti-apoptotic

genes Bc1-2 and Bclxl (Buteau et al., 2004). This may be the result of nuclear factor kappa B (NF-kB)-dependent transcription of Bc12 as well as lap2 (Li et al., 2005). Interestingly, GLP-1 also appears to reduce endoplasmic reticulum stress as indicated by the overproduction of misfolded protein aggregates (Yusta et al., 2006; Tsunekawa et al., 2007). Such an action in peripheral cells could have a clear relevance to neurodegenerative disorders such as Parkinson's disease (PD), where protein misfolding may be a significant aetiological factor.

Effects on food intake

GLP-1 is an anorexigen peptide, which inhibits food and drink intake upon intracerebroventricular injection in animal models and after intravenous infusion in normal weight and obese humans via direct modulation of the brain centres that control food intake such as the nucleus tractus solitarii and hypothalamus (Tang-Christensen et al., 1996; Turton et al., 1996; Flint et al., 1998; Naslund et al., 1999; Verdich et al., 2001; Gutzwiller et al., 2006). This effect might be also mediated through its incretin properties and/or via inhibition of gastric emptying (Flint et al., 1998; Gutzwiller et al., 1999; Van Dijk & Thiele, 1999; Larsen et al., 2001).

The anorectic effect of GLP-1 appears to be mediated by the paraventricular nucleus, as direct injections of GLP-1 into this nucleus cause anorexia without concomitant taste aversion, suggesting a specific action upon neuronal circuits involved in the regulation of feeding (Baumgartner et al., 2010). Moreover the GLP-1- receptor antagonist, exendin (9-39) stimulates feeding in satiated animals, and daily administration augmented food intake and body weight gain. In addition the anorectic effects of GLP-1 may be mediated through NPY signalling because GLP-1 inhibits and exendin 9-39 augments NPY-induced feeding (Turton et al., 1996). The GLP-1 receptor antagonist also blocks the leptin-induced inhibition of food intake and body weight, indicating that the GLP-1 pathway may be one of the targets for the anorectic effects of leptin (Goldstone et al., 1997). Stimulation of peripheral GLP-1R delays stomach emptying, trigger the end of feeding, and cause satiety through the vagus nerve. Indeed vagotomy reduces the anorectic effect of GLP-1, thus indicating the significance of this anorectic signal transmitted to the hypothalamus (Abbott et al., 2005). In rats, subdiaphragmatic bilateral vagotomy or surgical transection of the brainstem-hypothalamic pathway precludes peripheral GLP-1–induced anorexia and blocks

neuronal activation of hypothalamic feeding circuits (Abbott et al., 2005). Likewise, systemic treatment with capsaicin, to selectively ablate nodose ganglionic neurons and the vagus nerve, completely blocks the anorectic effect of peripherally administered exendin-4 in mice (Talsania et al., 2005), then the vagus is required for peripheral GLP1-induced anorexia, which is abolished by vagal transection or deafferentation (Abbott et al., 2005; Talsania et al., 2005).

Importantly, both of liraglutide and exentide induce lose weight in healthy volunteers and in diabetic patients (Henry et al., 2006; Garber et al., 2009; Nauck et al., 2009; Zinman et al., 2009).

The underlying mechanisms that mediate the effects of liraglutide on body weight are incompletely understood, and most probably a combination of effects on the gastrointestinal tract and the brain are involved. Several clinical studies have demonstrated that chronic administration of liraglutide at doses greater than 1.2 mg causes weight loss and this has been attributed to a delay in gastric emptying and to sensations of early fullness or satiation (Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Zinman et al., 2009). In these studies, higher doses of liraglutide were also associated with gastrointestinal adverse effects such as nausea, diarrhea and vomiting (Astrupt et al., 2009; Garber et al., 2009; Nauck et al., 2009; Zinman et al., 2009). Because liraglutide causes a dose-dependent weight loss (Vilsboll et al., 2007), it may also provide an attractive treatment option for obesity.

Although pharmacologic use of exenatide can stimulate the illness pathway, nausea is not the only mechanism reducing food intake. There is little correlation between the severity of nausea and the amount of weight lost, and doses of exenatide too low to cause nausea do promote weight loss (Dushai et al., 2011; Kelly et al., 2011).

Effects on the Gastrointestinal tract

GLP-1 affects food intake through a direct interaction with neural circuits, but also by its effects on gastrointestinal motility (Deane et al., 2010).

GLP-1 inhibits gastric emptying in health and in obese individuals (Naslund et al.,1998; Wishart et al.,1998)**.** It is one of the mediators of the ileal brake (Shirra et al., 2006), reduces gut motility (Imeryuz et al., 1997; Tolessa et al., 1998b; Shirra et al., 2002; Miki et al., 2005), retards gastric emptying (Imeryuz et al., 1997; Anvari et al., 1998; Naslund et al., 1998; Tolessa et al., 1998; Tolessa et al., 1998b; Wettergren et al., 1998; Wettergren et al., 1998b; Schirra et al., 2000) and inhibits gastric acid secretion

(Wettegren et al., 1994; Wettergren et al., 1997). The rate of gastric emptying is highly correlated with circulating levels of GLP-1 and is correlated with the normalization of glycemia (Kolligs et al., 1995).

Interestingly, GLP-1 increases gastric accommodation (Delgado –Arros et al., 2002; Andrews et al., 2007; Andrews et al., 2007b). In fact in healthy volunteers during fasting state, peripherally administered synthetic GLP-1 dose-dependently increases fundic relaxation and, also, compliance of the proximal stomach (Schirra et al., 2002) with a significant reduction of feeling of hunger. This may further support the concept that GLP-1 reduces food intake also independently of a direct interaction with hypothalamic satiety centres (Schirra et al., 2006).

The mechanisms through which GLP-1 mediated inhibition of gastric emptying and gut motility remain incompletely understood but they seem likely to involve vagal nerve activation and NO release from enteric neurons (Imeryuz et al., 1997; Wettergren et al., 1997b; Tolessa et al., 1998; Tolessa et al., 1998b; Naslund et al., 2000; Daniel e al., 2002; Andrews et al., 2007b). Some studies have documented that the inhibitory effect on gastric emptying is lost in both rats after vagal deafferentation (Imeryuz et al., 1997) and in humans after truncal vagotomy (Wettergren et al., 1997b). However, although *in vitro* studies have shown that in human or rat gastric muscular strips GLP-1 does not affect the smooth muscle contractility of the proximal stomach (fundus and corpus) (Tolessa et al., 1998; Naslund et al., 2001), it remains controversy the existence of peripheral functional sites and the relative importance of the ENS in mediating GLP-1 action .

Other Effects

Neuroprotective effects

GLP-1R agonists also exert proliferative and anti-apoptotic actions on neuronal cells, stimulate neurite outgrowth, enhance nerve growth factor–induced differentiation. Hence, it has been proposed that GLP-1R agonists may be of therapeutic use for the treatment of neurodegenerative diseases and other neurologic disorders, including diabetic peripheral neuropathy, Alzheimer's or PD as well as other neuropathies. Interestingly, at least with regard to PD, many neurons in the area postrema have been found to express tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, as well as surface receptors for GLP-1 (Yamamoto *et al.*, 2003). In Alzheimer's

disease, lesions are caused by a toxic protein, amyloid β (Aβ), excess glutamine, and disruption of calcium homeostasis; the activation of GLP-1R by GLP-1 displays neurotrophic properties, protects against excess glutamine, (Greig et al., 2004) and reduces the amount of Aβ (Perry et al., 2003). This creates a possibility for the use of GLP-1 in the treatment of Alzheimer's disease (Perry et al., 2004). During research on GLP-1's effect on calcium canal regulation in the hippocampus neurons, a decrease in glutamine's toxic activity, reduced depolarization, and a higher cell survival rate were observed (Gilman et al., 2003).

GLP-1R–dependent pathways also may be important for learning and memory. A study on rats with the use of a floating platform showed a beneficial effect of GLP-1 on learning and spatial memory. Animals which had been treated with intracerebroventricular administration of GLP-1, or which had genetically enhanced GLP-1R expression, more easily, and via a shorter route, found their way to the floating platform (During et al., 2003).

Cardiovascular effects

GLP-1 effects on the cardiovascular system may include a direct effect on the cardiac muscle and the vascular system. Intravenous administration of GLP-1R agonists increases systolic, diastolic, and mean arterial blood pressures and heart rate in rodents (Barragan et al., 1996). However GLP-1 exhibits cardioprotective effects in experimental models of cardiac injury or heart failure; GLP-1 reduces infarct size and increases left ventricular function and myocardial glucose uptake after ischemia-reperfusion injury in isolated rat hearts (Zhao et al., 2006).

Liver effects

In liver GLP-1 exerts effect mainly concerning the glycogen pathway, indeed it promotes glycogen accumulation in cultured rat liver cells. It has been observed that GLP-1 increases the activity of glycogen synthase-A, decreases the activity of glycogen phosphorylase-A, and promotes the incorporation of glucose into glycogen in isolated rat hepatocytes. The insulin-independent effect of GLP-1 on hepatic glucose uptake is consistent with the presence of specific GLP-1R that could activate kinases and/or factors involved in glycogen synthesis and glucose uptake (Redondo et al., 2003). GLP-1 may also mediate the regulation of hepatic glucose output by insulin. The hepatic

insulin receptor and the hepatocyte membrane-bound glucose-transporter-2 (GLUT2) have been shown to be internalized into the endosomal compartment after feeding or insulin administration (Nathan et al., 2001) and the internalization of hepatocyte GLUT2 appears to mediate the suppression of hepatic glucose output by insulin (Andersen et al., 1994).

Renal effects

GLP-1 shows diuretic and natriuretic activity in both lean and obese subjects. Glomerular filtration in obese people decreases under the influence of GLP-1, while in lean people it does not change significantly (Gutzwiller et al., 2006). Of note is the fact that in experimental animals, GLP-1 increases glomerular filtration (Moreno et al. 2002) and potassium excretion. The latter phenomenon is not observed in humans (Moreno et al. 2002).

Effects on muscles and adipose tissue

GLP-1 increases glucose incorporation into glycogen in rat skeletal muscle and enhances insulin-stimulated glucose metabolism in cultures of adipocytes and primary rat adipocytes (Villanueva-Peñacarrillo et al., 1994). GLP-1 also stimulates glucose uptake in fat and muscle. GLP-1 and exendin-4 increase glycogen synthase activity and glucose metabolism in rat soleus muscle and human skeletal muscle (Villanueva-Peñacarrillo et al., 1994; Moreno et al. 2002). In addition, GLP-1 has lipolytic effects in rat adipocytes (Ruiz-Grande et al., 1992) and displays both lipolytic and lipogenic actions in human adipocytes. (Villanueva-Penacarrillo et al., 2001)

AIMS

The present research has been addressed mainly to explore the presence and the importance of peripheral action sites in mediating the motor effects of GLP-1 in the different regions of gastrointestinal tract (stomach, small and large intestine), using an animal model (mouse). Subsequently, in the attempt to correlate gastric motor responses with the regulation of food intake, the effects of stable GLP-1 analogue, liraglutide, were examined on gastric accommodation and satiation in humans.

In detail, we investigated:

- The effects of GLP-1 on spontaneous and evoked mechanical activities of longitudinal and circular muscle from mouse duodenun and colon *in vitro.*
- The mechanism of action responsible of the effects observed.
- The presence and distribution of GLP-1 receptor in the muscle coat by immunohistochemistry.

- The influence of exogenous GLP-1 on mouse gastric tone by detecting intraluminal pressure from the whole organ *in vitro.*
- The mechanism of action underlying the effects observed
- The regional activity of GLP-1 in the stomach using circular muscular strips from gastric fundus or antrum
- Mouse gastic, fundic and antral GLP-1 receptor expression by RT-PCR.

- The effect of liraglutide, stable analogue of GLP-1, on human gastric accommodation and satiation.

I° ARTICLE

PERIPHERAL MOTOR ACTION OF GLUCAGON-LIKE PEPTIDE-1 THROUGH ENTERIC NEURONAL RECEPTORS

Disclosure

The results included in this paper have been published in the Journal *Neurogastroenterology and Motility* (Amato et al., 2010b). Data concerning the immunohistochemical analysis have been obtained in collaboration with Prof. Vannucchi's research group (University of Florence). Some of the experiments described have previously been published in abstract form (Mulè et al., 2009; Rotondo et al., 2009).

Summary

The experiments described in this study examined the effects of GLP-1 on the spontaneous and evoked mechanical activity of mouse duodenum and colon using the organ bath technique. Furthermore, we evaluated the presence and distribution of GLP-1R in the muscle coat through immunohistochemical analysis. GLP-1 failed to affect spontaneous mechanical activity, however it caused a concentration-dependent reduction of the electrically-evoked cholinergic contractions in circular smooth muscle of both intestinal segments, without affecting the longitudinal muscle responses. The GLP-1 inhibitory effect was significantly antagonized by exendin (9-39) or L-NAME, while it was not affected by guanethidine, a blocker of adrenergic neurotransmission. Moreover GLP-1 failed to affect the contractions evoked by exogenous carbachol. Immunohistochemistry demonstrated GLP-1R expression in the enteric neurons; among them, 27% in the duodenum, 79% in the colon co-expressed NO synthase whereas very few co-expressed acetylcholine transferase. The present results suggest that GLP-1 is able to act in the enteric nervous system decreasing the excitatory cholinergic neurotransmission through presynaptic GLP-1Rs, which modulates NO release.

Introduction

Glucagon-like-peptide-1 (GLP-1) is a proglucagon-derived peptide expressed in the endocrine pancreas and in the L-type enteroendocrine cells mainly located in duodenum, distal small intestine and colon (Eissele et al., 1992; Theodorakis et al., 2006). GLP-1 is considered an important incretin which, at physiological concentrations, potentiates the glucose-induced insulin release and inhibits glucagon release, properties that provide the rationale for reducing glycaemia and for its potential use as a therapeutic agent in the treatment of diabetes (Sinclair & Drucker, 2005; Baggio & Drucker, 2007). However, several studies suggest multiple additional effects, including a GLP-1 physiological regulatory role in controlling appetite and energy intake (Flint et al., 1998; Gutzwiller et al., 1999). This hormone is thought to contribute to the so-called "ileal break", a mechanism by which the presence of nutrients in the distal small intestine causes inhibition of upper gastrointestinal motor activity (Giralt & Vergara, 1999). Moreover, it decreases the gastric emptying and the intestinal motility in humans and rodents (Imeryuz et al., 1997; Giralt & Vergara, 1998; Wettergren et al., 1998; Naslund et al., 1999; Schirra et al., 2000; Bozkurt et al., 2002; Miki et al., 2005; Schirra et al., 2006; McDonagh et al., 2007; Hellstrom et al., 2008) and it modifies the faecal pellet output in rat when administrated intra-cerebro-ventricularly (Gulpinar et al., 2000). Although experiments in rats and pigs suggest that the effects of GLP-1 on gastrointestinal motility are exerted through interaction with centres in the brain or afferent neural pathways (Imeryuz et al., 1997; Tolessa et al., 1998; Wettergren et al., 1998; Naslund et al., 1999; Gulpinar et al., 2000), the mechanisms by which the GLP-1 exerts its action in gut remain poorly understood. One major question regarding the actions of the GLP-1 on the gastrointestinal tract is the relative importance of the local enteric nervous system.

The effects of GLP-1 are exerted through the interaction with a G-protein-coupled specific receptor (GLP-1R). In rodents and humans, a single structurally identical GLP-1R was identified using molecular biology and its mRNA has been demonstrated in the stomach, small and large intestine as well as in the nodose ganglion of the vagus nerve (Campos et al., 1994; Bullock et al., 1996; Nakagawa et al., 2004; Baggio & Drucker, 2007).

Despite more than a decade of research, the sites and mechanisms of action of the GLP-1 within the intestine remain controversial and poorly understood. Therefore, the present study was undertaken to examine, in mouse duodenum and proximal colon,

possible peripheral effects of GLP-1 on the spontaneous and neurally-evoked mechanical activities. More specifically, we have used an *in vitro* experimental approach, which allows the simultaneous registration of the endoluminal pressure (index of the circular mechanical activity) and of the isometric tension (index of the longitudinal mechanical activity) from intestinal segments. Previously it has been reported that the changes in isometric tension and endoluminal pressure reflect the mechanical activity of longitudinal and circular muscle, respectively (Mulè et al., 1992; Mulè & Serio, 2002). In addition, another objective of this study was to determine the mechanism responsible for the GLP-1 actions in the small and large intestinal segments. At this aim we also used immunohistochemistry to identify first, the presence and distribution of the GLP-1R in the muscle coat and, second, its possible co-expression with excitatory and inhibitory neurotransmitters by labelling acetylcholine transferase (ChAT) and neuronal nitric oxide synthase (nNOS), respectively.

Materials end Methods

General

The experiments were authorised by Ministero della Sanità (Rome, Italy), following the guidelines of the European Communities Council Directive of 24 November 1986. For both physiology and immunohistochemistry, adult male mice weighing 25 ± 1.5 g (C57BL/10, Harlan, Italy) were killed by cervical dislocation. Animals were fed ad libitum prior to use. The abdomen was immediately opened and the duodenum and the proximal colon were removed from a position just distal to the pylorus and the caecum, respectively. The content of the excised segments was cleaned with Krebs solution and segments of about 2 cm in length were cut. For physiological experiments, preparations were mounted in a custom designed horizontal organ bath (volume=5 ml), which was continuously perfused with oxygenated (95% O_2 and 5% CO_2) and heated (37 °C) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11.1. For immunohistochemistry, duodenum and colon specimens taken from three mice were fixed in 4% paraformaldehyde for 4 hours at 4°C. The specimens were cryoprotected in 30% sucrose in PBS for approximately 12 hours at 4°C, and then frozen in Killik cryostat embedding medium (Bio-Optica, Italy) and stored at -80°C. Transverse cryosections, 8 μm thick, were cut and placed on glass slides previously treated with polylisine, and stored at -20°C.

Physiology

The mechanical activity was recorded as previously described (23). In brief, the distal end of each segment was tied around the mouth of a J-tube, which was connected *via* a T catheter to a pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA) and to a syringe for filling the preparation with Krebs solution. The ligated proximal end was secured with a silk thread to an isometric force transducer (Grass FT03; Grass Instruments Co., Quincy, MA) Each preparation was filled with 0.1 ml Krebs solution and subjected to an initial tension of 0.1 g. The mechanical signals were detected as changes in both endoluminal pressure and isometric tension, and recorded on an ink writer polygraph (Grass model 7D; Grass Instruments Co., Quincy, MA). To provide electrical field stimulation (EFS), was used an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled *via* a stimulus isolation unit (Grass SIU5) to a pair of platinum plates, which were placed in parallel on either sides of the intestinal segment. Preparations were allowed to equilibrate for about 60 min before starting the experiment. After the equilibration time, GLP-1 was added non-cumulatively to the bath at increasing concentrations (1 – 10 – 100 -1000 nM) and the effects on the baseline tone and amplitude of phasic contractions were recorded over a period of 10 min. The interval between single concentrations was 40 min to avoid tachyphylaxis. The influence of GLP-1 (0.1 nM – 1 μM) on the electrically-evoked contractions was evaluated in another set of preparations, because the spontaneous mechanical activity of circular muscle was reduced by the repetitive application of EFS. Trains of stimuli (duration 5 s, supramaximal voltage, 8 Hz and 0.5 ms pulse duration) were applied to duodenal and colonic preparations at intervals of 70 s and stable and reproducible responses for a time-period of 3 h were observed. Under basal condition, EFS induced a cholinergic muscular contraction which was abolished by atropine (1 μM) and TTX (1 μM). After stable control cholinergic contractions had been recorded, the responses evoked by EFS were analysed in the presence of cumulative increasing concentrations of GLP-1 (0.1 nM – 1 μM). The contact time for each concentration was 7 min. The effect of GLP-1 was evaluated after pre-treatment of intestinal preparations with exendin (9-39) (100 - 300 nM for 20 min), GLP-1 receptor antagonist, guanethidine (1 μM, for 60 min), a blocker of adrenergic transmission or L-NAME (300 μM, for 30 min), an inhibitor of nitric oxide (NO)-synthase. In separate experiments, tissues were exposed to cumulative increasing concentrations of carbachol (10 nM - 30

μM), and the myogenic contractions produced were evaluated in the absence or in the presence of GLP-1 (100 nM).

Data analysis and statistical tests

For data analysis, the mean value of 10 peaks of the twitch contractions following EFS were set as 100% contraction. The inhibitory effects of GLP-1 on the EFS-evoked cholinergic contractions were expressed as a percentage of this value. Contractile effects induced by carbachol were expressed as a percentage of the maximal response. All data are expressed as mean values ± SEM. The letter *n* indicates the number of experimental animals. The concentration (EC_{50}) with 95% confidence intervals (Cls) producing half maximum response was calculated using Prism 4.0, GraphPad (San Diego, CA, USA). Statistical analysis was performed by means of Student's *t*-test or ANOVA followed by Bonferroni *post hoc* test, when appropriate. A probability value of less than 0.05 was regarded as significant.

Drugs

The following drugs were used: atropine sulphate, guanethidine monosulphate, carbachol (CCh), Nω-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin (TTX) (Sigma-RBI, Milano, Italy), glucagon-like peptide-1 (7-36) amide, exendin (9-39) (Tocris-Bioscience, Bristol, UK). Each compound was prepared as a stock solution in distilled water. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs**.**

Immunohistochemistry

The slides were pre-incubated in 0,5% Triton (Sigma Aldrich, Italy) and 1,5% BSA (Sigma Aldrich, Italy) in PBS for 15 min at room temperature. Successively the sections were incubated in GLP-1R polyclonal antibody (Santa Cruz Biotechnology, USA) at final dilution of 1:100 in 0,5% triton and 1,5% BSA in PBS for 24h at 4°C. The immunoreaction was revealed by using the secondary antibody Alexa Fluor 488 Donkey anti Goat (Invitrogen, USA) 1:500. For GLP-1R/nNOS, GLP-1R/ChAT and ChAT/nNOS double labelling, the sections were pre-incubated as described above. For GLP-1R/nNOS, nNOS monoclonal antibody (BD Bioscience, USA) was applied firstly at final dilution of 1:1000 for 24h at 4°C. The immunoreaction was revealed with the secondary antibody Alexa Fluor 568 Goat anti Mouse (Invitrogen, USA) 1:333 for 2h at

room temperature. The sections were then incubated with the GLP-1R polyclonal antibody at final dilution of 1:100 for 24h at 4°C and immunoreaction was revealed with the secondary antibody Alexa Fluor 488 Donkey anti Goat 1:500 for 2h at room temperature. For GLP-1R/ChAT double labelling, ChAT polyclonal antibody (a generous gift of Dr M. Schemann who characterized this antibody, 25) was applied firstly at final dilution of 1:500 for 24h at 4°C. The immunoreaction was revealed with secondary antibody Alexa Fluor 568 Goat anti Rabbit 1:333 for 2h at room temperature. Then the sections were incubated in GLP-1R polyclonal antibody at final dilution of 1:100 for 24h at 4°C and immunoreaction revealed with the secondary antibody Alexa Fluor 488 Donkey anti Goat 1:500 for 2h at room temperature. For ChAT/nNOS double labelling, ChAT polyclonal antibody was applied firstly at final dilution of 1:500 for 24h at 4°C and the immunoreaction was revealed with the secondary antibody Alexa Fluor 568 Goat anti Rabbit 1:333 for 2h at room temperature. The sections were then incubated in nNOS monoclonal antibody (BD Bioscience, USA) at final dilution of 1:1000 for 24h at 4°C and the immunoreaction revealed with the secondary antibody Alexa Fluor 488 Donkey anti Goat 1:500 for 2h at room temperature.

The immunoreaction products were observed under an epifluorescence Zeiss Axioskop microscope.

Quantitative analysis

GLP-1R-IR neurons as well GLP-1R/nNOS-IR or GLP-1R/ChAT-IR neurons were counted on three adjacent slices taken at the distance of 50 μm from each other from the duodenum and the colon of three control mice. The immune-labelled slices were examined under fluorescence microscope at x40 magnification. The results were expressed as mean ± SEM.

Results

Physiology

Influence of GLP-1 on spontaneous mechanical activity

In our experimental conditions, mouse duodenum and proximal colon exhibited spontaneous mechanical activity consisting of phasic changes in both intraluminal pressure and isometric tension (duodenum: 1.4 ± 0.1 cm H_2O and 180 ± 20 mg; n=4,

respectively; colon: 11.1 ± 1.1 cm H_2O and 328.7 mg ± 40.3 mg; n=5, respectively). GLP-1 (up to 1 μM) did not show any influence on the spontaneous mechanical activity (baseline tone or amplitude of the contractions) of both preparations (duodenum: 1.5 ± 0.2 cm H_2O and 195 ± 22 mg; n=4 for circular and longitudinal muscle, respectively; colon: 10.8 ± 1.3 cm H_2O and 310.3 mg ± 37.3 mg; n=5; P>0.05). These results shifted our focus to examine possible GLP-1 effects on the EFS evoked responses.

Influence of GLP-1 on the evoked cholinergic responses

In the duodenal preparations, EFS (trains of 8 Hz for 5 s) induced a contraction with an amplitude of 2.8 ± 0.4 cm H_2O (n=15) in the circular muscle and 250 ± 30 mg (n=15) in the longitudinal muscle. The longitudinal muscle contraction appeared as an "on response" being simultaneous to the stimulation period, while the circular muscle contraction appeared as an "off response" occurring after the stimulation period. The circular and longitudinal muscle contractions were abolished by the muscarinic receptor blocker, atropine (1 μM), suggesting their cholinergic origin. GLP-1 (0.1 nM - 1 μM) caused a concentration-dependent reduction of the electrically-evoked cholinergic contractions of the circular muscle, without affecting significantly the contraction amplitude of the longitudinal muscle (Fig. 10). GLP-1 (1 μM) produced about 55% of reduction of the evoked contraction amplitude (EC_{50} = 2.1 nM, Cls=1-4 nM) (Fig. 10). Often, at the maximal concentrations tested a small relaxation with an amplitude of 0.4 + 0.1 cm H_2O (n=7) was unmasked during EFS. The inhibitory effects induced by GLP-1 on cholinergic-evoked contractions were reversible after washing out. Exendin (9-39) (300 nM), a GLP-1 receptor antagonist, *per se* did not affect the evoked contractions but it reduced significantly the inhibitory action of GLP-1 (EC_{50} = 17 nM, Cls = 4.8 – 60 nM) (Fig. 10).

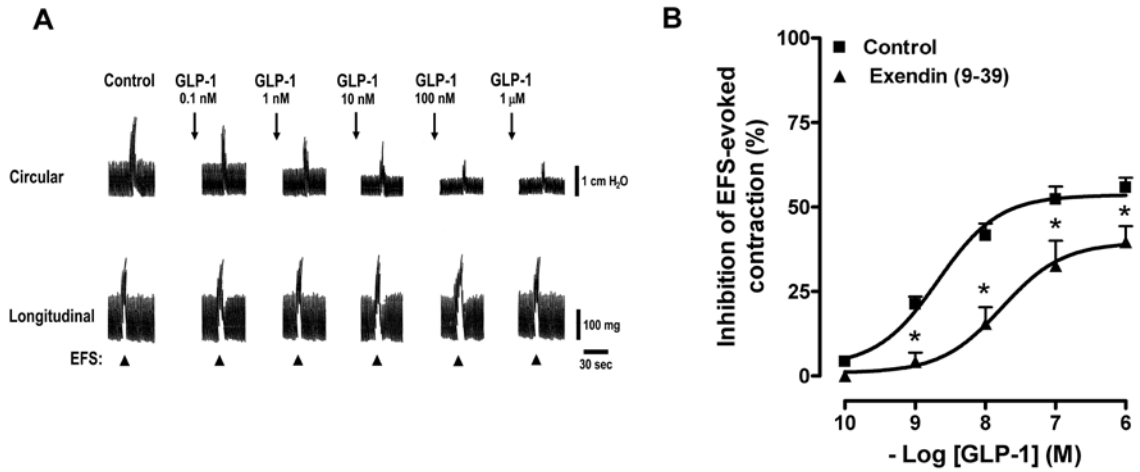


Fig.* 10** ***A*: Typical recordings showing the inhibitory effects of increasing concentrations of GLP-1 on cholinergic contractile response to EFS (train of 5 s, 0.5 ms, supramaximal voltage, 8 Hz) in circular muscle (upper trace) but not in longitudinal muscle (lower trace) of mouse duodenal preparations.* ***B****: Concentration-response curves for the inhibitory effects induced by GLP-1 alone or after treatment of the preparation with exendin (300 nM) on the neurally evoked cholinergic contraction of circular muscle of mouse duodenum. Data are expressed as a percentage of the response obtained in the absence of GLP-1. Each value is mean ± SEM from 6 animals. * P<0.05 for exendin relative to GLP-1 alone.*

In proximal colon preparations, EFS (trains of 8 Hz for 5 s) evoked a cholinergic contraction (blocked by atropine), with an amplitude of 12.6 ± 3.1 cm H_2O and 314.3 ± 74 mg (n=15) in circular and longitudinal muscle, respectively. In colonic circular muscle the contraction always followed a small relaxation (0.4 ± 0.2 cm H_2O; n=15). GLP-1 (0.1 nM – 1 µM) caused a significant concentration-dependent reduction of the electrically-evoked contraction amplitude only in the circular muscle, without affecting the longitudinal muscle response (Fig. 11). Moreover, GLP-1 (up to 1 µM) failed to affect the EFS-induced relaxation (0.4 ± 0.3 cm H_2O; n=6; P>0.05). The maximal effect was about 55% of reduction of the evoked contraction amplitude (EC_{50} = 1.8 nM, Cls = 0.7 – 4.7 nM) (Fig. 2). In the presence of exendin (9-39) (100 nM), which *per se* had no significant effect on the EFS-evoked responses, the inhibitory action of GLP-1 was significantly reduced (EC_{50} = 9.9 nM, Cls = 4.6 – 21 nM) (Fig. 11).

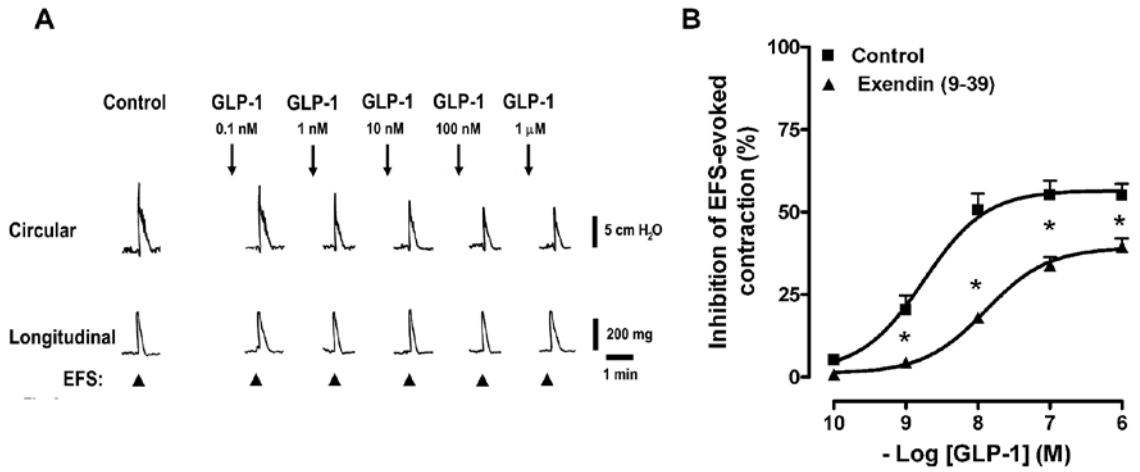


***Fig. 11 A**: Typical recordings showing the inhibitory effects of increasing concentrations of GLP-1 on cholinergic contractile response to EFS (train of 5 s, 0.5 ms, supramaximal voltage, 8 Hz) in circular muscle (upper trace) but not in longitudinal muscle (lower trace) of mouse proximal colon. **B**: Concentration-response curves for the inhibitory effects induced by GLP-1 alone or after treatment of the preparation with exendin (100 nM) on the neurally evoked cholinergic contraction of circular muscle of mouse proximal colon. Data are expressed as a percentage of the response obtained in the absence of GLP-1. Each value is mean ± SEM from 6 animals. * P<0.05 for exendin relative to GLP-1 alone.*

Both in duodenal and colonic preparations, the GLP-1 inhibitory effect on the cholinergic evoked contractions was unaffected by pre-treatment with guanethidine (1 μM), while it was significantly reduced in the presence of L-NAME (300 μM) (Fig. 12).

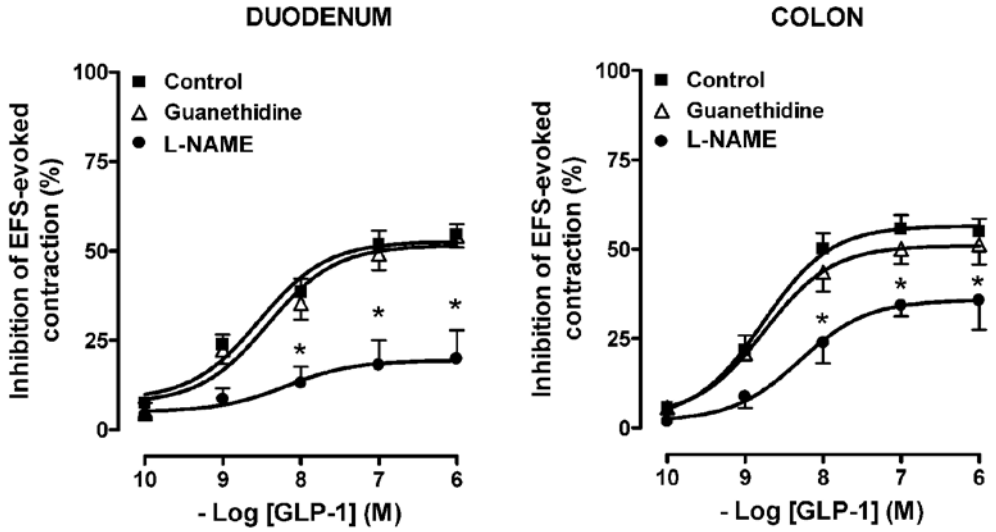


***Fig. 12.** Concentration-response curves for the inhibitory effects induced by GLP-1 alone and after treatment of the preparations with guanethidine (1 μM) or L-NAME (300 μM) on the neurally evoked cholinergic contraction of circular muscle of mouse duodenum and colon. Data are expressed as a percentage of the response obtained in the absence of GLP-1. Each value is mean ± SEM from at least 4 animals. * P<0.05 for L-NAME relative to GLP-1 alone.*

To explore if GLP-1 was able to alter the myogenic responses of duodenal and colonic circular muscle, a muscarinic agonist was used. In both intestinal segments, carbachol (10 nM - 30 μM) induced a concentration-dependent contractile response, which was not affected by GLP-1 (100 nM) (Fig. 13).

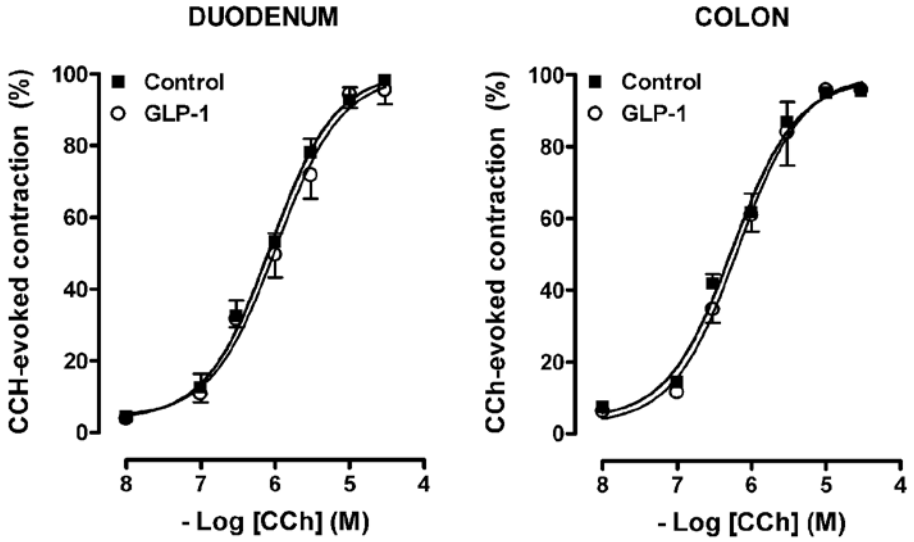


Fig.13. *Concentration-response curves for the contractile effects induced by carbachol in circular muscle of mouse duodenal or colonic preparations before and after pre-treatment with GLP-1 (1 μM). Contractile response is expressed as percent of the maximal response. Data are means ± SEM from 4 animals*

Immunohistochemistry

GLP-1R-immunoreactivity (IR) was detected in some myenteric neurons of both duodenum (Fig. 14 A) and proximal colon (Fig.14 B) and in few submucous neurons in the colon only (Fig. 14 C). These neurons had a round or piriform perikaryon; the labelling was diffuse throughout the perikaryon and had a granular aspect; clusters of granules more intensely labelled were also frequently observed (Fig. 14 A-C). IR intraganglіar varicosities were present, although very few, whereas no IR fibers were seen outside the ganglia, neither in the muscle layers nor at the deep muscular plexus of the duodenum. No other cell type showed GLP-1R-IR in both the duodenum and colon muscle coat.

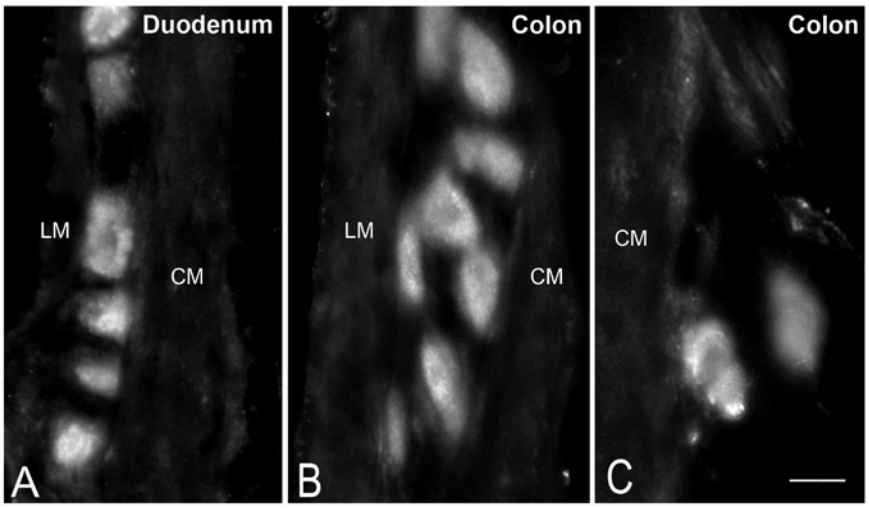


Fig. 14 *GLP-1R-immunoreactivity (IR) in the mouse duodenum and colon.* ***A*** *(duodenum) and* ***B*** *(colon), GLP-1R-IR neurons in the myenteric plexus.* ***C,*** *colon, GLP-1R-IR neurons in the submucous plexus. All these neurons have a round or piriform perikaryon and the labelling is diffuse throughout the cytoplasm and has a granular aspect; clusters of granules intensely labelled are present in most of the neurons (arrows). CM= circular muscle layer; LM=longitudinal muscle layer. Bar = 40 μm.*

GLP-1R/nNOS double labelling demonstrated either in the colon or in the duodenum that some of the neurons sharing GLP-1R were also nNOS-IR. In some of the double labelled neurons, the two stainings co-localized (see Fig. 15A) while in other neurons the GLP-1R-IR nNOS-IR were distributed in distinct areas (Fig. 15B). Furthermore, some nNOS-IR neurons appeared surrounded by GLP-1R-IR nerve varicosities (Fig. 15A).

GLP-1R/ChAT double labelling demonstrated that very few of the GLP-1R-IR neurons were also ChAT-IR. The latter labelling was preferentially distributed along the cell periphery (see Fig. 15C).

nNOS/ChAT double labelling demonstrated that nNOS-IR and ChAT-IR were never co-expressed (Fig. 15D).

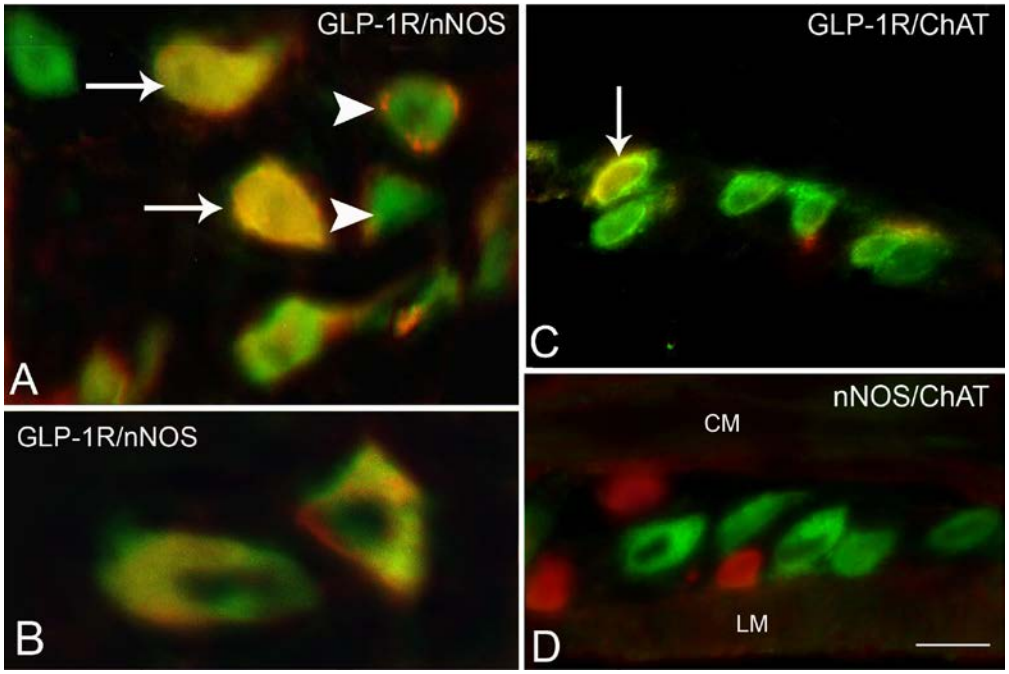


***Fig. 15. A** and **B,** GLP-1R/nNOS immunoreactivity (IR) in the myenteric neurons. **A**, mouse colon. Two neurons (arrows) co-express GLP-1R-IR (red) and nNOS-IR (green). Where the double labelling is co-localized it appears yellow. Two nNOS-IR neurons are surrounded by GLP-1R-IR varicosities (arrowheads). **B,** mouse duodenum. Two neurons GLP-1R/nNOS double labelled. In both cells, the labelling at the cell periphery is co-localized while within the cytoplasm the GLP-1R- and the nNOS-IR occupy distinct areas (asterisks). **C,** mouse colon. Only one neuron is GLP-1R/ChAT double labelled (arrow) and the labelling is mainly located along the cell contour. GLP-1R-IR (red), ChAT-IR (green). **D**, mouse colon. None of the myenteric neurons is nNOS/ChAT double labelled. nNOS-IR (red), ChAT-IR (green). CM= circular muscle layer; LM=longitudinal muscle layer. Bar A,C,D= 40 μm; B=25 μm.*

Quantitative analysis. The number of GLP-1R-IR neurons/slice in the duodenum and proximal colon, was 14.33 ± 1.43 and 16.1 ± 1.26, respectively. In duodenum 27.22 ± 3.14 % and in colon 79.67 ± 4.5% of GLP-1R-IR neurons expressed nNOS. The number of GLP-1R neurons that co-expressed ChAT-IR was less than 1/slice therefore, no quantification was done for GLP-1R/ChAT-IR neurons.

Discussion

The present *in vitro* study demonstrates that in mouse, activation of GLP-1 peripheral receptors can exert an inhibitory effect on motility of small and large intestine, independently on the neural extrinsic control. Furthermore, by

immunohistochemistry, we presently show that GLP-1R is expressed by the enteric neurons, both in duodenum and colon, and that some of these neurons co-expressed nNOS or ChAT. Taken together, immunohistochemical and physiological data suggest that GLP-1 is able to decrease the excitatory cholinergic neurotransmission in mouse duodenal and proximal colonic circular muscle, by acting on specific GLP-1R and this inhibitory effect seems to be mediated by a nitrergic pathway.

In addition to its principal effect as potent incretin (Baggio & Drucker, 2007), GLP-1 is able to exert other actions in gastrointestinal tract, such as decrease in the rate of gastric emptying and reduction of gut motility, in a number of species including man, pigs, rats and mice (Imeryuz et al., 1997; Giralt & Vergara, 1998; Naslund et al., 1999; Bozkurt et al., 2002; Miki et al., 2005; Sinclair & Drucker, 2005; Nagell et al., 2006; Schirra et al., 2006; Schirra et al. 2009). However, so far, few experimental studies have been performed in *in vitro* conditions and the involvement of GLP-1 in the regulation of the motility of the gastrointestinal tract has been mainly supported by experiments conducted in *in vivo c*onditions. Indeed, multiple findings suggest that GLP-1 effects on motor function of the gastrointestinal tract are exerted through the interaction with centres in the brain or afferent neural pathways (Imeryuz et al., 1997; Tolessa et al., 1998; Tolessa et al., 1998b; Wettergren et al., 1998; Gulpinar et al., 2000; Baggio et al., 2004; Nagell et al., 2006; Schirra et al., 2009). A role for sympathetic pathways was also claimed by Giralt and Vergara (Giralt & vergara 1998), who found that the inhibitory effect of GLP-1 on rat upper gut motility was mediated via adrenergic pathways.

Our results provide evidence that there are peripheral sites of action of GLP-1 at the level of the enteric nervous system. This conclusion appears in contrast with a previous study demonstrating that in rats GLP-1 does not affect spontaneous contractility in isolated gut muscular strips or the contractile responses induced by EFS or acetylcholine leading to the conclusion that this peptide does not modulate intestinal motility through a peripheral action (Tolessa et al., 1998b). A likely explanation for the discrepancy between the previous observations and our physiological results is the muscle layer used for the study. In our experiments the GLP-1 effect was observed only on the electrically evoked responses of the circular muscle, whereas Tolessa and coll. (Tolessa et al., 1998b) investigated the contractile activity of the longitudinal muscle, which actually was unaffected by GLP-1 also in our experimental conditions. Taken

together these findings turn out interestingly that GLP-1 has differential effects on the two muscular layers.

In mouse duodenal and colonic circular smooth muscle, activation of GLP-1R reduces electrically evoked cholinergic contractions. Since contraction of the circular muscle is dominant in peristalsis, the action of GLP-1 could contribute to the reduction of the intestinal transit and to retard the digestion and the absorption of the luminal content.

Just few works have suggested that GLP-1 may have a role also in the regulation of colonic motility (Gulpinar et al., 2000; Ayachi et al., 2005). GLP-1 increases the rat faecal pellet output via hypothalamic receptors activation (Gulpinar et al., 2000;) and can induce a weak contraction in isolated circular smooth muscle cells from human colon likely due to an increase of glucose energy disposal (Brubacker et al., 2002). Our findings emphasize the contribution of a GLP-1 peripheral action to delay the colonic transit and could explain the mechanism through which the peptide delays the flow of contents from colon to the rectum. Furthermore, our results are in agreement with the prolonged gastrointestinal transit and constipation associated with overexpression of GLP-1 and GLP-2 reported in patients having a glucagon-like peptide secretory tumour (Byrne et al., 2001; Brubacker et al., 2002).

The GLP-1 concentrations used in our experiments are in agreement with previous *ex vivo* study reporting that increasing concentrations of GLP-1 infused intra-arterially into the canine ileum, were able to inhibit phasic activity elicited by EFS (Daniel et al., 2002). The inhibitory responses induced by GLP-1 were significantly antagonized by exendin 9-39, a specific GLP-1R antagonist, indicating the specificity of the observed effect and in agreement with the GLP-1R presence in these organs. However, the observation that exendin 9-39 failed on its own to affect the evoked cholinergic contractions suggests that GLP-1R modulating intestinal neurotransmission is not tonically activated.

In our experimental model the mechanism by which GLP-1R activation influences the neural evoked contractions appears to be related to reduction of acetylcholine release from cholinergic nerves. In fact, even at the highest concentration used, GLP-1 failed to affect both in the duodenum and in the colon the responses evoked by exogenous carbachol, which works by activating muscarinic receptors on the smooth muscle, suggesting that the inhibition of the electrically evoked cholinergic contractions involves neural mechanisms. Consistent with our results, recent findings

have pointed out the importance of the enteric excitatory motoneurons in the downstream signalling of the glucagon-like peptides to inhibit mouse intestinal motility (McDonagh et al., 2007). In fact, they showed that in a murine animal model with a partial enteric nervous system deficit, characterized by a dramatic decrease of cholinergic neurons number, GLP-1 was not able to induce reduction of intestinal transit, as did in wild-type animals (McDonagh et al., 2007).

Because additional mediators have been reported to mediate the effects of the peptide in the gastrointestinal tract, the possible involvement of adrenergic transmitters or nitric oxide in the action of GLP-1 on small and large intestine motility was also investigated. However, in our experiments GLP-1 inhibitory effects in both duodenal and colonic preparations were unaffected by guanetidine, a blocker of adrenergic neurotransmission, ruling out involvement of an adrenergic pathway in the presynaptic inhibition of cholinergic transmission. Moreover, it has been shown in different part of the gastrointestinal tract of diverse species that the mechanical inhibitory effect of exogenous GLP-1 depends on endogenous nitric oxide (Kilbinger et al., 1996; Tolessa et al., 1998b; Daniel et al., 2002; Naslund et al., 2002; Andreaws et al., 2007). It seems likely that the action of the peptide is dependent on the production and release of NO because the inhibitory response of GLP-1 on the evoked-contractions in both intestinal preparations was significantly reduced by L-NAME, an NO synthase inhibitor. On the other hand, the nitrergic relaxation induced by EFS in colonic circular muscle was unaffected by GLP-1, suggesting that likely GLP-1 acts on nitrergic interneurons. Similarly, duodenal circular relaxation obtained in the presence of atropine was not modified by the peptide (*data not shown)* ruling out an action on nitrergic motor neurons. Quantification of the immunohistochemical findings, showing a consistent co-expression of GLP-1R and nNOS in the myenteric neurons (27% and 79%) together with a very sporadic co-expression with ChAT, is in agreement with a direct action of GLP-1 on the release of NO, which in turn would reduce the ACh release. In support of this hypothesis there is the observation that endogenous NO is able to decrease the evoked-release of acetylcholine from enteric neurons in the gut (Kilbinger et al., 1996; Mang et al., 2002). On the other hand, the proposed mechanism could represent the rationale to explain the selectivity of GLP-1 action toward the circular *vs* longitudinal muscle layers.

The localization of the GLP-1R suggests that the GLP-1 actions in the duodenum and colon are mediated via circulating GLP-1 in an endocrine manner.

Nevertheless, it remains possible that GLP-1 locally released from mucosal cells also acts on terminals of intrinsic primary afferent neurons (IPANs). Indeed, there is good evidence that the GLP-1R on vagal afferent nerves mediates sensory input from the gastrointestinal tract (Bucinskaite et al., 2009), while so far it is not known if GLP-1 can modulate IPANs. Although it can not be stated by our experiments, IPANs might be represent a target for GLP-1 paracrine action.

In summary, in mouse small and large intestine GLP-1 can reduce the gut motility acting peripherally through the presynaptic inhibition of the cholinergic neurotransmission, which is mediated by NO production. Slowed propulsion of intestinal contents through the gut caused by GLP-1 may be important for the pre-absorptive delivery of nutrients and for absorption of water and electrolytes.

II° ARTICLE

GLUCAGON-LIKE PEPTIDE-1 RELAXES GASTRIC ANTRUM THROUGH NITRIC OXIDE IN MICE

Disclosure

The experiments including in this paper have been published in the Journal *Peptides* (Rotondo et al., 2011). Some of the experiments described have previously been published in abstract form (Rotondo et al., 2010).

Summary

The purposes of this study were i) to examine exogenous GLP-1 effects on mouse gastric mechanical activity using isolated whole stomach ii) to clarify the regional activity of GLP-1 using circular muscular strips from gastric fundus or antrum iii) to analyse the mechanism of action underlying the observed effects; iv) to verify regional differences of GLP-1 receptors (GLP-1R) expression by RT-PCR. In the.whole stomach GLP-1 caused concentration-dependent relaxation significantly anatagonized by exendin (9-39), an antagonist of GLP-1R and abolished by tetrodotoxin (TTX) or N_{ω}-nitro-L-arginine methyl ester (L-NAME), inhibitor of nitric oxide (NO) synthase. GLP-1 was without any effect in fundic strips, but it induced concentration-dependent relaxation in carbachol-precontracted antral strips. The effect was abolished by TTX or L-NAME. RT-PCR analysis revealed a higher expression of GLP-1R mRNA in antrum than in fundus. These results suggest that exogenous GLP-1 is able to reduce mouse gastric motility by acting peripherally in the antral region, through neural NO release.

Introduction

Glucagon-like peptide-1 (GLP-1) is a gastrointestinal regulatory peptide secreted from the mucosal endocrine L cells in response to the nutrient ingestion (Schirra et al., 1996). GLP-1 acts mainly as an incretin enhancing glucose-stimulated insulin secretion and inducing insulin gene expression (Fehmann & Habener, 1992). It also stimulates somatostatin release and inhibits glucagon secretion (Sinclair & Drucker, 2005). These properties provide the rationale for its potential use as a therapeutic agent in the treatment of diabetes (Sinclair & Drucker, 2005; Baggio & Drucker, 2007; Holst, 2007). In addition, GLP-1 acts as an anorexigen peptide decreasing hunger feelings. In fact, GLP-1 inhibits food intake upon intracerebroventricular injection in animal models and after intravenous infusion in normal weight and obese humans (Flint et al., 1998; Gutzwiller et al., 1999; Van Dijk & Thiele, 1999;Larsen et al., 2001).

GLP-1 reduces postprandial glycemia not only by its hormonal effects (i.e., reduced glucagon and increased insulin release) but also by its effects on gastrointestinal motility (Deane et al., 2010). In fact, GLP-1, which is one of the mediators of the ileal brake (Giralt & Vergara, 1999), reduces gut motility (Imeryuz et al., 1997; Tolessa et al., 1998; Schirra et al., 2002; Miki et al., 2005), retards gastric emptying of liquid and solid meals, inhibits antro-pyloro-duodenal motility (Imeryuz et al., 1997; Anvari et al., 1998; Tolessa et al., 1998; Wettregreen et al., 1998; Wettergreen et al., 1998b; Schirra et al., 2000; Naslund et al., 2001) and increases gastric accommodation (Delgado-Arros et a., 2002; Andrews et al., 2007; Andrews et al., 2007b). However, the GLP-1 action mechanism on the gastrointestinal motility is unclear yet.

The peptide actions are initiated by activation of specific G protein-coupled receptors (GLP-1 receptor: GLP-1R) [19]. GLP-1R has been identified in several regions of central nervous system that control feeding behaviour as brainstem and hypothalamus and in the nodose ganglion of the vagus nerve [7,27]. In rodents and humans GLP-1R is also expressed in pancreatic islets, brain, heart, kidney, small and large intestine and in the stomach [7,8,12,21,27,39].

The distribution of the GLP-1R in the central nervous system (Goke et al., 1995) and in the nodose ganglion (Holst, 2007) together with functional evidence suggest that GLP-1 effects on gastrointestinal motility are exerted through the interaction with centres in the brain or afferent neural pathways (Imeryuz et al., 1997; Tolessaa et al., 1998; Gulpinar et al., 2000; Schirra et al., 2000; Baggio & Drucker, 2007; Bucinskate et

al., 2009). However, recently we have shown that GLP-1 can act through a peripheral mechanism by binding GLP-1R present in murine colonic and duodenal myenteric neurons (Amato et al., 2010b

Therefore, it is likely to hypothesize that GLP-1 may have also a direct influence on the gastric mechanical activity since the major part of experimental studies about its involvement in the regulation of gastric motility have been conducted in *in vivo* conditions (Imeryuz et al., 1997; Tolessa et al., 1998; Wettergren et al., 1998b; Gulpinar et al., 2000; Naslund et al., 2002).

The present study was undertaken to examine the effects of exogenous GLP-1 on mouse gastric spontaneous mechanical activity and to analyse the mechanism of action responsible for the observed effects. The muscle function of whole stomach was examined *in vitro* where the influence of external factor is removed, but the muscle performs in a manner analogues to its *in vivo* capacity (Mulè & Serio, 2002). Furthermore, to clarify the regional activity of GLP-1, the effects of the peptide were tested on circular muscular strips from gastric fundus or antrum and by RT-PCR was investigated the possible regional differences in the expression of GLP-1R in mouse stomach.

Materials and methods

All animal procedures were in conformity with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Adult male mice (C57BL/10SnJ) (Harlan Laboratories, San Pietro di Natisone Udine, Italy) were housed under controlled conditions of temperature (22 ± 2 C°) and humidity (55 ± 5 %) until used. Animals, fed ad libitum prior to use, were killed by cervical dislocation. The abdomen was immediately opened, the oesophagus was tied just below the lower oesophageal sphincter, and the entire stomach was excised.

Isolated stomach

The entire stomach was mounted in custom-designed horizontal organ bath (volume=5 ml), which was continuously perfused with oxygenated (95% O_2 and 5% CO_2) and heated (37°C) Krebs solution with the following composition (in mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11.1. The

pyloric end was cannulated and connected to a standard pressure transducer (Statham Mod. P23XL) in order to detect the changes of intraluminal pressure. The mechanical activity was recorded on ink-writer polygraph (Grass model 7D). Preparations were allowed to equilibrate for about 60 min before starting the experiment.

At the beginning of each experiment, the preparation was challenged with isoproterenol (1 μM) until reproducible responses were obtained, to ensure that a stable and acceptable level of sensitivity had been reached before the experimental procedure was begun. The responses to non-cumulative concentrations of GLP-1 (10 nM - 3 μM) were examined. The peptide was added into the bath at increasing concentrations in volumes of 50 μl after switching off the perfusion. Each concentration was left in contact with the tissue for 4 min. The interval between two subsequent application of GLP-1 was not less than 30 min. The response to GLP-1 was also tested in the presence of exendin (9-39) (300 nM), GLP-1R antagonist, or in presence of N_{ω}-nitro-L-arginine methyl ester (L-NAME) (300 μM), an inhibitor of nitric oxide (NO) synthase (NOS) or tetrodotoxin (TTX) (1 μM), a voltage-dependent Na^{+}-channel blocker. All agents were added to perfusing solution at least 30 min before the peptide was tested.

Antral and fundic muscular strips

Circular muscle strips (2 x 10 mm) of fundus or antrum were suspended in a vertical organ bath containing 6 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained to 37°C. One end of each strip was tied to organ holders, and the other end was secured with silk thread to an isometric force transducer (FORT 25, 2Biol, Besozzo VA, Italy). Mechanical activity was digitized on an A/D converter, visualized, recorded, and analyzed on a personal computer with the use of the PowerLab7400 system (2Biol, Italy). The strips were placed under an initial tension of 0.5 g and allowed to equilibrate for 60 min before the start of each experiment. During this period the Krebs solution in the organ bath was changed every 15 min. The preparations were repeatedly tested with carbachol (CCh, 10 μM) until constant responses were obtained. The interval between two subsequent application of CCh was not less that 15 min. Non-cumulative concentration-response curves of GLP-1 (10 nM - 3 μM) were performed when the contraction elicited by CCh reached a plateau. Each concentration of GLP-1 was left in the organ bath until the effect reached its maximum.

Data analysis and statistical tests

All data are mean values ± SEM. The letter *n* indicates the number of experimental animals. The relaxing responses to GLP-1 on the whole stomach have been expressed as a percentage of the maximum response obtained in the same tissue. In experiments using muscular strips, the entity of relaxation was expressed as percentage inhibition of contractile response to carbachol (10 μM). Concentration-response curves were computer fitted to a sigmoidal curve using non-linear regression (Prism 4.0, GraphPad Software, San Diego, CA, USA) and the concentration (EC_{50}) with 95% confidences intervals (Cls) producing half maximum response was calculated. Statistical analysis was performed by means of Student's *t*-test or 2-way ANOVA followed by Bonferroni post hoc test, when appropriate. A probability value less than 0.05 was regarded as significant.

Drugs

The following drugs were used: carbachol (CCh), N_{ω}-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin (TTX) (Sigma-RBI, Milano, Italy), glucagon-like peptide-1 (7-36) amide, exendin (9-39) (Tocris-Bioscience, Bristol, UK). Each compound was prepared as a stock solution in distilled water. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs.

GLP-1R expression analysis

Total RNA from mouse whole stomach, and from fundic and antral region was extracted using PureLink™ RNA Mini Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. After quantification by spectrophotometry 1 μg of total RNA was reverse-transcribed in a final volume of 50 μL using the High Capacity c-DNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The GLP-1R and β-actin were amplified using 5 μl of cDNA (30 ng total RNA equivalents) per reaction. The oligonucleotide primers for mouse GLP-1R and β–actin were as follows: GLP-1R (forward) 5'-AGG AAC CCT ACG CTT CGT CAAG-3', (reverse) 5'- TTT GGC AGG TGG CTG CAT ACAC-3'; β–actin (forward) 5'-CGG GAT CCC CGC CCT AGG CAC CAG GGT-3', (reverse) 5'-GGA ATT CGG CTG GGG TGT TGA AGG TCT CAAA-3'. The thermal cycle profile employed a 3-min denaturing step at 94 °C followed by 40 cycles at 95 °C for 45 s, 55°C for 45 s and 72 °C for 75 s, and a final

extension step of 10 min at 72°C. The amplimers were separated on a 1% agarose gel containing 0.5 μg mL^{-1} of ethidium bromide for visualization and the gel was scanned under UV light. Expected length of the PCR products for GLP-1R was 251 bp, and 286 bp for β–actin. Semi-quantitation of the GLP-1R mRNA expression levels was obtained using densitometry. The corrected band intensities of the specific GLP-1R amplicon, obtained using the ImageJ software were normalized with the corresponding β-actin mRNA expression. It was ensured that the band intensities of β-actin mRNA were similar before the comparison of the expression levels. The expression levels of GLP-1R mRNA in the different gastric region are represented as the ratio of mean optical density of GLP-1R to β-actin and shown as arbitrary units.

Results

Effects of GLP-1 on whole stomach

GLP-1 (10 nM - 3 μM) induced a relaxation that developed slowly, persisted throughout the contact time (Fig. 16), and was reversible after washing out.

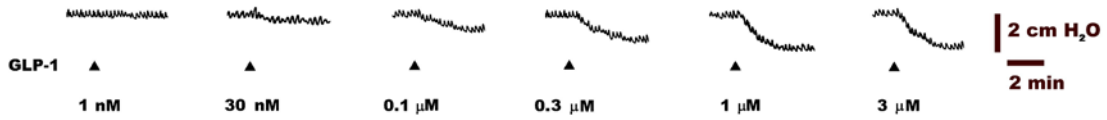


Fig. 16*. Original tracings showing the relaxation induced by increasing concentrations of GLP-1 on isolated mouse gastric preparations.*

The effect enhanced by increasing the concentration and the maximal response (2.2 ± 0.2 cm H_2O) was obtained at 1 μM GLP-1 (EC_{50} 0.1 μM; Cls= 0.06 – 0.15 μM, n=5) (Fig. 17).

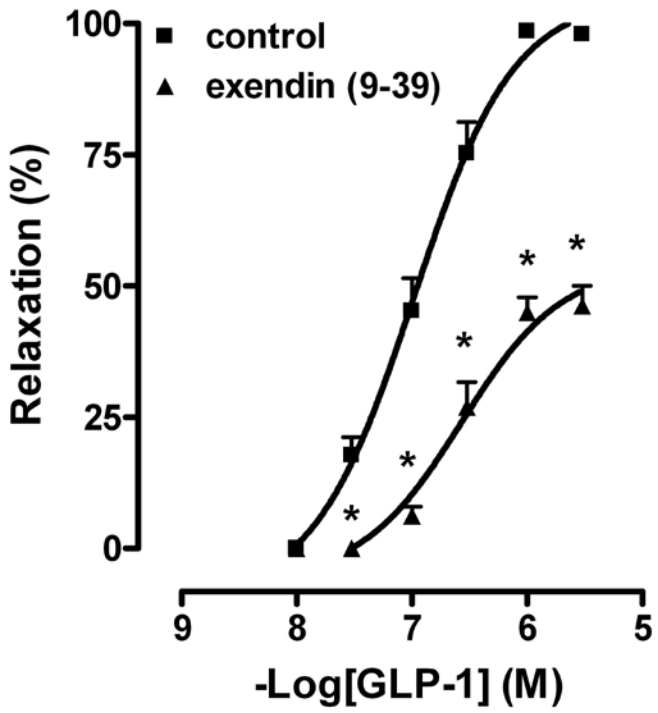


Fig. 17. *Concentration-response curves for the relaxant effects induced by GLP-1 alone or after treatment of the preparation with exendin (9-39) (300 nM) on isolated stomach. Data are expressed as a percentage of the maximum response obtained in the same tissue. Each value is mean ± SEM of 5 experiments. * P<0.05*

After pre-treatment with exendin (9-39) (300 nM), a GLP-1R antagonist, which per se did not affect the gastric mechanical activity, the relaxation induced by the peptide was significantly reduced (Fig. 17). Exendin (9-39) (300 nM) failed to affect the relaxation induced by isoproterenol (1 μM), being 3.9 ± 0.2 cm H_2O and 4.1 ± 0.1 cm H_2O (n=5) before and after treatment, respectively. Moreover, the effect of a submaximal concentration of GLP-1 (0.3 μM) was abolished in presence of TTX (1 μM), blocker of neuronal voltage-dependent Na^+ channels, or L-NAME (300 μM), an inhibitor of the NOS (Fig.18).

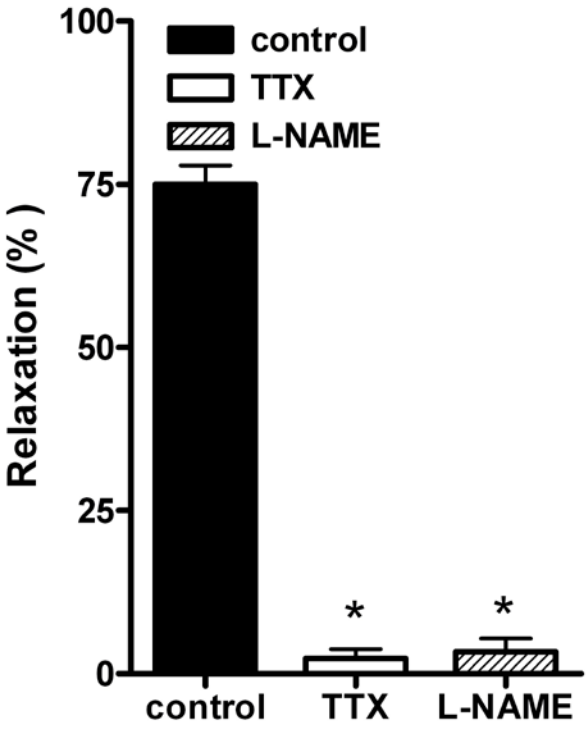


Fig. 18. *Effects of TTX (1 µM) or L-NAME (300 µM) on the relaxation induced by GLP-1 (0.3 µM). Data are means ± SEM (n= 4 for each treatment) and are expressed as a percentage of the 1 µM GLP-1 response induced in the same tissue. *P< 0.05 when compared to control.*

Effects of GLP-1 on antral and fundic muscular strips

Because GLP-1 did not induce any effect on mechanical activity of circular antral or fundic strips, gastric muscular strips were precontracted with CCh. In both preparations, CCh (10 µM) caused a sustained contraction with a plateau that persisted until washout. In CCh-precontracted fundic strips GLP-1 (1 µM) had no effects, while in CCh-precontracted antral strips GLP-1 (1 µM) was able to induce concentration-dependent inhibition of the response to CCh, which was antagonized by exendin (9-39) (300 nM) (Fig. 19). The GLP-1 effect on antral strips was abolished by TTX (1 µM) or L-NAME (300 µM) (Fig. 20).

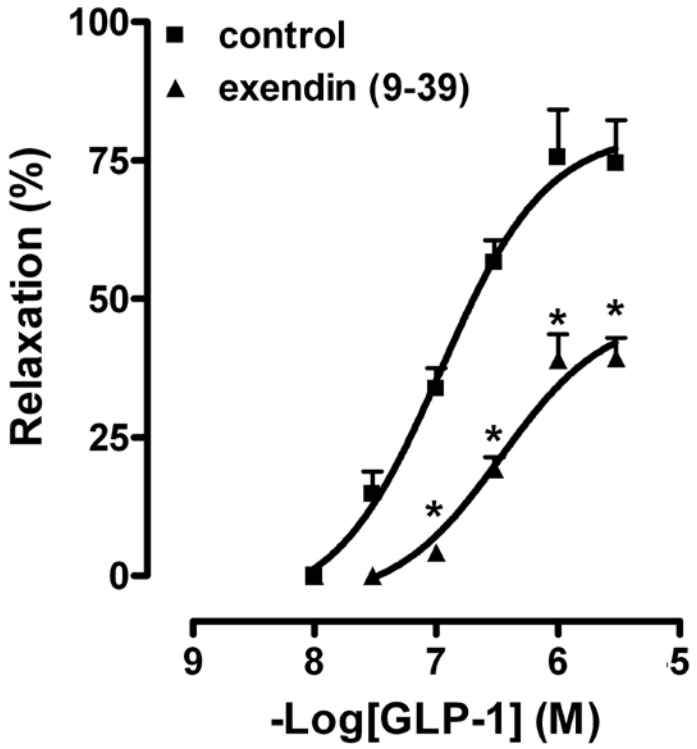


Fig. 19. *Concentration-response curves for the relaxation induced by GLP-1 in CCh-precontracted antral muscular strips before and after exendin (9-39) (300 nM). The response is expressed as percentage inhibition of the contractile response to CCh (10 μM). Each value is the mean ± SEM (n=5). *P< 0.05 compared with the respective control conditions.*

GLP-1R gene expression

RT-PCR performed on whole stomach, antrum and fundus revealed the presence of a 251 bp mRNA encoding the GLP-1R with expression of a β–actin PCR product (286 bp) as standard. RT-PCR analysis revealed a greater expression of GLP-1R mRNA in antrum than in fundus (Fig.21).

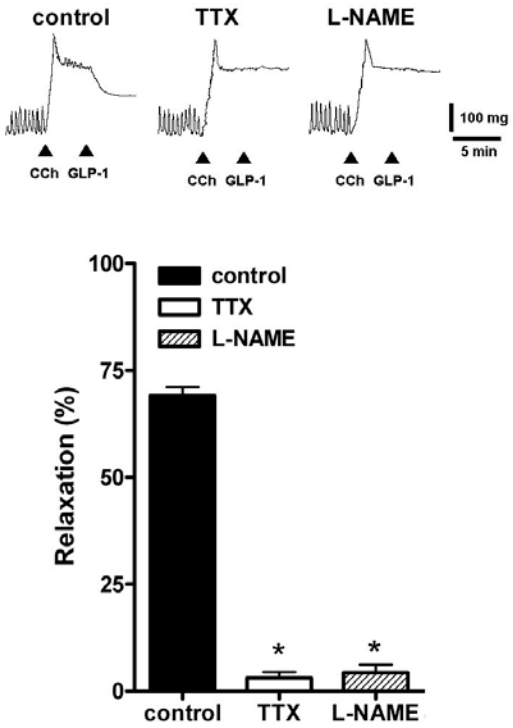


Fig. 20. *Relaxation induced by GLP-1 (1 µM) in antral circular strips precontracted by carbachol (CCh) (10 µM) before and after treatment with TTX (1 µM) or L-NAME (300 µM). The response is expressed as percentage of inhibition of the contraction evoked by CCh. Each value is the mean ± SEM of 4 experiments. *P< 0.05 when compared to control.*

Discussion

The results of the present study provide evidence for ability of exogenous GLP-1 of reducing mouse gastric motility, through a peripheral action on the antral region. The effect is mediated by neural NO release.

It is well recognized that exogenous GLP-1 and GLP-1R agonists slow gastric emptying (Schirra et al., 2000; Schirra et al., 2002; Meier et al., 2003; Little et al., 2006) and recent findings indicate that also endogenous GLP-1 plays a physiological role to slow gastric emptying (Shirra et al., 2006; Shirra et al., 2009; Deane et al., 2010). However, it is not clear how this effect is brought about. Experiments in rats (Imeryuz et al., 1997; Tolessa et al., 1998; Gulpinar et al., 2000; Naslund et al., 2002) and pigs (Wettregren et al., 1998; wettergren et al., 1998b) suggest that effects of GLP-1 on gastrointestinal motility are exerted through interaction with centres in the brain or afferent neural pathways, which in turn reduce the vagal efferent output. Moreover,

hypothesis for GLP-1 mechanisms acting directly on the enteric nervous system has also been advanced (Tolessa et al., 1998; Amato et al., 2010b)

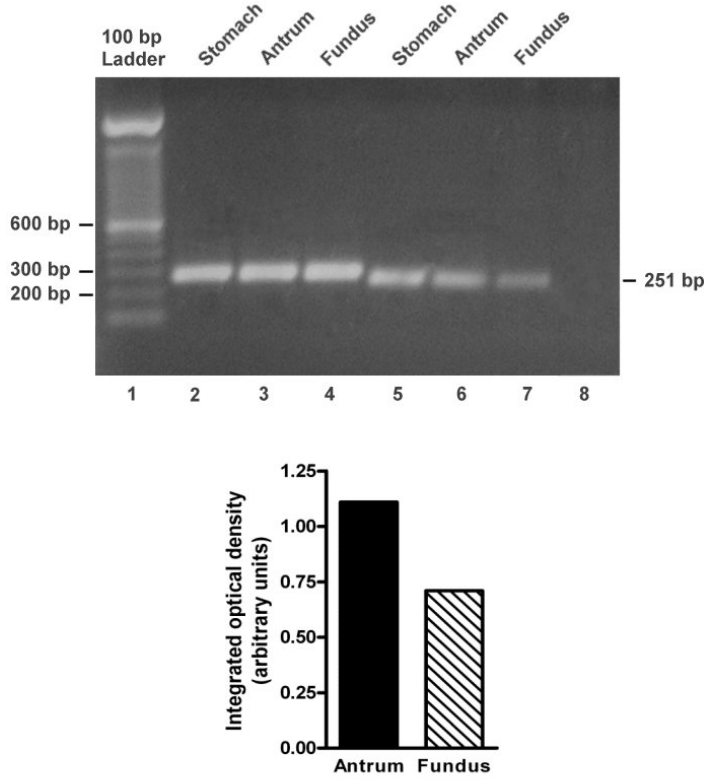


Fig. 21. *Detection of GLP-1R mRNA expression levels in mouse whole stomach, antrum and fundus by RT-PCR. A product of 251 bp mRNA corresponding to GLP-1R was detected in each sample. The expression of β-actin (286 bp) was used as a common reference PCR product. Lane 8 shows negative control obtained without addition of cDNA. Densitometric analysis showed GLP-1R mRNA higher expression levels in antrum compared to fundus. Data are represented as the ratio of optical density of GLP-1R to β-actin. One of three identical experiments is shown.*

Our results show that GLP-1 is able to induce a concentration-dependent reduction of mouse gastric tone in absence of extrinsic hormonal or neural influence. This observation confirms that GLP-1 action is not mediated by insulin or somatostatin release, as reported by Tolessa and collaborators in the small bowel motility (Tolessa et al., 1998b), and suggests the occurrence of peripheral action sites in the mouse stomach. This conclusion is in agreement with our recent studies showing that activation of peripheral GLP-1 receptors, expressed in mouse duodenal and colonic enteric neurons, mediates an inhibitory effect on motility of small and large intestine, independently on the extrinsic neural control (Amato et al., 2010bOn the other hand, the GLP-1-induced gastric relaxation was significantly antagonized by exendin 9-39, a

specific GLP-1R antagonist, suggesting the specificity of the observed effect. Moreover, the observation that exendin 9-39 *per se* failed to affect the gastric tone implies that GLP-1R is not tonically activated at least in the mouse stomach.

Indeed previous studies in vitro have shown that in human or rat gastric strips GLP-1 did not affect the smooth muscle contractility of the proximal stomach (fundus and corpus) (Tolessa et al., 1998; Naslund et al., 2001). The discrepancy between the previous observations and our results could be due to the different species analysed (mouse vs human or rat) or differences in the gastric region analysed. In fact, a peripheral direct mechanism could be a peculiarity of the mouse since recent findings suggested the involvement of stomach GLP-1 receptors in the inhibitory action of GLP-1 on gastric emptying and provided evidence for a genetic component in the regulation of gastric emptying in this animal model (Kumar et al., 2008). Moreover, previous studies leading to the conclusion that the peptide does not modulate gastric motility through a peripheral action, analysed GLP-1 effects in vitro in strips from gastric fundus or corpus (Tolessa et al., 1998; Naslund et al., 2001). Indeed, our experiments using muscular strips showed that GLP-1 failed to affect the spontaneous mechanical activity of fundus, but it was able to relax pre-contracted antral strips suggesting that the site of GLP-1 action may be represented by gastric antrum. To substantiate our findings we performed expression analysis of GLP-1R mRNA in wall tissue from gastric antrum and fundus. We found GLP-1R mRNA in both regions of stomach, which could be due to the expression of the receptor on gastric pits and parietal cells, as reported in rat stomach (Schmidtler et al., 1994; Bullock et al., 1996). However, the increased presence of GLP-1R in the distal part of the stomach might reflect the GLP-1 ability to inhibit antral motility and, consequently, the gastric emptying. In fact, whereas the gastric fundus and proximal gastric body largely serve as a passive reservoir, the distal stomach is more electrically active, generating intense peristaltic contractions, responsible for the gastric emptying rate. Therefore, we hypothesize that the slowing of gastric emptying induced by GLP-1 previously reported (Imeryuz et al., 1997; Anvari et al., 1998; Tolessa et al., 1998; Wettergen et al., 1998; Schirra et al., 2000; Naslund et al., 2001) may be due to peripheral inhibitory action of GLP-1 on antral contractility besides the modulation of neurons in the central nervous system or vagal neural pathways.

Moreover, we analysed also the mechanism by which GLP-1 was able to inhibit gastric mechanical activity. The observation that TTX, a blocker of neuronal voltage-dependent Na^+ channels, abolished the inhibitory effects of the peptide in whole

stomach or in pre-contracted antral strips suggests that neurons within the intramural plexuses are responsible for the action of GLP-1. Our finding is in agreement with other studies which show GLP-1 acts by neural mechanisms (Imeruyz et al., 1997; Tolessa et al., 1998; Daniel et al., 2002). Because NO is the main inhibitory neurotransmitter mediating gastric muscular relaxation in the mouse (Mulè & serio, 2002) we examined the possible involvement of nitrergic pathway in the mechanism underlying the inhibitory responses induced by GLP-1 using L-NAME, a blocker of NO synthesis. The inhibitory effect of the peptide was abolished in the presence of L-NAME, suggesting that it was mediated by NO production. Indeed recent evidence suggest that several effects of GLP-1 e.g., inhibition of fasting motility in rat small bowel small bowel motility (Tolessa et al., 1998b; Naslund et al., 2002), inhibition of mouse small and large intestine evoked mechanical responses (Amato et al., 2010b and increase in human postprandial gastric accommodation (Andrews et al., 2007b)are mediated by NO.

In conclusion, exogenous GLP-1 is able to act peripherically in mouse stomach and to inhibit antral propulsion through neural NO release. This action might contribute to slow gastric emptying and to cause indirectly reduction of food intake in well agreement with its anorexigen effects (Flint et al., 1998; Gutwiller et al., 1999).

III° ARTICLE

EFFECT OF THE GLP-1 ANALOGUE LIRAGLUTIDE ON SATIATION AND GASTRIC SENSORIMOTOR FUNCTION DURING NUTRIENT DRINK INGESTION

Disclosure

The experiments including in this paper have been submitted for publication in the Journal *Alimentary Pharmacology and Therapeutics* (Rotondo et al., 2011). Some of the experiments described have previously been published in abstract form (Rotondo et al., 2011; Rotondo et al., 2011b; Rotondo et al., 2011c).

Summary

The purpose of this paper was to verify if its stable analogue, liraglutide, can influence gastric motility and satiation as GLP-1 by measuring the intragastric pressure (IGP) during nutrient drink consumption by High Resolution Manometry technique and using barostat technique. 10 healthy volunteers (HVs) were tested after placebo, 0.3, 0.6 or 1.2 mg liraglutide administration. After a stabilization period intragastric infusion of a nutrient drink (1.5 kcal/ml) started at 60 ml/min and the HVs scored their satiation on a graded scale until the infusion was stopped. Our results showed that the highest dose of liraglutide induced nausea, while the lower doses were well-tolerated. During nutrient drink infusion IGP decreased indicating gastric relaxation. The average IGP decrease was dose-dependently suppressed by liraglutide and this value was significant after 0.6 mg treatment. Although liraglutide did not affect satiation feelings, the maximum tolerated volume of nutrient drink was significantly different in 1.2 mg liraglutide treatment (695±135 ml) compared to placebo (912±133 ml).

Barostat study showed that liraglutide does not affect perception or compliance, but significantly decreases gastric accommodation to the meal (168±27 ml for placebo vs. 78.8±36.4 ml for 0.6 mgliraglutide, $p<0.05$). In conclusion, we have demonstrated using

two different technique that liraglutide inhibits gastric accommodation and this could represent the mechanism by which GLP-1 analogues induce loss weight.

Introduction

Food intake can be regulated via satiety signals from the gut that directly influence brain centres, as the hypothalamus and the brainstem (Cone et al., 2001; Delgado-Arros et la., 2005; Schwartz, 2006; Wise, 2006). These signals include circulating levels of orexigenic and anorexigenic gut peptides, and neural signals through vagal afferents(Janssen et al., 2011). Mechanosensitivity of the stomach is believed to play an important role in the regulation of food intake (Janssen etal., 2011). During food intake the muscles of the stomach relax so that the intragastric pressure (IGP) does not increase despite the large volumes of food we sometimes consume. This reflex relaxation, also referred to as gastric accommodation is believed to play a role in the regulation of food intake. Indeed, we previously showed that food intake is decreased in functional dyspeptic patients with impaired gastric accommodation (Tack et al., 2003).

Glucagon-like peptide-1 (GLP-1) is a gastrointestinal regulatory peptide secreted from the mucosal endocrine L cells in response to the nutrient ingestion (Schirra et al., 1996). GLP-1 acts mainly as an incretin, enhancing glucose-stimulated insulin secretion and glucose homeostasis (Fehmann & Habener, 1992). In addition, GLP-1 acts as an anorexigenic peptide decreasing hunger feelings. In animal models, intracerebroventricular injection of GLP-1 inhibits food intake, and intravenous infusion decreases food intake in normal weight and obese humans (Flint et al., 1997; Gutzwiller et l., 1998; Van Dijk & Thiele, 1999). GLP-1 reduces postprandial glycemia, not only through its hormonal effects, but also by its effects on gastrointestinal motility. GLP-1 is a mediator of the ileal brake (Giralt & Vergara, 1999), and administration of liraglutide slows solid and liquid gastric emptying, reduces gut motility, inhibits antro-pyloro-duodenal motility, enhances gastric accommodation (Wettergren et al., 1993; Imeryuz et al., 1997; Anvari et al., 1998; Tolessa et la., 1998; Schirra et al., 2000; Naslund et al., 2001; Delgado-Arros et al., 2002; Miki et al., 2005; Andrews et al., 2007; Andrews et al., 2007b; Amato et al., 2010b; Rotondo et al., 2011).

Since native GLP-1 has a short elimination half-life of 1–2 min due to rapid metabolism by the widely distributed enzymes dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase 24.11 (NEP) (Drucker & Nauck, 2006), one method currently being

pursued for the therapeutic targeting of GLP-1 receptors is the production of GLP-1 analogs that are resistant to degradation by DPP-IV and NEP.

Liraglutide is a GLP-1 analog with an additional 16-carbon fatty acid and a small amino acid based spacer which confers reversible binding of the agonist to albumin and increases resistance to DPP-IV activity, providing liraglutide with a half-life of approximately 13 hours (Knudsen et al., 2000). Although *in vitro* studies suggest that liraglutide binds to GLP-1 receptors with similar potency as native GLP-1(Knudsen et al., 2000), clinical data indicate that the spectrum and magnitude of actions of GLP-1 and liraglutide are not identical. These differences may be attributed to differences in concentrations but potentially also to different binding profiles (Knodsen, 2000). Several studies have shown that daily administration of liraglutide (1.8 mg/day) is associated with decreased food intake and weight loss (Astrupt et a., 2009; Garber et al., 2009; Naucke et al., 2009; Zinman et al., 2009). The mechanism underlying weight loss with liraglutide treatment remains to be elucidated, as delay in gastric emptying, sensations of early satiation and a decreased sense of appetite have all been implicated (Janssen et al., 2011).

The aim of this study was to clarify the mechanisms underlying liraglutide's effect on food intake by studying the influence of acute administration of liraglutide on gastric sensorimotor function and meal-induced satiation in humans. We measured the effects of liraglutide on intragastric pressure (IGP) during intragastric nutrient drink infusion and on the compliance and the relaxation of the proximal stomach during a barostat study.

Material and methods

Study subjects:

10 Healthy volunteers (HVs) (7 men, age 33±9.7 years, BMI 23.2±2.2 kg/m2) participated in the study. All study procedures were approved by the Ethics Committee of the Leuven University Hospital, Belgium. All partecipants gave written, informed consent. Exclusion criteria included the presence of symptoms or a history of gastrointestinal diseases, diabetes, any other significant disease or psychological disorder. Moreover, since liraglutide could decrease blood glucose concentrations we especially excluded volunteers that suffered from hypoglycemia or took any drugs that are known to decrease blood glucose concentration. HV's came to the motility unit after at least 6 hours fasting. HV's were asked to refrain from alcohol, the and coffee at

least 12 hours before participation, moreover they were asked to refrain from smoking cigarettes at least 1 hour before the start of the experiment.

Treatments

Liraglutide (Victoza®, Novo Nordisk, Belgium; 0.3, 0.6 and 1.2 mg) was administered as an abdominal subcutaneous (SC) injection 12-20 hours before the start of each experiment, hence on the day preceding the experiment (Malm-Erjefalt et la., 2010; Kapitza et al., 2011). A washout period of at least 1 week was respected between treatments and the order of the respective treatments was determined at random. For the placebo arm of the study we used saline (SC injection in the abdomen).

IGP measurement during intragastric nutrient drink infusion

IGP was assessed using a high-resolution solid-state manometry system (36 channels, 1 cm in between each channel, Manoscan 360; Sierra Scientific Instruments, Los Angeles, CA, USA; Manoview analysis software v2.0.1). Upon arrival in the clinic the manometer was positioned through the nose so that at least one sensor was positioned in the lower esophageal sphincter (LES; detected as a clearly elevated pressure zone compared to oral and aboral areas), while IGP was measured using the average pressure of the first five pressure channels that were clearly positioned below the LES or the pressure area influenced by the LES (approximately 3–8 cm under the position of the LES determined from the high-resolution plot) (Janssen et al., 2011b). An infusion catheter (Flocare; Nutricia, Bornem, Belgium) was positioned in the stomach through the mouth. The tip of the infusion catheter was positioned approximately 5 cm under the LES and its position was verified by fluoroscopy. From preliminary experiments we found that the position of the infusion catheter (with the tip in the proximal or distal stomach) does not affect the IGP during intragastric nutrient drink infusion.

After positioning the catheters, and after a stabilization period of at least 30 minutes intragastric infusion of a nutrient drink (Nutridrink, Nutricia; 630 KJ, 6g proteins, 18.4g carbohydrates, and 5.8g lipids/100mL) started at a constant speed of 60 mL per minute (determined by an automated system using a peristaltic pump) (Janssen et al., 2011b). At 1-minute intervals, the subjects were asked to score their satiation using a graphic rating scale that combines verbal descriptors on a scale graded of 0–5 (1, threshold; 5, maximum satiety). In addition, at 5-minute intervals the HVs were asked to fill out a

visual analogue scale for 11 epigastric symptoms. Intragastric infusion was stopped as soon as the volunteers scored maximal satiation. Ten minutes thereafter the catheters were disconnected and removed and the volunteers could leave the unit.

Sensitivity to gastric distention and gastric accommodation assessed with a barostat

Based on the IGP experiments we selected the 0.6 mg liraglutide dose for further investigations with the barostat. Sensitivity to gastric distention and gastric accommodation to a meal were studied using a gastric barostat, as previously described (Tack et al., 1998; Tack et al., 2001). Upon arrival in the clinic, a double lumen polyvinyl tube (Salem sump tube 14Ch; Sherwood Medical, Petit-Rechain, Belgium) with an adherent plastic bag (1200 ml capacity; 17 cm maximal diameter) finely folded, was introduced through the mouth and secured to the subject's chin with adhesive tape. The position of the bag was checked fluoroscopically. The polyvinyl tube was then connected to a programmable barostat device (Synectics Visceral Stimulator, Stockholm, Sweden). To unfold the bag, it was inflated with a fixed volume of 300 ml of air for two minutes with the study subject in a recumbent position, and again deflated. Subjects were then positioned in a comfortable sitting position with knees bent (80°) and trunk upright in a specifically designed bed.

After a 30 minute adaptation period, minimal distending pressure (MDP) was first determined by increasing intrabag pressure by 1 mmHg every 3 minutes until a volume of 30 ml or more was reached(Notivol et al., 1995). This pressure level equilibrates the intra-abdominal pressure. Subsequently, isobaric distentions were performed in stepwise increments of 2 mmHg starting from MDP, each lasting two minutes, while the corresponding intragastric volume was recorded. Subjects were instructed to score their perception of upper abdominal sensations at the end of every distending step, using a graphic rating scale that combined verbal descriptors on a scale graded 0–6. Additionally we evaluated the intensity of 9 upper abdominal symptoms (discomfort, fullness, nausea, bloating, heartburn, belching, epigastric pain, satiety and abdominal cramps) using 10 cm visual analogue scale (VAS). The end point of each sequence of distentions was established at an intrabag volume of 1000 ml or when the subject reported discomfort or pain (score 5 or 6).

After a 30 minute adaptation period with the bag completely deflated, the pressure level was set at MDP + 2 mmHg for at least 90 minutes. After 30 minutes, a liquid meal (200

ml, 300 kcal, 13% proteins, 48% carbohydrates, 39% lipids; Nutridrink, Nutricia) was administered. Gastric tone measurement was continued for 60 minutes after the meal. At 5 min intervals VAS scores were completed to evaluate the intensity of 9 abdominal symptoms. Hereafter the balloon was retracted and the volunteers could leave the hospital.

Data analysis:

IGP: The original data were imported from the recording software to Excel®. We were primarily interested in slow IGP changes that could reflect changes in gastric muscle tone. Therefore, and in order to avoid influence from movement artifacts and artifacts caused by coughing, sneezing, moving, or swallowing a moving median was calculated per channel from the original data (median value over 1 min of original data). Per channel, a baseline value was calculated from the moving median data as the average pressure in the last 5 min of the stabilization period. Data were presented per minute as the difference of the minimum moving median value in that minute and the baseline value. Data were presented as mean± SEM and compared with a two-way ANOVA ($P<0.05$ was considered significant).

Barostat: The perception threshold was defined as the first level of the intraballoon pressure (during stepwise ramp distentions) and the corresponding volume that evoked a perception score of 1 or more. The discomfort threshold was defined as the first level of the intraballoon pressure and corresponding volume that provoked a perception score of 5 or more (Tack et al., 2001). Pressure thresholds were expressed as pressures relative to MDP. Based on previous studies, gastric compliance was calculated as the slope of the pressure–volume curve obtained by stepwise ramp distentions ($mL*mmHg^{-1}$) (Tack et al., 1998). Similarly, pressure–perception curves were obtained from the stepwise distentions. Comparisons were perfomed using a t-test and mixed model analysis for the accommodation data. Data are presented as mean±SEM ($P<0.05$ was significant).

During the evaluation of meal-induced fundic relaxation at MDP+2mmHg, the mean intraballoon volume was calculated over consecutive 5-min intervals. To quantify the effect of liraglutide on gastric accommodation, gastric relaxation was quantified as the difference between the average volume during the 60min after the meal and the last 10min preprandially, during drug administration. For each symptom evaluated during barostat studies and meal-induced satiety testing, the cumulative symptom score was obtained by adding scores at each individual time point and expressed as area under the curve (AUC). The symptom scores were compared using Student's t-test.

Results

Conduct of the study

The lower doses of liraglutide were well-tolerated, but the highest dose tested (1.2 mg) induced nausea and vomiting in 4/5 volunteers initially dosed during the IGP studies. This dose was therefore eliminated for all subsequent volunteers.

Manometry

IGP during nutrient drink infusion

0.3 and 0.6 mg liraglutide did not affect meal-induced satiation, while 1.2 mg tended to increase satiation scores in the 5 subjects receiving this dose (P=0.45; Fig.22).

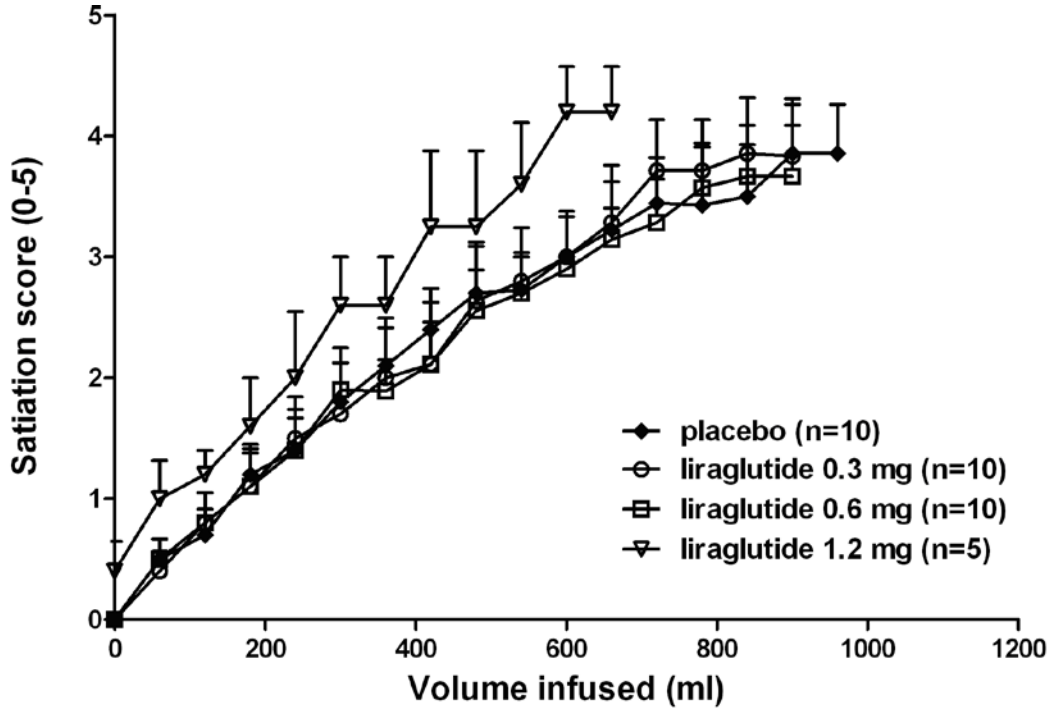
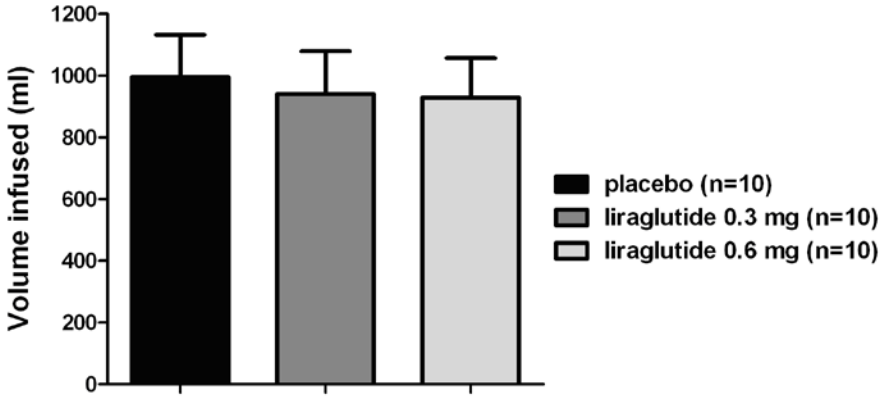


Fig.22. *Influence of liraglutide (0.3 or 0.6 or 1.2 mg) and placebo on meal-induced satiety scores during nutrient drink infusion. Results presented as mean ± SEM*

The maximum nutrient volumes tolerated by the volunteers were 912±133, 851±144, 847±116 after placebo, 0.3 and 0.6 mg liraglutide, respectively (Fig. 23A). When separately analyzing the maximum tolerated volume in the 5 subject that received 1.2

mg liraglutide vs. placebo a significant difference could be observed (694±135 vs 1008±197 ml, respectively, P<0.05; Fig. 23B).

A



B

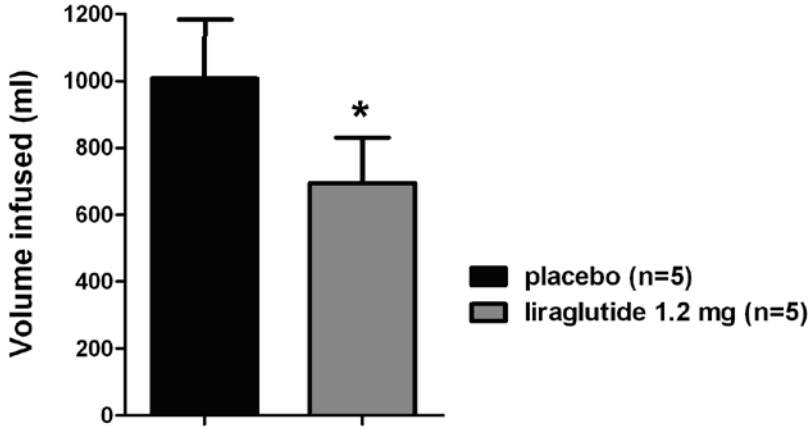


Fig.23 A *Maximum tolerated volume during intragastric nutrient drink infusion. Results presented as mean ± SEM.* ***B*** *Maximum tolerated volume during intragastric nutrient drink infusion. Results presented as mean ± SEM. *P<0.05 compared to the placebo value in the 5 subjects receiving 1.2 mg.*

In all treatment groups IGP decreased initially during nutrient drink infusion and gradually increased thereafter (Fig. 24). The average pressure during nutrient drink infusion was 4.1±0.7, 3.0±0.4, 2.1±0.3 and 2.6±0.4 mmHg after placebo, 0.3, 0.6 and 1.2 mg liraglutide respectively (placebo vs 0.6 mg liraglutide P<0.05; Fig.25).

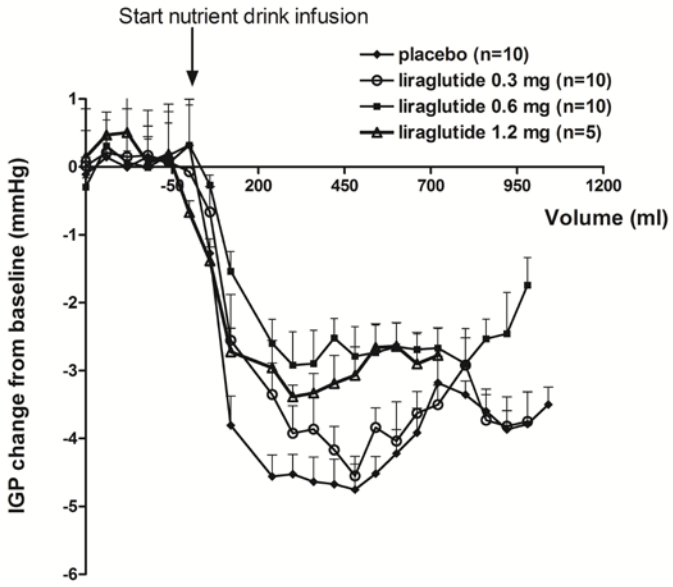


Fig. 24 *Intragastric pressure changes during intragastric nutrient drink infusion. Intragastric pressure (IGP) change from baseline pressure during intragastric nutrient drink infusion. Nutrient drink started at T=0 minutes.*

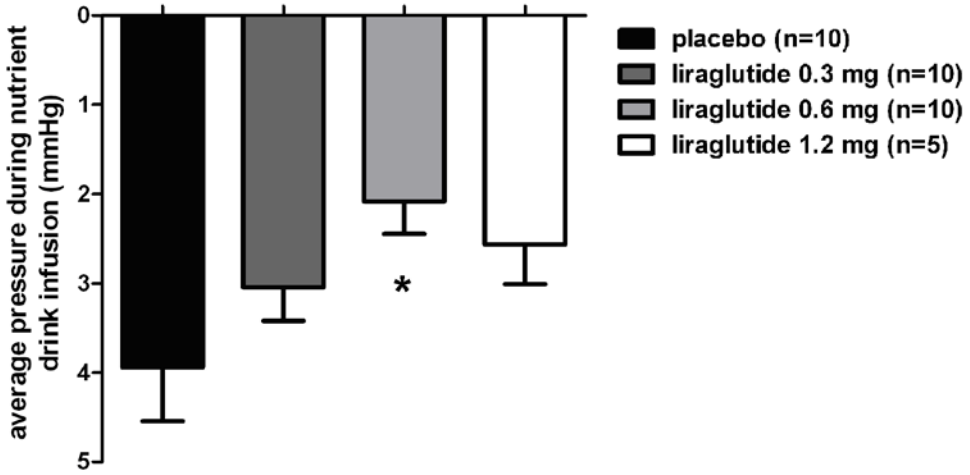


Fig. 25*. Average pressure during intragastric nutrient drink infusion. *P<0.05*

The pressure drop during nutrient drink infusion was 5.5±0.7, 4.9±0.6, 3.8±0.5 and 4.1±0.8 mmHg after placebo, 0.3, 0.6 and 1.2 mg liraglutide respectively (Fig.26).

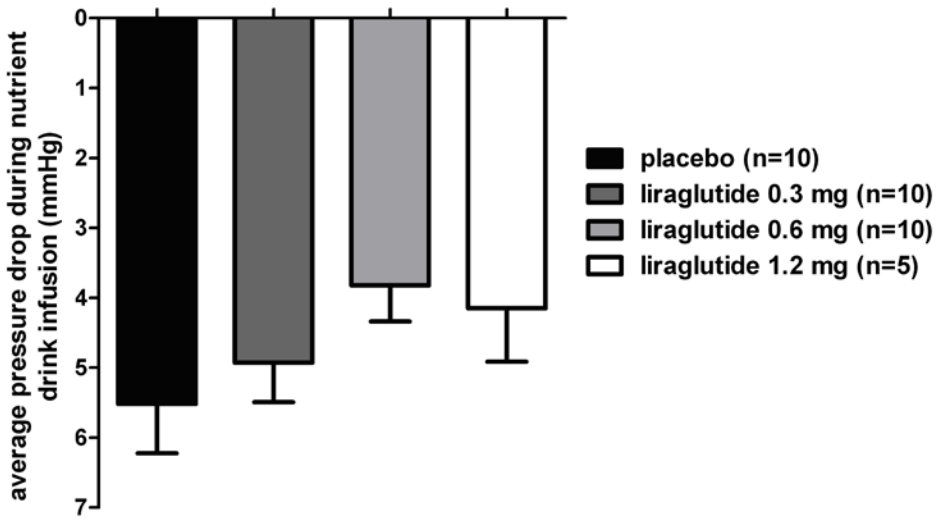


***Fig. 26**. Average pressure drop during intragastric nutrient drink infusion.*

During intragastric nutrient drink infusion 0.3 and 0.6 mg liraglutide did not significantly influence VAS scores for any of the 7 abdominal symptoms. 1.2 mg liraglutide was associated with higher scores for nausea, vomiting, early satiation and decreased appetite scores (Table 1; P<0.05).

	BEFORE NUTRIENT DRINK			AFTER NUTRIENT DRINK		
	Placebo	LG	P-value	Placebo	LG	P-value
Appetite	63.2 ± 13.6	45.3 ± 7.9	P=0.19	24.1 ± 9.6	11.3 ± 6.4	P=0.50
Satiation	55.5 ± 4.4	33.3 ± 8.5	P=0.39	65.5 ± 4.4	73.8 ± 12.5	P=0.88
Vomiting	4.5 ± 0.2	3.6 ± 2.7	P=0.34	40.1 ± 10.2	48.6 ± 10.5	P=0.7
Nausea	0.4 ± 0.4	2.5 ± 1.7	P=0.41	1.1 ± 2.9	11.1 ± 1.9	P<0.05

Table 1 Influence of placebo and 1.2 mg liraglutide (LG) on appetite, satiation, vomiting and nausea in 5 healthy volunteers before and directly after nutrient drink infusion (P<0.05). Data are expressed as percentage (%) of a VAS scale (10 mm =100%)

Barostat

The MDP did not differ between both study conditions (7.0±0.4 vs. 7.7±0.4 mmHg after placebo and liraglutide 0.6 mg pretreatment respectively). Liraglutide pretreatment tended to enhance fasting gastric compliance (P=0.05), but the pressure thresholds for first perception or discomfort during gastric distentions, and the corresponding intra-balloon volumes, were similar for both study conditions (Table 2).

	Placebo	LG	P-value
MDP (mmHg)	7 ± 0.4	7.7 ± 0.4	P=0.35
Gastric compliance premeal (mL $mmHg^{-1}$)	51.8 ±10.1	78.6 ± 7	P=0.05
Preprandial intraballon volume (mL)	236.8 ± 24.7	211.6 ± 24.04	P=0.57
Postprandial intraballon volume (mL)	362.9 ± 41.06	274.8 ± 33.9	P<0.0001
Perception threshold (mmHg above MDP)	2±0.42	2±0.37	P=0.35
Discomfort threshold (mmHg)	10.8 ± 0.8	9.6 ± 0.8	P=0.77
Volume at first perception (mL)	157.6± 17.8	132.6 ±17.3	P=0.32
Volume at discomfort (mL)	588.4 ±68.3	645.10 ± 45.7	P=0.50

Table 2 Influence of placebo and 0.6 mg liraglutide (LG) on gastric compliance, accommodation and perception of gastric distention in 10 healthy volunteers. Data are expressed as percentage (%) of a VAS scale (10 mm =100%)

Preprandial balloon volumes did not differ between both conditions. However, the postprandial volume was lower after liraglutide (P<0.0001; table 2). Gastric accommodation, was significantly inhibited by liraglutide (168±27 vs. 78.8±36.4 mL, P<0.05; Fig.27).

Liraglutide did not significantly influence the VAS scores for any of the 9 epigastric symptoms during the postprandial period or during isobaric pressure distentions (details not shown).

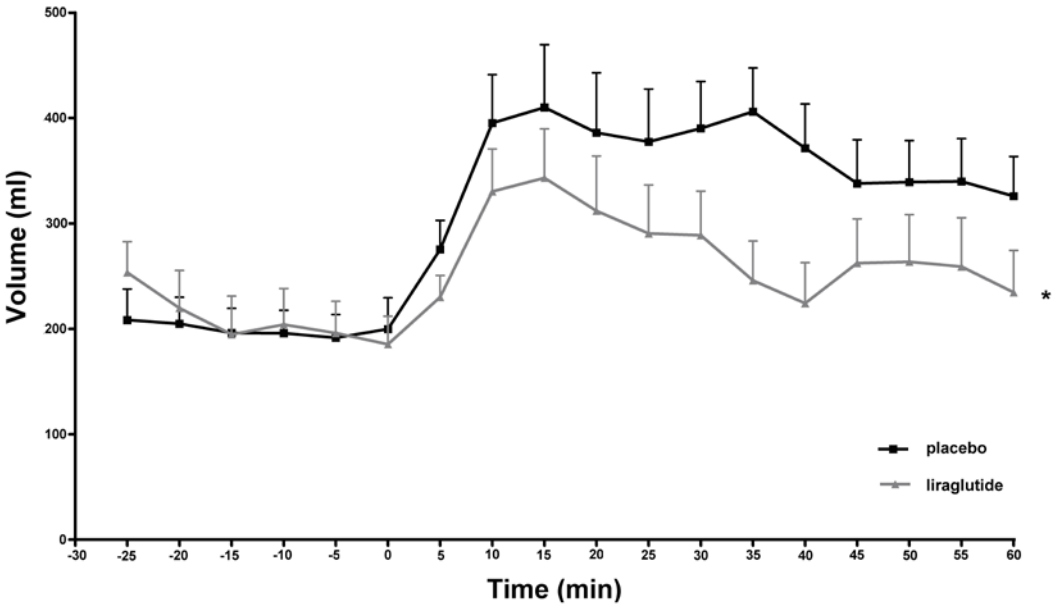


Fig. 27 *Influence of liraglutide or saline on intraballoon volumes before and after the meal. Meal-induced relaxation was significantly inhibited by liraglutide. *P< 0.05.*

Discussion

In this study we used 2 different techniques to demonstrate for the first time that low doses of the GLP-1 analogue liraglutide inhibit gastric accommodation in man without significantly affecting satiation. 1.2 mg liraglutide significantly increased satiation, but this is most likely due to the side effects observed with this dose.

Gastric accommodation is a reflex that enhances the storage capacity of the stomach during food intake. Indeed, in between meals the proximal stomach is characterized by a basal muscle tone, however during food intake the muscle tone decreases, a reflex that is mainly mediated via parasympathetic nerves, decreasing the contractile cholinergic input while activating the release of nitric oxide (NO). Gastric accommodation increases the compliance of the stomach muscles and thereby increases the storage capacity of the stomach while keeping IGP low (Moragas et al., 1993; Kindt & Tack 2006). The gastric barostat is regarded the golden standard to assess gastric accommodation (Azpiroz & Malageda , 1985; Azpiroz & Malageda , 1987; Azpiroz & Malageda , 1990) in healthy subjects and dyspeptic patients. However it should be emphasized that the procedure is invasive, time consuming, and uncomfortable, limiting its feasibility in routine clinical practice. In addition the presence of a gastric balloon has been shown to interfere with normal gastric physiology, as the direct stimulus

imposed by the balloon on the proximal stomach wall may alter intragastric distribution of the meal and may result in exaggeration of antral relaxation (Mundt et al., 2002). These limitations have served as an impetus to develop various alternative, less invasive diagnostic techniques which assess gastric accommodation.

We previously showed that gastric accommodation can be assessed using IGP measurement during intragastric nutrient infusion. This method was perceived as less invasive compared to the barostat and provides a more physiological alternative for the barostat, especially to assess gastric accommodation during food intake (Janssen et al., 2011bis). IGP during nutrient infusion decreases rapidly and gradually recovers upon continuous nutrient infusion, indicating gastric relaxation upon nutrient infusion (Janssen et al., 2011bis).

Liraglutide dose-dependently reduced the maximum IGP decrease upon nutrient infusion, indicating impaired gastric accommodation. This finding is confirmed using the barostat where we indeed showed that 0.6 mg liraglutide significantly decreased the postprandial balloon volume increase. These results were unexpected since it was previously shown that GLP-1 increased both fasting and postprandial gastric volume in humans (Delgado-Aros et al., 2002; Andrews et al., 2007; Andrews et al., 2007bis) using single photon emission computed tomography (SPECT) (Delgado-Aros et al., 2002; Andrews et al., 2007; Andrews et al., 2007bis). SPECT combines imaging of the gastric wall using intravenously administered ^{99m}Tc pertechnetate. The image analysis obtained provides a measure of gastric volume however it is unclear whether volume changes represent changes in gastric tone. A validation study where subjects underwent SPECT and barostat on separate occasions showed poor correlation between the two techniques with respect to meal induced accommodation (Van den Elzen et al., 2003). In addition, meal-induced accommodation assessed by SPECT did not differ from ingested meal volumes. It was concluded that in the absence of a distending pressure, gastric volumes determined by SPECT scanning reflect ingested volumes rather than gastric relaxation and this is likely to account for the poor correlation between the two techniques (Van den Elzen et al., 2003). Moreover some authors suggested that GLP-1 may increase postprandial gastric volume by slowing gastric emptying, however it is known that GLP-1 reduces gastric secretion (Wettergren et al., 1994; Wettergren et al., 1997) which would be anticipated to reduce and not increase gastric volumes. Either

way, differences observed between the SPECT study and the present results might well be explained by these methodological differences.

Alternatively, differences between liraglutide and GLP-1 can be attributed to a slightly different pharmacological profile: the studies which analyse the effect of GLP-1 on gastric accommodation used physiological and supraphysiological doses (Delgado-Aros et al., 2002; Andrews et al., 2007; Andrews et al., 2007bis) , indeed although studies *in vitro* suggest that liraglutide binds GLP-1 receptors with similar potency as native GLP-1 (Knudsen et al., 2000), clinical data indicate that the spectrum and magnitude of actions of GLP-1 and liraglutide are not identical (Knudsen et al., 2000) then it is difficult to confirm if the used doses are similar regarding the binding with GLP-1 receptors.

A second readout of this study was satiation. We observed that the acute administration of low doses of liraglutide (0.3 mg or 0.6 mg) did not significantly alter meal-induced satiation during a nutrient meal challenge. Our results are in agreement with other studies demonstrating that chronic administration of low dose of liraglutide (0.6 mg daily) does not affect the appetite while higher doses (higher than 1.2 mg) decreased appetite in healthy volunteers and diabetic patients (Lee et al., 2004; Nauck et al., 2009; Zinman et al., 2009). In these studies, high doses of liraglutide were also associated with gastrointestinal adverse effects such as nausea, diarrhea and vomiting (Astrupt et al., 2009; Garber et al., 2009; Zinman et al., 2009). Also in our study 1.2 mg liraglutide significantly decreased the maximum tolerated volume of the nutrient test meal, most likely since this dose was associated with major nausea and vomiting.

Dyspeptic symptoms as nausea, vomiting, early satiation and decreased appetite are linked to weight loss (Lee et al., 2004). Several clinical studies have demonstrated that chronic administration of liraglutide at doses greater than 1.2 mg caused weight loss and this has been attributed to a delay in gastric emptying and to sensations of early fullness or satiation (Harder et al., 2004; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009). The effect of liraglutide on gastric motility has not been well studied, but some studies have demonstrated that low doses of liraglutide (0.6 mg/d and 6 μg/kg/d) had no significant effect on gastric emptying (Degn et al., 2004; Harder et al., 2004) whereas higher doses (1.8 mg/d and 10 μg/kg/d) caused a slight delay (Jhul et al., 2002; Malm-Erjefalt et al., 2010). On the other hand, it has not been established that delayed gastric emptying contributes to weight loss and abnormalities of gastric reservoir

capacity (gastric accommodation) were shown to be more closely associated with weight loss (Janssen et al., 2011). Indeed, we have previously shown that impaired gastric accommodation in functional dyspeptic patients is associated with early satiation and weight loss (Tack et al., 2003; Janssen et al., 2011). In the present study, impaired gastric accommodation was not correlated to increased satiation while 1.2 mg liraglutide decreased the volume consumed but also induced nausea. This study does not confirm a possible relationship between gastric accommodation and satiation.

In conclusion, low doses of liraglutide inhibit gastric accommodation in HVs while this was not associated with altered perception, discomfort thresholds to gastric distention or satiation. Administration of 0.3 or 0.6 mg of liraglutide was well tolerated however 1.2 mg induced nausea and vomiting and decreased the maximum volume nutrients consumed.

GENERAL DISCUSSION

In general, the experiments described in this thesis have provided evidence for the existence of GLP-1 functional receptors throughout the mouse gut.

Although it has been reported that the action of GLP-1 on food intake and intestinal motility involves neural central mechanisms and activation of reflex circuits (Imeryuz et al., 1997; Wettergren et al., 1997b; Tolessa et al., 1998; Tolessa et al., 1998b; Naslund et al., 2000; Daniel e al., 2002; Andrews et al., 2007b) the scarce information on the peripheral effects of the peptide on GI smooth muscle contractility, on the precise location of the receptors led us to investigate the presence of GLP-1 sites of action within the gut wall. Consequently, we chose to perform our experiments *in vitro,* using animal model (mouse) to examine motor effects of GLP-1 in the absence of any external influence.

Activation of peripheral GLP-1R by the exogenous peptide is able to induce gastric relaxation and reduction of cholinergic evoked contractions of the duodenal and colonic circular muscle suggesting an inhibitory role on gastrointestinal motility. This peripheral action could represent a further mechanism by which GLP-1 exerts its anorexigenic properties.

Indeed, the intestinal segments were mounted in a custom-designed horizontal organ bath, in order to record the distal endoluminal pressure (index of circular muscle mechanical activity) and the isometric tension (index of longitudinal muscle mechanical activity). The correspondence of the endoluminal pressure and tension recordings to the mechanical activity of circular and longitudinal muscle, respectively, has been previously demonstrated (Mulè et al., 1992; Mulè et al., 1999). In a such way the effects induced by exogenous application of GLP-1 on both muscular layer could be detected simultaneously. We found that activation of GLP-1R reduces electrically-evoked cholinergic contractions in mouse duodenal and colonic circular smooth muscle, without affecting longitudinal contractions. In addition, the experimental results have suggested that GLP-1 effect on circular muscular layer is mediated by nitrergic pathway stimulation that in turn inhibits acetylcholine release.

Although controversy (Smith & Robertson, 1998), the two muscular layers show different behavior during the propulsive activity (*peristalsis*), circular relaxation coupled with longitudinal contraction (Wood, 1998). Peristalsis, that is defined as a motor pattern involving partial or total occlusion of the lumen that moves content in the anal direction, is initiated by circular smooth muscles contracting behind the luminal material to prevent it from moving back, followed by contraction of longitudinal smooth muscles which pushes the digested food forward (Huizinga & Lammers, 2009). Peristaltic movement is orchestrated by the enteric nervous system in response to a bolus, where contraction is evoked oral to the bolus and relaxation is observed anal to the bolus. The peristaltic reflex has been studied extensively experimentally (Costa et al., 2000) and directional neural pathways (ascending excitatory reflexes that evoke contractions above a bolus; descending inhibitory reflexes which cause relaxations below) to elicit this response have been firmly established. Since contraction of the circular muscle is dominant in peristalsis, the action of GLP-1 could contribute to the reduction of the intestinal transit and, consequently, to retard the digestion and the absorption of the luminal content. In fact, a slow intestinal transit prolongs the time contact of the chyme with intestinal juices and with the absorptive surface of the small intestine. This can induce stimulation of chemosensory activity linked to central control of food intake by signalling the energy needs of the organism. Therefore modulation of peristaltic events by GLP-1 can be involved in its action of modulation of feeding behaviour. In our experimental preparations, results have suggested that GLP-1 effect on circular muscular layer is mediated by nitrergic pathway stimulation that in turn inhibits acetylcholine release.

Very interesting and novel appear results obtained with immunohunohistochemical analysis. Indeed other authors used molecular techniques such as RNAse protection, Northern blotting, RT-PCR and in situ hybridation to determine the tissue distribution of GLP-1R in rodents (Bullock et al., 1996 ; Campos et al., 1994; Dunphy et al., 1998), but no information there was about the cellular type (enteroendocrine cells, enteric neurons, smooth muscle and/or pacemaker cells) which express GLP-1R. We found GLP-1R-immunoreactivity only in some myenteric neurons of both duodenum and proximal colon and in few submucosal neurons in the colon, while no other cell type showed GLP-1R-IR in both duodenum and colon muscle coat, suggesting that the ENS can play a role major in the peptide motor action than previously hypothesized. Recent work, using immunofluoroscence, showed that GLP-

1R is expressed in guinea-pig submucosal plexus and co-localizes with choline acetyltransferase, confirming the relative importance of peripheral GLP-1R (Baldassano et al., 2011). So far study on human isolated gastrointestinal tissue are lacking, therefore, future research efforts should aim at providing new information on the presence and roles of GLP-1R within the human gut wall.

Also the analysis of GLP-1 effects, using the mouse isolated whole stomach *in vitro,* has been helpful to clarify if the peptide can modulate gastric motility, through a peripheral site of action. We found that exogenous GLP-1 induced gastric relaxation mediated by neural release of NO. Indeed, previous studies *in vitro* had shown that in human or rat gastric strips GLP-1 did not affect the smooth muscle contractility of the fundus and corpus (Tolessa et al., 1998b; Naslund et al., 2001). However, it is important to consider the experimental method. In our experimental conditions, gastric preparations develops tone and spontaneous mechanical which allow us to detect relaxation in the absence of contractile agents (Mulè & Serio, 2002). In fact, it is evident that the smooth muscle outside sphincteric regions is capable of developing tonic contractions (Gregersen & Christensen, 2000 et al., 2000), in particular, in the gastric region where the relaxation plays a role in establishing the reservoir function of the stomach (Azpiroz & Malagelada, 1990; Bayguinov et al., 1999). Previous experimental data had suggested that the smooth muscle of the murine stomach shows a tone that is dependent on acethylcholine acting on muscarinic receptors and on NO synthesis, because it was reduced by atropine and increased by L-NAME (Mulè & Serio, 2002). Nitric oxide is a key mediator of relaxation (Desai et al., 1991); however, other inhibitory transmitters such as vasoactive intestinal polypeptide (VIP) and adenosine triphosphate (ATP) may contribute to the gastric inhibitory neurotransmission in mouse stomach (Mulè & Serio, 2003).

The activation of the nitrergic pathway induced by GLP-1R activation could lead to improvement of gastric accommodation since NO has been demonstrate to be the main neurotransmettitor involved in gastric accommodation events (Tack & Demedts, 2002). However, our functional results, supported by the analysis of expression of GLP-1R, shown that the effect of GLP-1 was confined to the antral region. This could reflect the GLP-1 ability to inhibit antral motility and, consequently, to retard gastric emptying.

Functionally it is possible to distinguish two gastric portions: the proximal stomach, consisting of the fundus and the proximal part of the corpus, and the distal

stomach consisting of the distal part of the corpus and the antrum. Concerning the motor activity, the proximal stomach is mainly involved in the gastric accommodation, occurring during the early food intake, in which the mechanical distension of the gastric wall increase the gastric capacity and induced satiety effect. The distal stomach contracts to mix and grind the food and also generate a flow of food to the duodenum. Therefore, the GLP-1 local inhibitory action on antral region would reflect slowing of gastric emptying and it would represent a further mechanism of action in addition to GLP-1 modulation of neurons in the central nervous system or vagal neural pathways previously reported (Imeryuz et al., 1997; Anvari et al., 1998; Tolessa et al., 1998; Wettergreen et al., 1998; Schirra et al., 2000; Naslund et al., 2001).

On the other hand, control of gastric emptying is a relevant factor for postprandial blood glucose concentrations. Perturbations in gastric emptying may have significant impact on postprandial glycemia in health and diabetes (Jones et al., 1995; O'Donovan et al., 2004). Hence, GLP-1-induced slowing of gastric emptying could contribute to its action to attenuate postprandial glycemic excursion, as reported in healthy humans (Deane et al., 2010).

It is interesting to note that also GLP-2, peptide derived from the proglucagon gene and released from enteroencrine L-cells after nutrient intake together GLP-1 (Brubaker, 2006), is able to induce relaxation of mouse stomach, but its action is direct to the proximal stomach region (Amato et al., 2009) differently to GLP-1. Accordingly, GLP-2 inhibitory action is mediated by neural release of VIP (Amato et al., 2009). Therefore, it is possible to speculate that GLP-1 and GLP-2 act sinergically on gastric motility slowing the gastric emptying and increasing gastric distension, respectively. The combined actions of both peptides on the stomach could increase the satiety sensation. In fact, increase in the gastric volume might mean activation of stretch receptors and greater satiety signals to the brain, but electrophysiological experiments in vivo are necessary to confirm this hypothesis.

Our results *in vitro* do not allow to delineate any conclusion about the action mode (endocrine, paracrine) of GLP-1 to decrease gastric emptying and intestinal motility. The localization of the GLP-1R in the enteric nervous system suggests that only circulating GLP-1 would reach the neuron cell bodies and, thus acting in an endocrine manner. Nevertheless, it remains possible that at least in the intestinal regions, GLP-1 locally released from mucosal cells also acts on terminals of intrinsic

primary afferent neurons (IPANs). IPANs might represent a target for GLP-1 paracrine action, but although there is good evidence that the GLP-1R on vagal afferent nerves mediates sensory input from the gastrointestinal tract (Bucinskaite et al., 2009) so far it is not known if GLP-1 can modulate IPANs.

In the second part of my PhD work, I switched my attention on GLP-1, motor gastric function and satiation feelings in humans n the attempt to correlate gastric motor responses with the regulation of food intake.

Since GLP-1 short half-life represents a serious problem about its use, one strategy for circumventing the GLP-1 short half-life problem is represented by use of mimetics of GLP-1, designated as receptor agonists, resistant to inactivation by DPP-4, thus prolonging and enhancing the effect of the hormone, as liraglutide. Another strategy involves inhibition of DPP-IV, hereby prolonging the effect of endogenously secreted GLP-1 with some drugs like vildagliptin and sitagliptin.

Thus, we studied the effect of the GLP-1 stable analogue, liraglutide, on gastric accommodation and on satiety in humans and measured the intragastric pressure changes during food intake. The gastric barostat has been the gold technique to study gastric accommodation in human (Tack et al., 2001) and consist of a computer-driven air pump connected to an oversized balloon, which can be positioned in the proximal or distal stomach. The barostat maintains a fixed pressure level within the stomach by adapting the intraballoon volume. Measurements of volume changes at a costant low pressure allow quantification of changes in gastric tone, and measurements of perception at various distending pressure levels allow quantification of sensitivity to gastric distention. Measurements of volume changes at constant distending pressure, traditionally, have been used to quantify gastric accommodation or to study factors involved in the control of gastric accommodation (Azpiroz & Malagelada, 1985). However the barostat represents an invasive technique because of uncomforted and stressed feelings. Then, now, it is possible to study gastric accommodation with a new methodology proposed as a minimally invasive and more physiological alternative to the barostat for the assessment of gastric accommodation and satiation during food intake (Janssen et al., 2011b). This new technique engages the intragastric pressure (IGP) measurement during an intragastric nutrient drink infusion as an indirect parameter to evaluate the accommodation. It allows to analyse what physiologically is happening during food intake. In detail, initially the intragastric pressure decreases to

gradually recover thereafter and this latter phenomenon is correlated with satiation increase, indicating that IGP is a determinant of satiation (Janssen et al., 2011b). Surprisingly, results showed that liraglutide inhibited human gastric accommodation, without affecting satiation feelings. Therefore, the drugs does not mimic the action of GLP-1 on gastric accommodation in human (Delgado-Arros et al., 2002; Andrews et al., 2007; Andrews et al., 2007b). The discrepancy between GLP-1 and liragluitde activity might be attributed to liraglutide doses or its variable ability to gain access to GLP-1 receptors in different human tissue (Malm-Erjefält et al., 2010).

Therefore, we plan to use inhibitors of DPP-IV to increase plasma concentrations of active GLP-1 and verify the potential role of GLP-1 on gastric accommodation and food intake in humans.

In summary, the research performed during my PhD has evidenced the presence and the importance of peripheral action sites in mediating the motor effects of GLP-1 in the different regions of mouse gastrointestinal tract. Further studies have to be performed in order to correlate human gastric motor responses with the regulation of food intake.

REFERENCES

Aaboe K, Krarup T, Madsbad S, Holst JJ. 2008. GLP-1: physiological effects and potential therapeutic applications. *Diabetes Obes Metab* 10: 994–1003.

Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, Ghatei MA, Bloom SR. 2005. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem- hypothalamic pathway. *Brain Res* 1044:127–131.

Amato A, Baldassano S, Serio R, Mulè F. 2009. Glucagon-like peptide-2 relaxes mouse stomach through vasoactive intestinal peptide release. *Am J Physiol Gastrointest Liver Physiol* 296: G678-84.

Amato A, Rotondo A, Cinci L, Baldassano S, Vannucchi MG, Mulè F. 2010 . Role of cholinergic neurons in the motor effects of glucagon-like peptide-2 in mouse colon. *Am J Physiol Gastrointest Liver Physiol* 299:G1038-44.

Amato A, Cinci L, Rotondo A, Serio R, Faussone-Pellegrini MS, Vannucchi MG, Mulè F. 2010biPeripheral motor action of glucagon-like peptide-1 through enteric neuronal receptors. *Neurogastroenterol Motil* 22:664-72. bis

Andersen DK, Ruiz CL, Burant CF. 1994. Insulin regulation of hepatic glucose transporter protein is impaired in chronic pancreatitis. *Ann Surg* 219:679– 686

Andrews CN, Bharucha AE, Camilleri M, Low PA, Seide BM, Burton DD, Baxter KL, Zinsmeister AR. 2007. Nitrergic contribution to gastric relaxation induced by glucagon-like peptide-1 (GLP-1) in healthy adults. *Am J Physiol Gastrointest Liver Physiol* 292:G1359-65.

Andrews CN, Bharucha AE, Camilleri M, Low PA, Seide BM, Burton DD, Nickander KK, Baxter KL, Zinsmeister AR. 2007. Effects of glucagon-like peptide-1 and sympathetic stimulation on gastric accommodation in humans. *Neurogastroenterol Motil* 19:716-23.bis

Anvari M, Paterson CA, Daniel EE, Mcdonald T. 1998. Effects of GLP-1 on gastric emptying, antropyloric motility, and transpyloric flow in response to a nonnutrient liquid. *Dig Dis Sci* 43:1133-40.

Astrup A, Rossner S, Van Gaal L, Rissanen A, Niskanen L, Al Hakim M, Madsen J, Rasmussen MF, Lean MEJ on behalf of the NN8022-1807 study group. 2009. Effects of liraglutide in the treatment of obesity: a randomized, double-blind, placebo controlled study. *Lancet* 374:1606-16

Azpiroz F, Malagelada JR. 1985. Physiological variations in canine gastric tone measured by an electronic barostat. *Am J Physiol* 248(2 Pt 1):G229-37.

Azpiroz F & Malagelada JR. 1987. Importance of vagal input in maintaining gastric tone in the dog. J Physiol 384: 511-24

Azpiroz F, Malagelada J-R. 1990. Perception and reflex relaxation of the stomach in response to gut distension. *Gastroenterology* 98: 1193–8;

Ayachi SE, Borie F, Magous R, Sasaki K, le Nguyen D, Bali JP, Millat B, Jarrousse C. 2005. Contraction induced by glicentin on smooth muscle cells from the human colon is abolished by exendin (9-39). *Neurogastroenterol Motil* 17: 302–9

Badman MK & Flier JS. 2005. The gut and energy balance: visceral allies in the obesity wars. *Science* 307:1909-14.

Baggio LL, Huang Q, Brown TJ, Drucker DJ. 2004. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. *Gastroenterology* 127:546–58.

Baggio LL & Drucker DJ. 2007. Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132:2131-57

Baldassano S, Wang GD, Mule F, Wood JD. 2011. Glucagon-Like Peptide-1 Modulates Neurally-Evoked Mucosal Chloride Secretion in Guinea Pig Small Intestine In Vitro. *Am J Physiol Gastrointest Liver Physiol* doi:10.1152/ajpgi.00333.2011.

Baldassano S, Mulè F. 2011. Peripheral Glucagon Like Peptide 2 Analogue administration reduces food Intake in lean and diet-induced obese mice *Gastroenterology*, 140 (5, Supplement 1), Sa1813.

Balks HJ, Holst JJ, Von Zur Muhlen A, Brabant G. 1997. Rapid oscillations in plasma glucagon-like peptide-1 (GLP-1) in humans: cholinergic control of GLP-1 secretion via muscarinic receptors. *J Clin Endocrinol Metab* 82:786–90.

Barragán JM, Rodríguez RE, Eng J, Blázquez E. 1996. Interactions of exendin-(9-39) with the effects of glucagon-like peptide-1-(7-36) amide and of exendin-4 on arterial blood pressure and heart rate in rats. *Regul Pept* 67:63-8

Batterham R.L., Le Roux CW, Cohen MA, Park AJ, Ellis SM, Patterson M, Frost GS, Ghatei MA, Bloom SR. 2003. Pancreatic polypeptide reduces appetite and food intake in humans. *J Clin Endocrinol Metab* 88:3989–92 bis

Baumgartner I, Pacheco-López G, Rüttimann EB, Arnold M, Asarian L, Langhans W, Geary N, Hillebrand JJ. 2010. Hepatic-portal vein infusions of glucagon-like peptide-1 reduce meal size and increase c-Fos expression in the nucleus tractus solitarii, area postrema and central nucleus of the amygdala in rats. *J Neuroendocrinol* 22:557-63.

Bayguinov O, Keef KD, Hagen B, Sanders KM. 1999. Parallel pathways mediate inhibitory effects of vasoactive intestinal polypeptide and nitric oxide in canine fundus. *Br J Pharmacol* 126: 1543–52

Berthoud HR. 2008. Vagal and hormonal gut-brain communication: from satiation to satisfaction. *Neurogastroenterol Motil (Suppl. 1)* 20: 64-72

Blundell JE, Halford JC. 1994. Regulation of nutrient supply: the brain and appetite control. *Proc Nutr Soc* 53:407–18.

Bornstein JC, Costa M, Grider JR. 2004. Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil* 16 Suppl 1:34-8.

Bozkurt A, Naslund E, Holst JJ, Hellström PM. 2002. GLP-1 and GLP-2 act in concert to inhibit fasted, but not fed, small bowel motility in the rat. *Regul Pept* 107: 129-35.

Brennan IM, Feltrin KL, Horowitz M, Smout AJPM, Meyer JH, Wishart J, Feinle-Bisset C. 2005. Evaluation of interactions between CCK and GLP-1 in their effects on appetite, energy intake, and antropyloroduodenal motility in healthy men. *Am J Physiol Regulat Integrat Comp Physiol* 288: R1477–85

Brubaker PL, Drucker DJ, Asa SL, Swallow C, Redston M, Greenberg GR. 2002. Prolonged gastrointestinal transit in a patient with a glucagon-like peptide (GLP)-1- and -2-producing neuroendocrine tumor. *J Clin Endocrinol Metab* 87: 3078–83.

Brubaker PL. 2006. The glucagon-like peptides: pleiotropic regulators of nutrient homeostasis. *Ann N Y Acad Sci* 1070:10–26.

Bucinskaite V, Tolessa T, Pedersen J Rydqvist B, Zerihun L, Holst JJ, Hellström PM. 2009. Receptor-mediated activation of gastric vagal afferents by glucagonlike peptide-1 in the rat. *Neurogastroenterol Motil* 21: 978–85.

Bullock BP, Heller RS, Habener JF. 1996. Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. *Endocrinology* 137:2968–78.

Bunck MC, Diamant M, Cornér A, Eliasson B, Malloy JL, Shaginian RM, Deng W, Kendall DM, Taskinen MR, Smith U, Yki-Järvinen H, Heine RJ. 2009. One-year treatment with exenatide improves beta-cell function, compared with insulin glargine, in metformin-treated type 2 diabetic patients: a randomized, controlled trial. *Diabetes Care* 32:762-8.

Buse JB, Bergenstal RM, Glass LC, Heilmann CR, Lewis MS, Kwan AY, Hoogwerf BJ, Rosenstock J. 2011. Use of twice-daily exenatide in Basal insulin-treated patients with type 2 diabetes: a randomized, controlled trial. *Ann Intern Med* 154:103-12.

Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, Prentki M. 2004. Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia* 47: 806–15.

Byrne MM, McGregor GP, Barth P, Rothmund M, Go¨ke B, Arnold R. 2001. Intestinal proliferation and delayed intestinal transit in a patient with a GLP-1-, GLP-2- and PYY-producing neuroendocrine carcinoma. *Digestion* 63: 61–8.

Camilleri M. 2006. Integrated upper gastrointestinal response to food intake. *Gastroenterology* 131:640-58

Campos RV, Lee YC, Drucker DJ. 1994. Divergent tissue-specific and developmental expression of receptors from glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology* 134: 2156–64

Carney BI, Jones KL, Horowitz M, Sun WM, Penagini R, Meyer JH. 1995. Gastric-emptying of oil and aqueous meal components in pancreatic insufficiency – effects of posture and on appetite. *Am J Physiol Gastrointest Liver Physiol* 31:G925–G932.

Chaudhri O, Small C, Bloom S. 2006. Gastrointestinal hormones regulating appetite. *Philos Trans R Soc B Biol Sci* 361:1187-209

Chisholm C & Greenberg GR. 2002. Somatostatin-28 regulates GLP-1 secretion via somatostatin receptor subtype 5 in rat intestinal cultures. *Am J Physiol Endocrinol Metab* 283:E311-7.

Cinci L, Faussone-Pellegrini MS, Rotondo A, Mulè F, Vannucchi MG. 2011. GLP-2 receptor expression in excitatory and inhibitory enteric neurons and its role in mouse duodenum contractility. *Neurogastroenterol Motil* 23:383-92.

Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ. 2001. The arcuate nucleus as conduit for diverse segnals relavant to energy homeostasis. *Int J Obes Health Metab* Disord 25 Suppl 5:S63-7

Costa M, Brookes SJ, Hennig GW. 2000. Anatomy and physiology of the enteric nervous system. *Gut* 47Suppl: iv15–iv19,

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50:1714-9.

Cummings DE, Foster-Schubert KE, Overduin J. 2005. Ghrelin and energy balance: focus on current controversies. *Curr Drug Targets* 6:153–69.

Cummings DE & Overduin J. 2007. Gastrointestinal regulation of food intake. *J Clin Invest* 117:13-23

Cuomo R & Sarnelli G. 2004. Food intake and gastrointestinal motility. A complex interplay. *Nutr Metab Cardiovasc Dis* 14:173-9

Dakin CL, Gunn I, Small CJ, Edwards CM, Hay DL, Smith DM, Ghatei MA, Bloom SR. 2001. Oxyntomodulin inhibits food intake in the rat. *Endocrinology* 142:4244–50.

Dakin CL, Small CJ, Batterham RL, Neary NM, Cohen MA, Patterson M, Ghatei MA, Bloom SR. 2005. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. Endocrinology 145:2687–95.

Daniel EE, Anvari M, Fox-Threlkeld JE, McDonald TJ. 2002. Local, exendin (9-39)-insensitive, site of action of GLP- in canine ileum. *Am J Physiol Gastrointest Liver Physiol* 283:G595-602.

Deane AM, Nguyen NQ, Stevens JE, Fraser RJL, Holloway RH, Besanko LK, Burgstad C, Jones KL, Chapman MJ, Rayner CK, Horowitz M. 2010. Endogenous glucagons-like peptide-1 slows gastric emptying in healthy subjects, attenuating postprandial glycemia. *J Clin Endocrinol Metabol* 95:215-21.

Degn KB, Juhl CB, Sturis J, Jakobsen G, Brock B, Chandramouli V, Rungby J, Landau BR, Schmitz O. 2004. One week's treatment with the long-acting glucagon-like peptide 1 derivative liraglutide (NN2211) markedly improves 24-h glycemia and alpha- and beta-cell function and reduces endogenous glucose release in patients with type 2 diabetes. *Diabetes* 53:1187-94.

Delgado-Arros S, Kim DY, Burton DD, Thomforde GM, Stephens D, Brinkmann BH, Vella A, Camilleri M. 2002. Effect of GLP-1 on gastric volume, emptying, maximum volume ingested, and postprandial symptoms in humans. *Am J Physiol Gastrointest Liver Physiol* 282:G424-31

Delgado-Aros S, Camilleri M, Castillo JE, Cremonini F, Stephens D, Ferber I, Baxter K, Burton K, Burton D, Zinsmeister AR. 2005. Effect of gastric volume or emptying on meal-related symptoms after liquid nutrients in obesity: a pharmacologic study. *Clin Gastroenterol Hepatol* 3:997-1006

Desai KM, Sessa WC, Vane JR. 1991. Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature* 351:477-9.

Dhanvantari S, Seidah NG, Brubaker PL. 1996. Role of prohormone convertases in the tissue-specific processing of proglucagon. *Mol Endocrinol* 10:342–55

Drucker DJ & Brubaker PL. 1989. Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. *Proc Natl Acad Sci* 86: 3953–7

Drucker DJ & Nauck MA. 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 368:1696-705.

Dunphy JL, Taylor RG, Fuller PJ. 2008. Tissue distribution of rat glucagons receptor and GLP-1 receptor gene expression. *Mol Cell Endocrinol* 141:179–86

During MJ, Cao L, Zuzga DS, Francis JS, Fitzsimons HL, Jiao X, Bland RJ, Klugmann M, Banks WA, Drucker DJ, Haile CN. 2003. Glucagon-like peptide-1 receptor is involved in learning and neuroproection. *Nat Med* 9:1173-9.

Dushay J, Gao C, Gopalakrishnan GS, Crawley M, Mitten EK, Wilker E, Mullington J, Maratos-Flier E. 2011. Short-Term Exenatide Treatment Leads to Significant Weight Loss in a Subset of Obese Women Without Diabetes. *Diabetes Care*

Eissele R, Goke R, Willemer Harthus HP, Vermeer H, Arnold R, Göke B. 1992. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *Eur J Clin Invest* 22:283-91

Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, Bertolotto C, Di Mario U, Harlan DM, Perfetti R. 2003. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology* 144:5149-58

Fehmann HC & Habener JF. 1991. Functional receptors for the insulinotropic hormone glucagon-like peptide-I(7-37) on a somatostatin secreting cell line. *FEBS Lett* 279:335–340.

Fehmann HC & Habener JF. 1992. Insulinotrophic hormone glucagons-like peptide-1 (7-37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma TC-1 cells. *Endocrinology* 130:156-66.

Flint A, Raben A, Astrup A, Holst JJ. 1998. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. *J Clin Invest* 101:515-20.

Folli F, Guardado Mendoza R. 2011. Potential use of exenatide for the treatment of obesity. *Expert Opin Investig Drugs* 2011 Oct 24

Fried M, Erlacher U, Schwizer W, Lochner C, Koerfer J, Beglinger C, Jansen JB, Lamers CB, Harder F. 1991. Bischof-Delaloye A. Role of cholecystokinin in the

regulation of gastric emptying and pancreatic enzyme secretion in humans. Studies with the cholecystokinin-receptor antagonist loxiglumide. *Gastroenterology* 101:503-11

Funahashi H, Hori T, Shimoda Y, Mizushima H, Ryushi T, Katoh S, Shioda S. 2000. Morphological evidence for neural interactions between leptin and orexin in the hypothalamus. *Regul Pept* 92:31-5

Garber A, Henry R, Ratner R, et al, for the LEAD-3 (Mono) Study group. 2009. Liraglutide versus glimepiride monotherapy for the type 2 diabetes (LEAD-3 Mono): a randomized; 52-week, phase III, double blind, parallel-treatment trial. *Lancet* 374:473-81

Gilman CP, Perry T, Furukawa K, Grieg NH, Egan JM, Mattson MP. 2003. Glucagon-like peptide 1 modulates calcium responses to glutamate and membrane depolarization in hippocampal neurons. J *Neurochem* 87:1137-44

Giralt M & Vergara P. 1998. Sympathetic pathways mediate GLP-1 actions in the gastrointestinal tract of the rat. *Regul Pept* 74: 19–25.

Giralt M & Vergara P. 1999. Glucagonlike Peptide-1 (GLP-1) participation in ileal brake induced by intraluminal peptones in rat. *Dig Dis Sci* 44:322-9.

Goetze O, Steingoetter A, Menne D, van der Voort IR, Kwiatek MA, Boesiger P, Weishaupt D, Thumshirn M, Fried M, Schwizer W. 2007. The effect of macronutrients on gastric volume responses and gastric emptying in humans: A magnetic resonance imaging study. *Am J Physiol Gastrointest Liver Physiol* 292:G11-7.

Goke R, Larsen PJ, Mikkelsen JD, Sheinkh SP. 1995. Distribution of GLP-1 binding sites in the rat brain: evidence that exendin-4 is a ligand of brain GLP-1 binding sites. *Eur J Neurosci* 7:2294-300

Goldstone AP, Mercer JG, Gunn I, Moar KM, Edwards CM, Rossi M, Howard JK, Rasheed S, Turton MD, Small C, Heath MM, O'Shea D, Steere J, Meeran K, Ghatei MA, Hoggard N, Bloom SR. 1997. Leptin interacts with glucagon-like peptide-1 neurons to reduce food intake and body weight in rodents. *FEBS Lett* 415**:**134–8

Gregersen & Christensen, 2000 H & Christensen J. 2000. Gastrointestinal tone. *Neurogastroenterol Motil* 12: 501–8

Greig NH, Mattson MP, Perry T, Chan SL, Giordano T, Sambamurti K, Rogers JT, Ovadia H, Lahiri DK. 2004. New therapeutic strategies and drug candidates for neurodegenerative diseases: p53 and TNF-alpha inhibitors, and GLP-1 receptor agonists. *Ann N Y Acad Sci* 1035:290-315.

Gribble FM, Williams L, Simpson AK, Reimann F. 2003. A novel glucose- sensing mechanism contributing to glucagon-like peptide- 1 secretion from the GLUTag cell line. *Diabetes* 52:1147–54.

Gülpinar MA, Bozkurt A, Coşkun T, Ulusoy NB, Yegen BC. 2000. Glucagon-like peptide (GLP-1) is involved in the central modulation of fecal output in rats. *Am J Physiol Gastrointest Liver Physiol* 278: G924-9.

Gutzwiller JP, Göke B, Drewe J, Hildebrand P, Ketterer S, Handschin D, Winterhalder R, Conen D, Beglinger C. 1999. Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut* 44:81–6

Gutzwiller JP, Hruz P, Huber AR, Hamel C, Zehnder CE, Drewe J, Gutmann H, Stanga Z, Vogel D, Beglinger C. 2006. Glucagon-like peptide-1 is involved in sodium and water homeostasis in humens. *Digestion* 73:142-50

Hansen L, Hartmann B, Bisgaard T, Mineo H, Jørgensen PN, Holst JJ. 2000. Somatostatin restrains the secretion of glucagons like peptide-1 and 2 from isolated perfused porcine ileum. *Am J Physiol* 278:E1010–8

Harder H, Nielsen L, Tu DT, Astrup A. 2004. The effect of liraglutide, a long-acting glucagon-like peptide 1 derivative, on glycemic control, body composition, and 24-h energy expenditure in patients with type 2 diabetes. Diabetes Care. 27:1915-21.

Hayes MR, Bradley L, Grill HJ. 2009. Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. *Endocrinology* 150:2654-9.

Heine RJ, Van Gaal LF, Johns D, Mihm MJ, Widel MH, Brodows RG; GWAA Study Group. 2005. Exenatide versus insulin glargine in patients with suboptimally controlled type 2 diabetes: a randomized trial. *Ann Intern Me*d 43:559-69.

Heller RS, Kieffer TJ, Habener JF. 1997. Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas. *Diabetes* 46:785–91.

Hellstrom PM, Naslund E, Edholm T Schmidt PT, Kristensen J, Theodorsson E, Holst JJ, Efendic S. 2008. GLP-1 suppresses gastrointestinal motility and inhibits the migrating motor complex in health subjects and patients with irritablebowel syndrome. *Neurogastroenterol Motil* 20: 649–59.

Hellström PM. 2011. GLP-1 playing the role of a gut regulatory compound. *Acta Physiol (Oxf).* 201:151-6.

Hermann C, Goke R, Richter G, Fehmann HC, Arnold R, Goke B. 1995. Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. *Digestion* 56:117–26.

Holst JJ. 2007. The physiology of glucagon-like peptide 1. *Physiol Rev* 87:1409-39

Holz GG, Kuhtreiber WM, Habener JF. 1993. Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon- like peptide-1(7-37). *Nature* 361:362–5.

Huizinga JD & Lammers WJ. 2009. Gut peristalsis is governed by a multitude of cooperating mechanisms. *Am J Physiol Gastrointest Liver Physiol* 296:G1-8.

Imeryüz N, Yeğen BC, Bozkurt A, Coşkun T, Villanueva-Peñacarrillo ML, Ulusoy NB. 1997. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. *Am J Physiol Gastrointest Liver Physiol* 273:G920-7.

Janssen P, Vanden Berghe P, Verschueren S, Lehmann A, Depoortere I, Tack J. 2011. Article review: the role of gastric motility in the control of food intake. *Aliment Pharmacol Ther* 33:880-94

Janssen P, Verschueren S, Ly G, Vos R, Van Oudenhove L, Tack J. 2011 bis. Intragastric pressure during food intake: a physiological and minimally invasive method to assess gastric accommodation. *Neurogastroenterol Motil* 23:316–e154

Jiang G & Zhang BB. 2003. Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* 284: E671–8.

Jones KL, Horowitz M, Wishart MJ, Maddox AF, Harding PE, Chatterton BE. 1995. Relationships between gastric emptying, intragastric meal distribution and blood glucose concentrations in diabetes mellitus. *J Nucl Med* 36:2220-8.

Juhl CB, Hollingdal M, Sturis J, Jakobsen G, Agersø H, Veldhuis J, Pørksen N, Schmitz O. 2002. Bedtime administration of NN2211, a long-acting GLP-1 derivative, substantially reduces fasting and postprandial glycemia in type 2 diabetes. *Diabetes* 51:424-9.

Kapitza C, Zdravkotic M, Zijlstra E, Segel S, Heise T, Flint A. 2011. Effect of three different injection sites on the pharmacokinetics of the once-daily human GLP-1 analogue liraglutide. *J Clin Pharmacol* 51:951-5.

Kelly AS, Metzig AM, Rudser KD, Fitch AK, Fox CK, Nathan BM, M Deering M, Schwartz BL, Abuzzahab MJ, Gandrud LM, Moran A, Billington CJ, Schwarzenberg SJ. 2011. Exenatide as a Weight-Loss Therapy in Extreme Pediatric Obesity: A Randomized, Controlled Pilot Study. *Obesity (Silver Spring).*

Kendall DM, Riddle MC, Rosenstock J, Zhuang D, Kim DD, Fineman MS, Baron AD. 2005. Effects of exenatide (Exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. *Diabetes Care* 28:1083–91.

Kilbinger H. 1996. Modulation of acetylcholine release by nitric oxide. *Prog Brain Res* 109:219–24.

Kindt S & Tack J. 2006. Impaired gastric accommodation and its role in dyspepsia. *Gut* 55: 1685–91

Kissileff HR, Carretta JC, Geliebter A, Pi-Sunyer FX. 2003. Cholecystokinin and stomach distension combine to reduce food intake in humans. *Am J Physiol Regul INtegr Comp Physiol* 285:R992-8.

Knudsen LB, Nielsen PF, Huusfeldt PO, Johansen NL, Madsen K, Pedersen FZ, Thøgersen H, Wilken M, Agersø H. 2000. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J Med Chem* 43:1664-9.

Kolligs F, Fehmann HC, Goeke R, Goeke B. 1995. Reduction of the incretin effect in rats by the glucagon-like peptide1receptor antagonist exendin(9–39)amide. *Diabetes* 44:16–9.

Konturek PC, Konturek JW, Cześnikiewicz-Guzik M, Brzozowski T, Sito E, Konturek SJ. 2005. Neuro-hormonal control of food intake; basic mechanism and clinical implications. *J Physiol Pharmacol* 56:5-25

Kumar KG, Byerley LO, Volaufova J, Drucker DJ, Churchill GA, Li R, York B, Zuberi A, Richards BK. 2008. Genetic variation in Glp1r expression influences the rate of gastric emptying in mice. *Am J Physiol Regul Integr Comp Physiol* 294:R362–71.

Kunze WA, Furness JB. 1999. The enteric nervous system and regulation of intestinal motility. *Annu Rev Physiol* 61:117-42.

Lal S, McLaughlin J, Barlow J, D'Amato M, Giacovelli G, Varro A, Dockray GJ, Thompson DG. 2004. Cholecystokinin pathways modulate sensations induced by gastric distension in humans. *Am J Physiol Gastrointest Liver Physiol* 287:G72-9

Larsen J, Fledelius C, Knudsen LB, Tang-Christensen M. 2001. Systemic administration of the long-acting GLP-1 derivative NN2211 induces lasting and reversible weight loss in both normal and obese rats. *Diabetes* 50:2530–9

Lavin JH, French SJ, Read NW. 2002. Comparison of oral and gastric administration of sucrose and maltose on gastric emptying rate and appetite. *Int J Obes* 26: 80–6.

Lee KJ, Vos R, Janssens J, Tack J. 2004. Differences in the sensorimotor response to distension between the proximal and distal stomach in humans. *Gut* 53:938-43.

Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ. 2005. Beta-cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. *Diabetes* 54:482–91

Lin HC & Taylor IL. 2004. Release of peptide YY by fat in the proximal but not distal gut depends on an atropine-sensitive cholinergic pathway. *Regul Pept* 117:73–6.

Little TJ, Pilichiewicz AN, Russo A, Phillips L, Jones KL, Nauck MA Wishart J, Horowitz M, Feinle-Bisset C 2006. Effects of intravenous glucagon-like peptide-1 on gastric emptying and intragastric distribution in healthy subjects: relationships with

postprandial glycemic and insulinemic responses. *J Clin Endocrinol Metabol* 91:1916-23.

Lovshin J, Estall J, Yusta B, Brown TJ, Drucker DJ. 2001. Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signalling. *J Biol Chem* 276:21489-99.

Madsbad S. 2009. Exenatide and liraglutide: different approaches to develop GLP-1 receptor agonists (incretin mimetics)--preclinical and clinical results. *Best Pract Res Clin Endocrinol Metab* 23:463-77.

Maljaars J, Peters HP, Masclee AM. 2007. Review article: The gastrointestinal tract: neuroendocrine regulation of satiety and food intake. *Aliment Pharmacol Ther* 26 Suppl 2:241-50.

Malm-Erjefält M, Bjørnsdottir I, Vanggaard J, Helleberg H, Larsen U, Oosterhuis B, van Lier JJ, Zdravkovic M, Olsen AK. 2010. Metabolism and excretion of the once-daily human glucagon-like peptide-1 analog liraglutide in healthy male subjects and its in vitro degradation by dipeptidyl peptidase IV and neutral endopeptidase. *Drug Metab Dispos* 38:1944-53.

Mang CF, Truempler S, Erbelding D, Kilbinger H. 2002. Modulation by NO of acetylcholine release in the ileum of wild-type and NOS gene knockout mice. *Am J Physiol Gastrointest Liver Physiol* 283: G1132–8.

Marre M, Shaw J, Brändle M, Bebakar WM, Kamaruddin NA, Strand J, Zdravkovic M, Le Thi TD, Colagiuri S; LEAD-1 SU study group. 2009. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with Type 2 diabetes (LEAD-1 SU). *Diabet Med* 26:268-78.

Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B, Drucker DJ. 2003. International Union of Pharmacology. XXXV. The glucagon receptor family. *Pharmacol Rev* 55:167–94.

McDonagh SC, Lee J, Izzo A, Brubaker PL. 2007. Role of glial cell-line derived neurotropic factor family receptor α2 in the actions of the glucagon-like peptides on the murine intestine. *Am J Physiol Gastrointest Liver Physiol* 293: G461-8

Meier JJ, Gallwitz B, Salmen S, Goetze O, Holst JJ, Schmidt WF, Nauck MA. 2003. Normalization of glucose concentration and deceleration of gastric emptying after solid meals during intravenous glucagons-like peptide 1 in patients with type 2 diabetes. *J Clin Endocrinol Metab* 88:2719-25.

Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, Gaeckler D, Schmidt WE, Gallwitz B. 2004. Secretion, degradation, and elimination of glucagonlike peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects. *Diabetes* 53:654–62

Mikhail NE. 2010. Is liraglutide a useful addition to diabetes therapy? *Endocr Pract* 16:1028-37

Miki T, Minami K, Shinozaki H, Matsumura K, Saraya A, Ikeda H, Yamada Y, Holst, JJ, Seino S. 2005. Distinct effects of glucose-dependent insulinotropic polypeptide and glucagons-like peptide-1 on insulin secretion and gut motility. *Diabetes* 54:1056-63

Moragas G, Azpiroz F, Pavia J, Malagelada JR. 1993. Relations among intragastric pressure, postcibal perception, and gastric emptying. *Am J Physiol* 264:G1112–7.

Moreno C, Mistry M, Roman RJ. 2002. Renal effects of glucagon‐like peptide in rats. *Eur J Pharmacol* 434: 163-7.

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. 2006. Central nervous system control of food intake and body weight. *Nature* 443:289–95

Mulè F, Postorino A, Geraci A, Serio R. 1992. Neurotensin: dual effect on the motor activity of rat duodenum. *Eur J Pharmacol* 212: 215–24.

Mulè , F., D'angelo, S., Amato, A., Contino, I. & Serio, R. 1999. Modulation by nitric oxide of spontaneous mechanical activity in rat proximal colon. J. Auton. Pharmacol., 19: 1 - 6.

Mulè F & Serio R. 2002. Spontaneous mechanical activity and evoked responses in isolated gastric preparations from normal and dystrophic (mdx) mice. *Neurogastroenterol Motil* 14:667-75

Mule` F, Baffi MC, Cerra MC. 2002. Dual effect mediated by protease-activated receptors on the mechanical activity of rat colon. *Br J Pharmacol* 136: 367–74. bis

Mulè F & Serio R. 2003. NANC inhibitory neurotransmission in mouse isolated stomach: involvement of nitric oxide, ATP and vasoactive intestinal polypeptide. *Br J Pharmacol* 140: 431-7

Mundt MW, Hausken T, Samsom M. 2002. Effect of intragastric barostat bag on proximal and distal gastric accommodation in response to liquid meal. *Am J Physiol Gastrointest Liver Physiol* 283: G681–6.

Munroe DG, Gupta AK, Kooshesh P, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A. 1999. Prototypic G protein–coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc Natl Acad Sci USA* 96:1569–73

Nakagawa H, Satake H, Nakabayashi M, Nishizawa K, Furuya K, Nakano S. 2004. Receptor gene expression of glucagon-like peptide-1, but not glucose dependent insulinotropic polypeptide, in rat nodose ganglion cells. *Auton Neurosci* 110: 36–43

Nagell CF, Wettergren A, Ørskov C, Holst JJ. 2006. Inhibitory effect of GLP-1 on gastric motility persists after vagal deafferentation in pigs. *Scand J Gastroenterol* 41: 667–72.

Näslund E, Gutniak M, Skogar S, Rössner S, Hellström PM. 1998. Glucagon-like peptide-1 increasese the period of postprandial satiety and slows gastric emptying in obese men. *Am J Clin Nutr* 68:525-30

Naslund E, Barkeling B, King N, Gutniak M, Blundell JE, Holst JJ, Rossner S, Hellstrom PM. 1999. Energy intake and appetite are suppressed by glucagon-like peptide-1(GLP-1) in obese men. *Int J Obes Relat Metab Disord* 23:304–11.

Näslund E, Bogefirs J, Gryback P, Bjellerup P, Jacobsson H, Holst JJ, Hellström PM. 2001. GLP-1 inhibits gastric emptyng of water but does not influence plasma vasopressin, sodium or osmolarity. *Scand J Gastroent* 2:156-62

Naslund E, Skogar S, Efendic S, Hellstrom PM. 2002. Glucagon-like peptide-1 analogue LY315902: effect on intestinal motility and release of insulin and somatostatin. *Regul Pept* 106: 89–95.

Naslund E & Hellstrom PM. 2007. Appetite signalling: from gut peptides and enteric nerves to brain. *Physiol Behav* 92:256-62

Nathan JD, Zdankiewicz PD, Wang J, Spector SA, Aspelund G, Jena BP, Seymour NE, Geibel JP, Andersen DK. 2001. Impaired hepatocyte glucose transport protein (GLUT2) internalization in chronic pancreatitis. *Pancreas* 22:172–8

Nauck M, Frid A, Hermansen K, Shah NS, Tankova T, Mitha IH, Zdravkovic M, Düring M, Matthews DR; LEAD-2 Study Group. 2009. Efficacy and safety comparison of liraglutide, glimiperide and placebo, all in combination with metformin, in type 2 diabetes. The LEAD (Liraglutide effect and action in diabetes)-2 study. *Diabetes Care* 32:84-90

Notivol R, Coffin B, Azpiroz F, Mearin F, Serra J, Malagelada JR. 1995. Gastric tone determines the sensitivity of the stomach to distention. *Gastroenterology* 108:330–6.

O'Donovan DG, Doran S, Feinle-Bisset C, Jones KL, Meyer JH, Wishart JM, Morris HA, Horowitz M. 2004. Effect of variations in small intestinal glucose delivery on plasma glucose, insulin, and incretin hormones in healthy subjects and type 2 diabetes. J *Clin Endocrinol Metab* 89:3431-5.

Olsson C, Holmgren S. 2001. The control of gut motility. *Comp Biochem Physiol A Mol Integr Physiol*. 2:481-503.

Ørskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. 2005. GLP-2 stimulates colonic growth via KGF released by subepithelial myofibroblasts with GLP-2 receptors. *Regul Pept* 124: 105-12

Osaka T, Endo M, Yamakawa M, Inoue S. 2005. Energy expenditure by intravenous administration of glucagon-like peptide-1 mediated by the lower brainstem and sympathoadrenal system. *Peptides* 26:1623-31

Park MI & Camilleri M. 2005. Gastric motor and sensory functions in obesity. *Obes Res* 13:491-500

Perry T, Lahiri DK, Sambamurti K, Chen D, Mattson MP, Egan JM, Greig NH. 2003. Glucagon-like peptide-1 decreases endogenous amyloid-beta peptide (Abeta) levels and protects hippocampal neurons from death induced by Abeta and iron. *J Neurosci Res* 72:603-12.

Perry T & Greig NH. 2004. A new Alzheimer's disease interventive strategy: GLP-1. *Curr Drug Targets* 5:565-71.

Raybould HE & Tache Y. 1988. Cholecystokinin inhibits gastric motility and emptying via capsaicin-sensitive vagal pathway in rats. *Am J Physiol* 255:G242-6

Redondo A, Trigo MV, Acitores A, Valverde I, Villanueva-Peñacarrillo ML. 2003. Cell signalling of the GLP-1 action in rat liver. *Mol Cell Endocrinol* 204:43-50.

Reeve JRJr, Green GM, Chew P, Eysselein VE, Keire DA. 2003. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. *Am J Physiol Gastrointest Liver Physiol* 285:G255–65.

Rehfeld JF. 2004. A centenary of gastrointestinal endocrinology. *Horm Metab Res* 36:735–41

Roberge JN & Brubaker PL. 1991. Secretion of proglucagon-derived peptides in response to intestinal luminal nutrients. *Endocrinology* 128:3169–74.

Rocca AS & Brubaker PL. 1999. Role of the vagus nerve in mediating proximal nutrient-induced glucagon- like peptide-1 secretion. *Endocrinology* 140:1687–94.

Rotondo A, Amato A, Lentini L, Baldassano S, Mulè F. 2011. Glucagon-like peptide-1 relaxes gastric antrum through nitric oxide in mice. *Peptides* 32:60-4

Ruiz-Grande C, Alarcn C, Mzrida E, Valverde I. 1992. Lipolytic action of glucagon-like peptides in isolated rat adipocytes. *Peptides* 13:13–6.

Saïfia S, Chevrier AM, Bosshard A, Cuber JC, Chayvialle JA, Abello J. 1998. Galanin inhibits glucagon-like peptide-1 secretion through pertussis toxin-sensitive G protein and ATP-dependent potassium channels in rat ileal L-cells. *J Endocrinol* 157:33-41.

Sanders KM & Ward SM. 1996. Electrical rhythmicity in gastrointestinal muscles. In Bolton, T.B. , Tomita, T. (Eds.), Smooth Muscle Excitation. Academic Press Ltd, London, 417-26

Schemann M, Sann H, Schaff C, Mader M. 1993. Identification of cholinergic neurons in enteric nervous system by antibodies against choline acetyltransferase. *Am J Physiol* 265: G1005–9.

Schirra J, Katschinski M, Weidmann C, Schafer T, Wank U, Arnold R, Göke B. 1996. Gastric emptying and release of incretin hormones after glucose ingestion in humans. *J Clin Invest* 97:92-103.

Schirra J, Houck P, Wank U, Arnold R, Goke B, Katschinski M. 2000. Effects of glucagon-like peptide-1 (7-36) amide on antro-pyloro-duodenal motility in the interdigestive state and with duodenal lipid perfusion in humans. *Gut* 46:622-31.

Schirra J, Wank U, Arnold R, Göke B, Katschinski M. 2002. Effects of glucagons-like peptide-1 (7-36)amide on motility and sensation of the proximal stomach in humans. *Gut* 50:341-8.

Schirra J, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ, Göke B. 2006. Endogenous glucagon-like peptide-1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. *Gut* 55:243-51

Schirra J, Nicolaus M, Woerle HJ, Struckmeier C, Katschinski M, Go¨ke B. 2009. GLP-1 regulates gastroduodenal motility involving cholinergic pathways. *Neurogastroenterol Motil* 21: 609–18.

Schmidtler J, Dehene K, Allescher HD, Schusdziarra V, Classen M, Holst JJ, Polack A, Schepp W. 1994. Rat parietal cell recptors for GLP-1(7-36)amide: Northern blot, cross-linking, and radioligand binding. *Am J Physiol Gastrointest Liver Physiol* 267:G423-32.

Schwarz MW, Woods SC, Porte DJr, Seeley RJ, Baskin DG. 2000. Central nervous system control of food intake. *Nature* 404: 661-71

Schwartz GJ. 2006. Integrative capacity of caudal brainstem in the control of food intake. *Philos Trans R Soc London Ser B* 361:1275-80

Sinclair EM & Drucker DJ. 2005. Proglucagon-derived peptides: mechanisms of action and therapeutic potential. *Physiology* 20:357-65.

Smith TK, Robertson WJ. 1998. Synchronous movements of the longitudinal and circular muscle during peristalsis in the isolated guinea-pig distal colon. *J Physiol* 506 (Pt 2):563-77

Strubbe JH & Woods SC. 2004. The timing of meals. *Psychol Rev* 111:128–41

Sturm K, Parker B, Wishart J, Feinle-Bisset C, Jones KL, Chapman I, Horowitz M. 2004. Energy intake and appetite are related to antral area in healthy young and older subjects. *Am J Clin Nut*r 80:656-67.

Tack J, Broeckaert D, Coulie B, Janssens J. 1998. The influence of cisapride on gastric tone and the perception of gastric distention. *Aliment Pharmacol Ther* 12:761-6

Tack J, Caenepeel P, Fischler B, Piessevaux H, Janssens J. 2001. Symptoms associated with hypersensitivity to gastric distention in functional dyspepsia. *Gastroenterology* 121:526-35.

Tack J & Demedts I. 2002. Role of nitric oxide in the gastric accommodation reflex and in meal induced satiety in humans. *Gut* 51: 219–24

Tack J, Caenepeel P, Piessevaux H, Cuomo R, Janssens J. 2003. Assessment of meal induced gastric accomodation by a satiety drinking test in health and in severe functional dyspepsia. *Gut* 52:1271-7

Talsania T, Anini Y, Siu S, Drucker DJ, Brubaker PL. 2005. Peripheral exendin-4 and peptide YY(3-36) synergistically reduce food intake through different mechanisms in mice. *Endocrinology* 146:3748–56

Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, Sheikh SP. 1996. Central administration of GLP-1-(7–36) amide inhibits food and water intake in rats. *Am J Physiol Regul Integr Comp Physiol* 271:R848–56

Tang-Christensen M, Larsen PJ, Thulesen J, Nielsen JR, Vrang N. 2001. Glucagon-like peptide 2, a neurotransmitter with a newly discovered role in the regulation of food ingestion. *Ugeskr Laeger* 163:287-91.

Tews D, Lehr S, Hartwig S, Osmers A, Paslack W, Eckel J. 2009. Anti-apoptotic action of exendin-4 in INS-1 beta cells: comparative protein pattern analysis of isolated mitochondria. *Horm Metab Res* 41: 294–301

Theodorakis MJ, Carlson O, Michipoulos S Doyle ME, Juhaszova M, Petraki K, Egan JM 2006. Human duodenal enteroendocrine cells :source of both incretin peptides, GLP-1 and GIP- *Am Physiol Endocrinol Metab* 290 :E550-9

Timmermans JP, Adriaensen D, Cornelissen W, Scheuermann DW. 1997. Structural organization and neuropeptide distribution in the mammalian enteric nervous system,

with special attention to those components involved in mucosal reflexes. *Comp Biochem Physiol A Physiol* 118:331-40.

Tolessa T, Gutniak M, Holst JJ, Efendic S, Hellström PM. 1998. Glucagon-like peptide-1 retards gastric emptying and small bowel transit in the rat: effect mediated through central or enteric nervous mechanisms. *Dig Dis Sci* 43:2284-90.

Tolessa T, Gutniak M, Holst JJ, Efendic S, Hellström PM. 1998b. Inhibitory effect of glucagon-like peptide-1 on small bowel motility. Fasting but not fed motility inhibited via nitric oxide independently of insulin and somatostatin. *J Clin Invest* 102:764-74.

Tornehave D, Kristensen P, Romer J, Knudsen LB, Heller RS. 2008. Expression of the GLP-1 receptor in mouse, rat and human pancreas. *J Histochem Cytochem* 56:841–51

Tsunekawa S, Yamamoto N, Tsukamoto K, Itoh Y, Kaneko Y, Kimura T, Ariyoshi Y, Miura Y, Oiso Y, Niki I. 2007. Protection of pancreatic beta-cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; in vivo and in vitro studies. *J Endocrinol* 193: 65–74.

Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 379: 69–72.

Van Dijk G & Thiele TE. 1999. Glucagon-like peptide-1 (7–36) amide: a central regulator of satiety and interoceptive stress. *Neuropeptides* 33: 406–14

Van den Elzen BD,Bennink RJ, Wieringa RE, Tytgat GNJ, Boeckxstaens GE. 2003. Fundic accommodation assessed by SPECT scanning: comparision with the gastric barostat. Gut 52:1548-54

Vanden Berghe P, Janssen P, Kindt S, Vos R, Tack J. 2009. Contribution of different triggers to the gastric accommodation reflex in humans. *Am J Physiol Gastrointest Liver Physiol* 297: G902–6.

Velasquez-Mieyer PA, Umpierrez GE, Lustig RH, Cashion AK, Cowan PA, Christensen M, Spencer KA, Burghen GA. 2004. Race affects insulin and GLP-1

secretion and response to a long-acting somatostatin analogue in obese adults. *Int J Obes Relat Metab Disord* 28:330-3

Verdich C, Flint A, Gutzwiller JP, Naslund E, Beglinger C, Hellstrom PM, Long SJ, Morgan LM, Holst JJ, AstrupA. 2001. A meta-analysis of the effect of glucagon-like peptide-1(7–36)amide on ad libitum energy intake in humans. *J Clin Endocrinol Metab* 86:4382–9.

Vergès B, Bonnard C, Renard E. 2011. Beyond glucose lowering: Glucagon-like peptide-1 receptor agonists, body weight and the cardiovascular system. *Diabetes Metab* 2011 Aug 24.

Villanueva-Peñacarrillo ML, Alcántara AI, Clemente F, Delgado E, Valverde I. 1994. Potent glycogenic effect of GLP-1(7-36) amide in rat skeletal muscle. *Diabetologia* 37 :1163-6.

Villanueva-Penacarrillo ML, Marquez L, Gonzalez N, Diaz-Miguel M, Valverde I. 2001. Effect of GLP-1 on lipid metabolism in human adipocytes. *Horm Metab Res* 33:73–7.

Vilsboll T, Zdravkovic M, Le-Thi T, Krarup T, Schmitz O, Courrèges JP, Verhoeven R, Bugánová I, Madsbad S. 2007. Liraglutide, a long-acting human glucagon-like peptide-1 analog, given as monotherapy significantly improves glycemic control and lowers body weight without risk of hypoglycemia in patients with type 2 diabetes. *Diabetes Care* 30: 1608-10

Wallis K, Walters JRF, Forbes A. 2007. Glucagon-like peptide 2 – Current applications and future directions. *Aliment Pharmacol Ther* 25:365-72

Wang Z, Wang RM, Owji AA, Smith DM, Ghatei MA, Bloom SR. 1995. Glucagon-like peptide-1 is a physiological incretin in rat. *J Clin Invest* 95:417–21.

Wettergren A, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ. 1993. Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man. *Dig Dis Sci* 38:665-73

Wettergren A, Petersen H, Orskov C, Christiansen J, Sheikh SP, Holst JJ. 1994. Glucagon-like peptide-1 7-36 amide and peptide YY from the L-cell of the ileal

mucosa are potent inhibitors of vagally induced gastric secretion in man. *Scand J Gastroenterol* 29:501-5.

Wettergren A, Maina P, Boesby S, Holst JJ. 1997. Glucagon-like peptide-1 7-36 amide and peptide YY have additive effect on gastric acid secretion in man. *Scand J Gastroenterol* 32:552-5.

Wettergren A, Wøjdemann M, Holst JJ. 1998. Glucagon-like peptide-1 inhibits gastropancreatic function by inhibiting central parasympathetic outflow. *Am J Physiol Gastrointest Liver Physiol* 275:G984–92

Wettergren A, Wøjdemann M, Holst JJ. 1998b. The inhibitory effect of glucagon-like peptide-1 (7–36) amide on antral motility is antagonized by its N-terminally truncated primary metabolite GLP-1 (9–36) amide. *Peptides* 19: 877–82.

Wise RA. 2006. Role of brain dopamine in food reward and reinforcement. *Philos Trans R Soc London Ser B* 361:1187-209

Wishart JM, Horowitz M, Morris HA, Kones KL, Nauck MA. 1998. Relation between gastric emptying of glucose and plasma concentrations of glucagon-like peptide-1. *Peptides* 19:1049-53

Wøjdemann M, Wettergren A, Hartmann B, Holst JJ. 1998. Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. *Scand J Gastroenterol* 33:828-32

Wood JD. 1998. Enteric neuropathobiology. In Functional disorders of the gut. ed. Phillips, S.F. & Wingate, D.L. pp. 19 - 42

Woods SC, Seeley RJ, Porte DJ, Schwartz MW. 1998. Signals that regulate food intake and energy homeostasis. *Science* 280:1378–83

Woods SC. 2004. Gastrointestinal satiety signals I. An overview of gastrointestinal signals that influence food intake. *Am J Physiol Gastrointest Liver Physiol* 286:G7-13.

Woods SC. 2005. Signals that influence food intake and body weight. *Physiol Behav* 86:709-16

Wren AM, Bloom SR. 2007. Gut hormones and appetite control. *Gastroenterology* 132:2116-30.

Wright RA, Krinsky S, Fleeman C, Trujillo J, Teague E. 1983. Gastric emptying and obesity. *Gastroenterology* 84(4):747-51.

Xing J & Cheng JDZ. 2004. Alterations of gastrointestinal motility in obesity. *Obes Res* 12:1723-32

Yamamoto H, Kishi T, Lee CE, Choi BJ, Fang H, Hollenberg AN, Drucker DJ, Elmquist JK. 2003. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. *J Neurosci* 23: 2939–46.

Yusta B, Huang L, Munroe D, Wolff G, Fantaske R, Sharma S, Demchyshyn L, Asa SL, Drucker DJ. 2000. Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology* 119:744-55

Yusta B, Baggio LL, Estall JL, Koehler JA, Holland DP, Li H, Pipeleers D, Ling Z, Drucker DJ. 2006. GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* 4: 391–406.

Zhao T, Parikh P, Bhashyam S, Bolukoglu H, Poornima I, Shen YT, Shannon RP. 2006. Direct effects of glucagon-like peptide-1 on myocardial contractility and glucose uptake in normal and postischemic isolated rat hearts. *J Pharmacol Exp Ther* 317: 1106–13

Zinman B, Gerich J, Buse LB, Lewin A, Schwartz S, Raskin P, Hale PM, Zdravkovic M, Blonde L; LEAD-4 Study Investigators. 2009. Efficacy and safety of the human glucagon-like peptide-1 analog liraglutide in combination with metformin and thiazolidinedione in patients with type 2 diabetes (LEAD-4 Met+ TZD). *Diabetes Care 32*:1224-30.