Incretin hormones as a target for therapy
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Incretin hormone physiology

The incretin effect

Incretin hormones are responsible for the incretin effect, which is the amplification of insulin secretion when nutrients are taken in orally, as opposed to intravenously. Strictly speaking, the incretin effect refers to the intake of glucose. Oral administration of glucose appears to engage a mechanism that allows the body to secrete insulin more efficiently than intravenous glucose administration [1]. The mechanism involves an augmented insulin release that is usually three-fold more than that observed in response to intravenous glucose. These figures can be derived from actual measurements of insulin secretion rates based on C-peptide concentrations, deconvolution, and C-peptide elimination kinetics [2]. The augmented insulin secretion is caused by incretin hormones released from the gut in response to the oral intake of glucose such as glucose-dependent insulinotropic polypeptide (GIP; previously known as gastric inhibitory polypeptide) and glucagon-like peptide-1 (GLP-1). There may be more contributory peptides (eg, secretin and oxyntomodulin), but GIP and GLP-1 are likely the most important [3].

The incretin-producing cells

GIP and GLP-1 are peptide hormones produced by endocrine cells located in the intestinal mucosal epithelium. GIP is known to be produced in the so-called K cells, which may be found all over the small intestine, but have the highest density in the proximal part, including the duodenum.
GLP-1 is produced in the L cells, which are found in all parts of the intestinal mucosa but with the highest densities in the ileum and the colon [4]. About 10–15% of the L cells express both GIP and GLP-1, but may also express other peptides including cholecystokinin (CCK), peptide YY (PYY), and neurotensin [5,6]. The nature of this apparent promiscuity is presently not clear but may have to do with the endocrine cell lifecycle, as the cells differentiate from the stem cell stage near the crypt villus transition, mature, move up the villus and, after just a few days, detach from the villus tip. Both L and K cells are open-type cells with a long cytoplasmic process reaching the gut lumen. The process is equipped with microvilli and it is thought that the cells may be able to sense nutrients because of the expression of molecular receptors and transporters in the microvillous cell membranes [7].

**Incretin hormone biosynthesis and structure**

GIP is formed from a precursor hormone, proGIP, from which the mature hormone, a 42-amino-acid peptide, is cleaved out by the enzyme pro-hormone convertase 1/3 [8,9]. GIP is released in response to nutrient ingestion, in particular glucose and lipids. The hormone binds to and activates a specific G protein-coupled receptor, the GIP-receptor [10]. Interestingly, the receptor is found in many tissues but more is known about its actions in the pancreatic islets and white adipose tissue [11]. In the pancreatic islets, the β cells, α cells, and somatostatin-producing δ cells all respond to GIP with stimulated secretion [12]. The mechanism involved is mainly activation of adenylate cyclase and accumulation of cyclic adenosine monophosphate (cAMP) [13].

GLP-1 is a product of the prohormone proglucagon, which is expressed in the pancreas, gut, and brainstem [14]. Phylogenetically, the L cells of the gut are related to pancreatic α cells. Both cells express the glucagon gene, which gives rise to the primary translation product proglucagon [15], but the common prohormone is differentially processed on the various tissues. In the α cells, proglucagon is cleaved by the enzyme prohormone convertase 2 (Figure 2.1). The products are:

- an N-terminal fragment (glicentin related pancreatic polypeptide [GRPP]);
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• glucagon; and
• the so-called major proglucagon fragment (MPF), which contains two additional glucagon-like sequences: GLP-1 and 2 [16].

They are ‘glucagon-like’ due to an approximately 50% sequence homology with glucagon, but in the pancreas, they remain assembled in the major proglucagon fragment, which is probably biologically inactive.

In the gut, proglucagon is cleaved by another processing enzyme, prohormone convertase 1/3 [17]. Here, the products include glicentin, a large peptide in which the glucagon sequence is buried. Not much is known about its possible actions but it may be broken further down to oxyntomodulin, which contains the full glucagon sequence plus a C-terminal octapeptide and is highly bioactive [14] (Figure 2.1). It is called oxyntomodulin because it was thought to influence gastric acid secretion but it turns out to be an agonist for both the glucagon and the GLP-1 receptor and is thought to play a role in appetite regulation [18–20]. The two glucagon-like sequences are cleaved out of the major proglucagon fragment and are released to the circulation. GLP-1 is a 30-amino-acid peptide, while GLP-2 has 33 amino acids, but is not known to influence glucose metabolism or appetite [21–23].

GIP and GLP-1 action

GLP-1 acts on the pancreatic islets, where the β and δ cells express the specific GLP-1 receptor. In addition, GLP-1 has important actions on gastrointestinal secretion and motility (eg, delaying gastric emptying)
and acts to inhibit appetite and food intake [14]. The GLP-1 and GLP-2 are released after nutrient intake, including carbohydrates, lipids, and proteins. The most important actions of GIP are thought to occur in the pancreatic islets, peripheral adipose tissue, and bones.

**Incretin secretion**

For both of the incretin hormones, nutrient-induced secretion is thought to involve proteins expressed on the L and K cell membranes (Figure 2.2). For glucose, there is evidence for expression of sodium-glucose cotransporters 1 (SGLT1) in membranes of the apical process [24-26]. For every glucose molecule that enters the cell via SGLT1, two sodium molecules with positive charge also enter, causing depolarization. The depolarization, in turn, leads to opening of voltage-gated calcium channels allowing calcium to enter the cells, with ensuing exocytosis of the hormone-containing granules.

Fructose also stimulates secretion of GLP-1, but not GIP, in agreement with a demonstrated expression of GLUT-5 in the L cells [27]. Once in the cell, metabolism of fructose seems to be responsible for GLP-1 secretion. Amino acids may also enter the cells in cotransport with sodium and may also be associated with depolarization and subsequent increase in intracellular calcium; however, a number of amino acids may also interact with G protein-coupled receptors on the cell surface, stimulating secretion [24]. Metabolism of the amino acids may also play a role; for instance glutamine, which is a preferred fuel for the small intestine, may be particularly effective [28]. The cells also express a number of lipid receptors, including the short-, medium-, and long-chain fatty acid receptors, which may activate specific intracellular pathways leading to secretion [29].

Like other small peptides, GIP and GLP-1 are eliminated in the kidneys by glomerular filtration, which would result in a half-life in the circulation of approximately 30 mins. However, both are eliminated much faster with (apparent) half-lives of less than 2 mins for GLP-1 and 7–8 min for GIP [30,31]. The immediate explanation for this is the actions of dipeptidyl peptidase-4 (DPP-4), an enzyme which cleaves off the two
The degradation of GLP-1 by the DPP-4 system

The peptide is stored in and released from the L cells in the intact form and diffuses from the epithelium to the capillaries of the villi but as soon as it enters the blood vessels, it is degraded by DPP-4 expressed by the endothelial cells (Figure 2.3). In this way, only about one-third to one-quarter of what was released from the L cells leaves the gut intact [35]. GLP-1 then reaches the liver but another DPP-4 system is ready to degrade approximately half of what reaches the liver; only approximately 12% is left to reach the systemic circulation [36] and, here, circulating DPP-4 may destroy the rest [37]. In fact, it has recently been demonstrated in an animal model that only about 8% of the newly released GLP-1 may
reach its peripheral targets with the arterial circulation [38]. It may seem paradoxical that a hormone is inactivated so rapidly and extensively after its release but the explanation seems to be that it is not acting only as a regular hormone via the circulation, but also interacts with afferent sensory nerve fibers in the gut [37].

**The neural pathway**

These nerve fibers signal to the brainstem and hypothalamus, where a reflex mechanism involving the efferent vagus regulates gastrointestinal motility and pancreatic secretion (Figure 2.4) [39,40]. Also, the appetite-regulating action of endogenous GLP-1 seems to involve the sensory vagal afferents [41], but high concentrations of active GLP-1 in plasma are also effective and may target GLP-1 receptors behind leaks in the blood–brain barrier (eg, in the postrema, subfornical organ, and median eminence). From here, GLP-1 may access the arcuate nucleus and reach appetite-regulating neurons, specifically the pro-opiomelanocortin (POMC)-expressing neurons, which express the GLP-1 receptor. This mechanism seems to be predominant for exogenous GLP-1 receptor agonists [42].
For GIP, a similar mechanism cannot be demonstrated and, although GIP is also a substrate for DPP-4, local degradation by DPP-4 does not seem to be important and there is no evidence that GIP acts on its target receptors by any other route than via the bloodstream.

**Measurement of the incretin hormones**

The rapid degradation of the two peptides has important consequences when one has to decide which assay to use for determination of their
plasma concentration. Regarding intact GLP-1 (7–36) amide, its concentrations are often undetectable and the rise after stimulation is small, whereas the sum of the concentrations of the metabolite plus the intact hormone (‘total-GLP-1’) gives a better reflection of L-cell secretion and GLP-1 action [43,44]. This is because the metabolite is derived from GLP-1, which was once secreted from the villi in the intact form, and as such, had a chance to interact with vagal and intestinal sensory afferents before it entered the capillaries and got degraded [44]. In contrast, the concentration of the intact hormone only provides information about the very small fraction of intact GLP-1 which reaches a target (ie, islets) via the circulation and, as previously mentioned, this may be as little as 8% [38].

**Incretin action in healthy individuals**

The incretin function of GIP and GLP-1 in humans has been probed in mimicry studies where their meal responses in plasma were copied by intravenous infusions during glucose clamping [45]. These studies clearly demonstrated that the incretin actions of the two hormones are about equal and that they efficiently stimulate insulin secretion even at fasting glucose concentrations, and that the effects are greatly augmented by increases in plasma glucose concentration within the usual postprandial interval [45]. Glucagon secretion was also measured but, in this case, it turned out that GLP-1 markedly inhibited glucagon secretion on top of the inhibition cause by glucose, whereas GIP actually slightly increased glucagon secretion [45]. For GLP-1, the existence of an antagonist for the GLP-1 receptor, exendin-(9,39), has allowed a direct investigation of the relative importance of GLP-1 for insulin secretion induced by glucose, which in the study (see below) was instilled intraduodenally. Here, a significantly lower insulin response was obtained after antagonist treatment [46]. The antagonist also brought about an increased glucagon secretion, suggesting that GLP-1 is responsible for part of the inhibitory effect of oral glucose on glucagon secretion.

**Incretin action in patients with type 2 diabetes**

In patients with type 2 diabetes, the picture is strikingly different. As demonstrated in the now classic study by Nauck and colleagues, most
of the gastrointestinal amplification of insulin secretion is lost in these patients and is one of the major mechanisms behind their prandial glucose intolerance [47]. This is even more clearly illustrated by calculation of the gastrointestinal-mediated glucose disposal (GIGD), which relates the amount of intravenous glucose required to copy the oral glucose tolerance test (OGTT) to the oral dose [48,49]. Normally, this is about 25g for a 50g OGTT, meaning that 50% of the glucose is removed from the circulation by the gastrointestinal mechanism, but this figure may fall to 0% in patients with type 2 diabetes [50,51]. Importantly, neither the incretin effect nor GIGD are constant but vary with the amount of glucose administered. Thus, while non-diabetic individuals are able to increase their GIGD (and their incretin effect) by as much as 70–80% and keep their plasma excursions after oral glucose ingestion relatively constant regardless of the dose, patients with type 2 diabetes are unable to do so. This results in their plasma glucose excursions rising proportionally with the glucose load, eventually reaching very high levels [51].

The mechanism underlying the normal adaptation of the incretin effect and GIGD according to glucose dose is an increased incretin hormone secretion, resulting in proportionately increased insulin secretion. Both GIP and GLP-1 secretion is rapidly increased after glucose ingestion and may reach a maximal value after 30–45 minutes [51]. With a low dose, secretion rapidly declines thereafter; with higher doses, secretion is maintained at the initial level and continues for longer time, depending on the dose. A correlation of the responses with gastric emptying rates reveals the mechanism behind the graded hormone response: gastric emptying is progressively retarded by higher doses of glucose. In other words, it is the gastric emptying that regulates the entry of glucose into the small intestine so that glucose is emptied at a rather constant rate; with larger doses, the duration of the emptying phase is simply prolonged and, as a result, the incretin hormone secretion is also prolonged. Interestingly, these findings suggest that the gastric emptying rate is one of the most important regulators of postprandial glucose profiles.

In the cited studies, there were no significant differences between the patients and the healthy controls regarding their GLP-1 and GIP responses to the varying doses of glucose, but this is not always the
case [50,51]. Obviously, one possible reason for the loss of the incretin effect in type 2 diabetes might be an impaired secretion of the two hormones. Indeed, a decreased GLP-1 response was reported in a large early study by Toft-Nielsen and colleagues, with similar findings made in several subsequent studies, particular with respect to the later phase of postprandial responses [52]; other, generally minor, studies have been unable to detect a significant differences, so the importance of an impaired GLP-1 response has been questioned [52,53].

However, for such comparisons, several important factors need to be considered. The most important is obesity, which is generally associated with impaired GLP-1 secretion for reasons that are unknown, but the impairment may be pronounced to the extent that a meal response may be missing altogether [54]. Secondly, as already alluded to, gastric emptying rates are extremely important for postprandial incretin responses, meaning that differences between controls and patients would have a major impact. In addition, differences in insulin sensitivity may play a role, and it has been demonstrated both directly and in meta-analyses that the severity and duration of diabetes influences secretion [55,56]. Finally, diabetes therapy may play a role. This is most importantly illustrated by metformin which stimulates GLP-1 secretion considerably, to an extent that increased GLP-1 secretion must be counted as one of the mechanisms whereby metformin lowers blood glucose in the patients [57]. In a recent large study of nearly 1500 individuals, it was clearly demonstrated that those with pre-diabetes or type 2 diabetes had 16–20% lower GLP-1 responses to an OGTT than people with a normal glucose test (NGT) [58]. Obese and overweight individuals had 25% and 15% lower GLP-1 responses, respectively, when compared to individuals with a normal weight, even after correction for age, sex, and glucose status. In addition, higher GLP-1 responses were associated with better insulin sensitivity and β-cell function and a lower degree of obesity. The conclusion, therefore, must be that an impaired secretion of GLP-1 is likely to contribute to the loss of the incretin effect in type 2 diabetes.
Therapeutic applications

Looking at the effects of GLP-1 and GIP, even more dramatic changes are seen. In one experiment, the hormones were infused into patients with poorly controlled type 2 diabetes during a hyperglycemic clamp (mainly established to ensure that all patients could be studied at the same clamp glucose level despite a wide range of fasting hyperglycemia levels), but neither the glucose clamp nor the hormone infusions had significant effects on insulin secretion, despite the same protocol having powerful effects on insulin secretion in healthy controls [59]. In further experiments, increasing the GIP infusion rate to supraphysiological levels did not help but actually stimulated glucagon secretion. On the other hand, increasing GLP-1 infusions to the therapeutic rate of 1.0–1.5 pmol/kg/min resulted in marked stimulation of insulin secretion, which reached similar levels as those seen in the controls during the same clamp conditions [60]. In addition, glucagon suppression in response to the glucose clamp, which was clearly impaired in the patients, was completely normalized. In other words, in these experiments, GLP-1 appeared to restore the sensitivity of both the $\beta$ cells and the $\alpha$ cells to glucose [59,60].

Given the general lack of effect of GIP in type 2 diabetes, it seems unlikely that GIP will be useful in relation to diabetes therapy, although some GIP effects may be regained over time after improving glucose control with insulin or DPP-4 therapy. GLP-1, on the other hand, is extremely attractive due its apparent ability to restore the incretin effect in patients with diabetes [61,62]. This raises the question of whether the incretin defect in type 2 diabetes may also play a role in the development of type 2 diabetes. As discussed above, an impaired secretion of GLP-1 is an early observation but it is unlikely to play a causative role [54]. Rather, the loss is a consequence of the development of insulin resistance and glucose intolerance, as indicated from studies of incretin secretion and effects in experimentally induced insulin resistance [63] and in patients with secondary diabetes, (eg, following chronic pancreatitis), characterized by similar lack of effect of GIP and a preserved effect of high
concentrations of GLP-1 [64]. Therefore, one should not expect incretin-based therapies to be able to cause a full resolution of diabetes, although the power of GLP-1 may be sufficient to near normalize metabolism in some patients for a period of time [65].

The development of GLP-1 based therapies

GLP-1 receptor agonists

Turning GLP-1 into a clinically useful therapeutic agent was initially hampered by its extensive and immediate metabolism and although continuous subcutaneous infusion of GLP-1 with insulin pumps was demonstrated to dramatically improve metabolic control in patients with rather advanced disease [66], other approaches have been more successful (although, interestingly, one promising recent approach involves insertion into the subcutis of a small osmotic mini-pump, which is capable of delivering a stabilized GLP-1 receptor agonist for periods of 6–12 months) [67]. The remarkable and apparently unique sensitivity of GLP-1 towards the enzyme DPP-4 (even the closely homologous hormone GLP-2 is not degraded nearly as rapidly or extensively as GLP-1) soon gave inspiration to the development of analogs with substitutions of the second amino acid, which directs the proline-directed enzymes like DPP-4 [32, 68]. It turned out that several substituted analogs were both resistant and retained potency, but the problem with rapid renal elimination remained [68].

Two approaches were made to circumvent this problem. The first followed from the identification of a full and at least equipotent agonist for the GLP-1 receptor in the saliva of the lizard *Heloderma suspectum* (Gila monster) [69]. The molecule, exendin-4, is a 39-amino-acid peptide with 53% homology to GLP-1 in its first 30 amino acid sequence (although, it is not lizard’s GLP-1; it has a sequence which more closely resembles GLP-1) and is resistant to DPP-4 but is not taken up by the kidneys and, thus, has an approximate 30-minute half-life in the circulation after intravenous administration [70,71]. This is sufficient to provide an exposure lasting for around 4–6 hours after a single subcutaneous injection, meaning that two daily injections might provide effective therapy.

The synthetic form, exenatide [72], shares most if not all of its actions with human GLP-1, including the effects on insulin and glucagon secretion,
gastric emptying, and appetite regulation, and has not generated any major surprises since its introduction in 2005. One might suspect that it would be antigenic, and indeed antibodies are formed in most patients, but the titers fall upon prolonged administration and are generally not associated with immunological problems or decreased efficacy [73]. Exenatide is also available in a slow-release formulation which provides very extended exposure and allows weekly administration [74].

Another approach has been to increase the dose in order to maintain adequate exposure for a longer period allowing once-daily dosing, and this turned out to be possible (lixisenatide) [75], although doses are limited by the gastrointestinal side effects shared by all GLP-1 receptor agonists (e.g., nausea, vomiting). Another approach has been to associate the GLP-1 molecule with larger molecules, thereby avoiding both DPP-4-mediated degradation (presumably because of steric hindrance) and renal clearance. Examples are covalent or noncovalent associations of GLP-1 with albumin or antibody Fc-fragments.

The first of these molecules to reach the market was liraglutide, consisting of the normal mammalian GLP-1 sequence to which is attached a fatty acid (palmitic acid) which causes it to bind to albumin [76]. The molecule now behaves like a protein-bound hormone with a long half-life, escaping renal elimination and with a free fraction (1–2% of total) and with a diffusion potential similar to that of the parent molecule which is responsible for most of the actions. In other cases, the attachment has been covalent, and this is interesting because it raises the question of whether or not these analogs can access receptors ‘behind’ the filter of the capillary fenestrae in the islets and in the central nervous system [77]. The answer is that dulaglutide, a stabilized GLP-1 sequence covalently attached to an antibody Fc fragment, has nearly the same effect and side effect profile as liraglutide in spite of the difference in molecular size, but a much longer survival in the body, allowing weekly administration [78].

DPP-4 inhibitors
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An entirely different approach consists of inhibiting the catalytic activity of the DPP-4 enzyme. Such inhibitors were originally developed for anti-immune therapies because DPP-4 is also expressed by certain cells of the
immune system, also known as CD26 [79]. However, very specific DPP-4 inhibitors turned out to have little or no effect on the immune system, so these projects were terminated. But when it was discovered that DPP-4 is responsible for the rapid degradation of GLP-1, it was also proposed that DPP-4 inhibitors could be used for diabetes therapy, analogous with the use of angiotensin-converting-enzyme (ACE) inhibitors for hypertension [80]. Subsequently, it was documented in animal models that a DPP-4 inhibitor available during GLP-1 degradation could enhance the survival of both endogenous and exogenous GLP-1 and result in much larger insulin responses [81]. This led to the development of new clinically useful DPP-4 inhibitors, the first of which was vildagliptin, as demonstrated in a clinical proof of concept study of patients with diabetes to lower HbA1c to a desired target of 7% over 52 weeks [82].

Subsequently, a large number of DPP-4 inhibitors have been developed and the first to reach the market was sitagliptin in 2006 [83]. The antidiabetic activity of the DPP-4 inhibitors is not quite as strong as that of the GLP-1 receptor agonists, but their great advantage is that they are orally available, most of them are suitable for once-daily administration, and that they have an extremely benign side effect profile, generally with frequencies similar to placebo. On the other hand, they have little effect on body weight. This may seem strange because they act by increasing the plasma concentrations of intact GIP and GLP-1, but the lack of an anorexic effect is most likely due to interference with the PYY system. The hormone PYY, co-stored with GLP-1 in many of the L cells, is also a substrate for DPP-4, converting it from PYY 1-36 to PYY 3-36. But PYY 1-36 is orexigenic and anorexigenic activity is gained only when it is converted to PYY 3-36 by DPP-4; this enables the molecule to interact with the Y-2 receptors which transmit appetite inhibitory effects in the hypothalamus. With the DPP-4 inhibitors, this conversion is prevented and so while the intact GLP-1 concentration is elevated, the PYY 3-36 concentration is simultaneously lowered, resulting in no or little net effect [84].

The antidiabetic action of DPP-4 inhibitors is consistent with their protective effect on GLP-1 and GIP, which both show significant and
approximate two- or three-fold elevations of their plasma concentrations of intact peptide [85]. The increase is somewhat lower than one would have expected from the concentration of the ‘total hormones’ as discussed above. There are probably two explanation for this: it turns out that the total hormone concentrations during therapy are generally lower than with placebo and these observations, with more direct measurements in experimental animal models [86,87], have suggested that the elevated concentrations of the intact hormones may feed back and inhibit the secretion of both GIP and GLP-1, perhaps by a mechanism involving local somatostatin secretion [88], thus limiting their secretion.

Another explanation is that degrading mechanisms other than DPP-4 (eg, neutral endopeptidase-22.11) may be involved in the degradation of at least GLP-1, which may reduce the concentration of the intact hormones relative to the concentration of total hormones [3,89]. Recently however, several groups have published studies of the incretin effect in humans, finding no difference between those treated with inhibitors and those treated with placebo [49,87,90]. This has generated theories of interference by the inhibitors with other systems unrelated to the incretins but with an effect on blood glucose.

Although this may be true (the list of potential substrates for DPP-4 is very long [32]), another explanation may be found in the proposed pathway of action for GLP-1 via the sensory afferents of the vagal system. As discussed above, this interaction takes place at a site (the lamina propria of the gut) where the hormone is still intact [37]; but if this is true, then DPP-4 inhibitors should not influence the interaction. Experiments to investigate this theory are currently ongoing.

Like GLP-1 receptor agonists, DPP-4 inhibitors are typically used on top of existing metformin therapy and in fixed combinations. The combination is associated with an apparently additive antihyperglycemic effect. This is important because metformin appears to enhance the secretion of GLP-1 [57]; in this way, one obtains an additional elevation of the intact GLP-1 concentrations and there is also experimental evidence that part of the antidiabetic action of metformin is exerted via stimulation of GLP-1 secretion.
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