

Physiological and pathophysiological aspects of incretin hormones and glucagon

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The thesis is based on the following three original papers

1. Bagger JI, Knop FK, Lund A, Vestergaard H, Holst JJ, Vilsbøll T. Impaired regulation of the incretin effect in patients with type 2 diabetes. *J Clin Endocrinol Metab* 2011 Mar;96(3):737-745
2. Bagger JI, Knop FK, Lund A, Holst JJ, Vilsbøll T. Glucagon responses to increasing oral loads of glucose and corresponding isoglycaemic intravenous glucose infusions in patients with type 2 diabetes and healthy individuals. *Diabetologia* 2014 Aug;57(8):1720-1725
3. Bagger JI, Holst JJ, Hartmann B, Andersen B, Knop FK, Vilsbøll T. Effect of Oxyntomodulin, Glucagon, GLP-1, and Combined Glucagon +GLP-1 Infusion on Food Intake, Appetite, and Resting Energy Expenditure. *J. Clin. Endocrinol. Metab.* 2015 Dec;100(12):4541-4552

INTRODUCTION

Worldwide, the number of obese individuals has more than doubled since 1980[1,2], and today it is more common to die from overweight than from underweight[2]. So far, weight loss is not easily accomplished. To date interventions have resulted in minor and rarely sustained results[3-5]. The risk of developing type 2 diabetes (T2D) escalates with increasing body weight. Thus, for each unit of increase in body mass index (BMI), the risk of T2D increases by approximately 12%[6]. The mortality rate more than doubles by having T2D and more than half of the patients with T2D die from cardiovascular disease[7].

The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are gut hormones secreted from enteroendocrine cells in the intestinal mucosa. They act as key regulators - in a glucose-dependent fashion - of pancreatic alpha and beta cell secretion in the pancreatic islets of Langerhans. In patients with T2D the effects of the incretin hormones on pancreatic beta cells[8] are markedly impaired, and also the regulation of alpha cell secretion seem to be altered[9]. In addition to GIP and GLP-1, the enteroendocrine cells produce a wide range of substances known to influence appetite and food intake (e.g. peptide YY (PYY), oxyntomodulin, cholecystokinin (CCK) and ghrelin)[10]. Interestingly, GLP-1 receptor agonists developed for the treatment of T2D induce weight loss and one was recently recommended for approval for the US Food and Drug Administration (FDA) for the treatment of obesity[11]. Additionally, glucagon has gained increased attention (again) since a recently developed GLP-1/glucagon receptor dual agonist has shown interesting results in relation to weight loss and food intake[12].

This thesis is based on three studies aiming to elucidate physiological and pathophysiological aspects of gastrointestinally mediated effects on alpha cell and beta cell secretion (study 1 and 2) and weight regulating properties of GLP-1 and glucagon (study 3).

INCRETIN EFFECT

The incretin effect refers to the phenomenon that oral glucose elicits a greater insulin response than intravenous (iv) glucose at identical plasma glucose (PG) profiles (isoglycaemia)[13,14]. The two incretin hormones GIP and GLP-1 convey the incretin effect. These hormones are secreted from intestinal endocrine cells in response to ingestion of nutrients, and, at their physiological plasma concentrations, both are highly insulinotropic in a strictly glucose-dependent manner[15,16]. Studies in mice with targeted deletions of the genes encoding the GLP-1 receptor, the GIP receptor or both of the receptors have demonstrated that both receptors are essential to maintain normal glucose tolerance[17]. The incretin effect is defined as the relative difference in the insulin responses following oral ingestion of glucose (oral glucose tolerance test (OGTT)) and isoglycaemic intravenous (iv) glucose infusion (IIGI), respectively (incretin effect = (area under the curve (AUCOGTT-AUCIIGI)/AUCOGTT), the latter representing the so-called the isoglycaemic clamp[8] (Fig. 1).

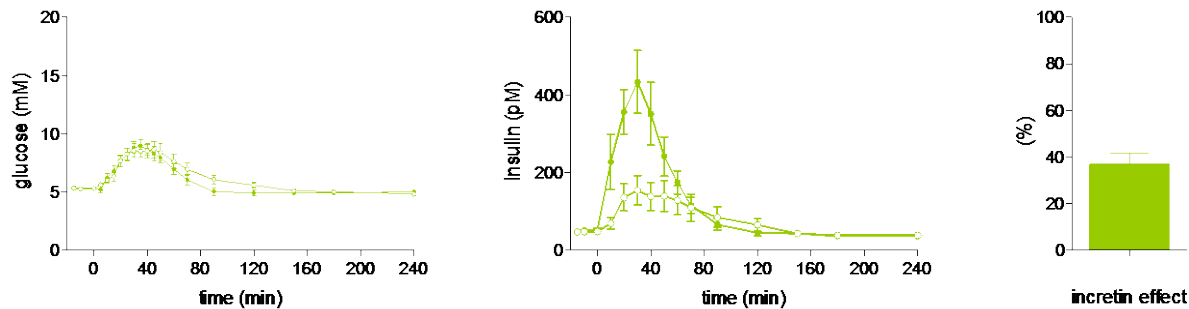


Figure 1

Glucose, insulin and incretin effect. Profiles are plasma glucose and plasma insulin (mean \pm SEM) following OGTT (25g) (closed symbols) and corresponding IIGI (15g) (open symbols) in healthy subjects. The bar plot shows the incretin effect calculated from the total area under the curves (tAUC) of the insulin profiles (mean \pm SEM) as described in the text (study 1).

Nauck et al. demonstrated the physiological importance of this phenomenon by estimating the incretin effect in healthy subjects during increasing glucose loads (25, 50 and 100 g)[18]. They showed that the incretin effect accounts for up to 70% of the insulin secretion following an OGTT. Remarkably, the PG excursions was very similar despite the fourfold increase in oral glucose load, probably caused by an accordingly increasing incretin effect[18]. By combining the isoglycaemic clamp with the infusion of physiological doses of GIP and GLP-1 during the IIGI, it was shown that GIP and GLP-1 was fully capable of restoring the insulin response to levels similar to the responses obtained by the OGTT[19]. Furthermore the effects of GLP-1 and GIP were found to be additive by the combined infusion during an IIGI[19].

It is now well established, that the incretin effect is reduced in patients with T2D[8,20,21]. The loss of incretin effect associated with T2D seems to be caused mainly by lost insulinotropic effect of GIP[22], combined with a reduced insulinotropic potency of GLP-1[23]. A reduction in the incretin effect is also found in other forms of diabetes, such as diabetes secondary to chronic pancreatitis, and gestational diabetes[20,24]. Interestingly, in gestational diabetes, the incretin effect is restored postpartum when normal glucose intolerance is re-established[24]. Tendencies towards re-establishment has also been shown in patients with T2D after treatment [25,26]. Højbjerg et al. enrolled a group of patients with dysregulated T2D to an intervention study employing aggressive insulin treatment for four weeks resulting in a near normalisation of PG[25,26]. The normalisation of PG did result in restoration of some insulinotropic potency of GIP and GLP-1[26]. Thus, the reduced incretin effect in T2D seems to be a consequence rather than a cause of the disease. Accordingly, using the opposite approach, i.e. by inducing glucose intolerance in young healthy subjects with per oral prednisolone, physical inactivity and increased intake of calories, reduced incretin effect was observed[27,28]. Further, Muscelli et al. found reduced incretin effect in individuals with impaired glucose tolerance (IGT)[29]. Investigating patients with maturity-onset diabetes of the young (MODY) type 2 and 3, we found reduced incretin effect only in patients with MODY-3[30]. MODY-2 is recognised by a mutation in the gene encoding glucokinase, which phenotypically causes a mild form of diabetes including elevated baseline PG but normal insulin responses although at higher glucose threshold[31]. MODY-3 on the other hand is characterized by a mutation in the hepatocyte nuclear factor 1 alpha (HNF1A), an essential regulator

of the pyruvate kinase activity leading to the formation of adenosine triphosphate (ATP)[32]. ATP deliverance is crucial for the effect of GLP-1 (as explained below) and therefore offers an explanation for the reduced incretin effect in those patients[30]. Both GLP-1 and GIP are released in response to ingestion of mixed meals in a load-dependent fashion according to caloric intake[21]. This incretin response results in a corresponding 'load-dependent' insulin secretion and consequently a strictly controlled PG[21]. However, the 'load-dependent' insulin secretion was found impaired in patients with T2D in response to the mixed meals[21]. Whether this defect in patients with T2D is caused by a failure to regulate the incretin effect sufficiently is unknown. This question was the main objective of study 1.

ENTEROENDOCRINE K AND L CELLS

The enteroendocrine cells are widely distributed throughout the mucosal lining of the gastrointestinal tract and produce a wide range of peptides[33]. Traditionally, the enteroendocrine cells are subdivided after granule morphology and the density of the different subtypes varies along the gastrointestinal tract[33]. For example, the GLP-1 producing L cells are mainly found in the distal part of the ileum and in the colon as opposed to the GIP producing K cells in the proximal part of the intestine[33]. In the K cells, processing of the prohormone proGIP is catalysed by the enzyme prohormone convertase (PC) 1/3 resulting in the formation of GIP[34]. Likewise, in the L cells, GLP-1 is produced by cleavage of proglucagon by PC1/3[35]. However, proglucagon is the parent peptide for a range of peptides and the end product (hormone) is depending on the enzymes present in the cells[15]. In the L cells, along with GLP-1, the processing of proglucagon by PC1/3 also results in formation of glucagon-like peptide 2 (GLP-2), glicentin and oxyntomodulin[35,36]. In the pancreatic alpha cells, PC2 processing of proglucagon leads to the formation of glucagon[37]. Apart from proglucagon products, the L cells also produce and secrete the anorectic hormone peptide YY (PYY)[38–40]. Interestingly, GLP-1 has also been found in GIP-producing K cells[41,42] and, additionally, it has been shown that a major lineage of enteroendocrine cells has the potential to produce a variety of peptides including cholecystokinin, PYY, secretin, neurotensin, GLP-1, GLP-2 and GIP[43]. This suggests that there may be more plasticity in the enteroendocrine cells than previously assumed[15]. The enteroendocrine 'L cells' or 'K cells' could therefore potentially

secrete a rather broad spectrum of potent substances upon stimulation. Further characterisation of the enteroendocrine cells is out of the scope of this study and has been dealt with elsewhere[44].

GIP

The 42 amino acid polypeptide GIP was the first discovered incretin hormone. Initially it was isolated as an inhibitor of acid secretion in preparations of dog stomachs in the late 1960's[45]. Later in the 1970's it was recognized as a potent insulinotropic peptide[46], and this is now thought to be the main action of the peptide. As aforementioned, it is evident that the insulinotropic action of GIP is almost abolished in patients with T2D whereas the effect of GLP-1 preserved, but reduced[22]. However, physiologically GIP has been suggested to be an even more important incretin than GLP-1[19]. Vilsbøll et al. showed that in healthy human volunteers clamped at three PG levels (fasting PG +0, +1 and +2 mM), the infusion of GIP and GLP-1, at concentrations corresponding to meal-induced responses, immediately and equally stimulated insulin secretion at all three PG levels[16]. Thus, GIP seems to be of considerable importance for normal glucose metabolism. This is supported by preclinical studies in GIP receptor knock-out mice. These mice show elevated glucose profiles in response to oral glucose, but not in response to intraperitoneal glucose administration[47]. Interestingly, the GIP receptor knock-out mice were also found to be resistant to diet-induced obesity[48]. Further, by reintroducing the GIP receptor in mice by targeted expression of the GIP receptor in adipocytes, the mice regain the ability to respond with obesity to high fat diet as opposed to global GIP receptor knock out littermates[49]. In line with this, studies in humans show that GIP may be involved in lipid metabolism by enhancing free fatty acid (FFA) re-esterification in peripheral adipose tissue[50]. This points to the GIP receptor as a target for obesity. Further research employing GIP antagonism would be interesting; however, so far no GIP receptor antagonists are available. Interestingly, GIP is most likely also important in the regulation of glucagon secretion as discussed below[51–54].

GLP-1

The mechanisms underlying the impaired incretin effect in type 2 diabetes are thought to include the abolished effect and the reduced potency of GIP and GLP-1, respectively. Also reduced secretion of GLP-1 has been suggested to contribute[55,56]. The initial rationale for conducting the first proof-of-concept clinical trials for the pharmacological use of GLP-1 included the assumptions that GLP-1 secretion was impaired in patients with T2D[55,56]. Later, clinical trials have shown both unchanged and decreased GLP-1 secretion[20,57]. Following secretion, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase 4 (DPP-4)[58]. This enzyme inactivates GLP-1 by cleaving off its two N-terminal amino acids, which results in a half-life of less than two minutes for the intact hormone [59]. DPP-4 is found first of all in the brush border of the ileal and renal epithelium, but also in a soluble form in plasma and in a membrane bound form in capillary endothelial cells in the lamina propria of the intestinal mucosa, adjacent to the cells secreting GLP-1 [60]. It is estimated that only 10% of the secreted GLP-1 reaches the peripheral circulation in its active form[61].

The GLP-1 receptor is found in many tissues including the brain[62], the pancreatic islets, the small and large intestine, the lungs, the kidneys, the heart, the liver and the portal vein[62–64]. The role of receptor stimulation in many of these tissues is not

understood. It is, however, known that the GLP-1 receptor is a G protein-coupled receptor[65], which is linked to an adenylate cyclase which upon activation produces cyclic adenosine monophosphate (cAMP) if ATP is available[66]. In the beta cells, increases in PG levels leads to formation of ATP, which is converted to cAMP by adenylate cyclase. Via protein kinase A and guanine nucleotide exchange factor (Epac) pathways, cAMP inhibits potassium out flux and promote calcium influx, which in turn leads to exocytosis of insulin granules[67–69]. Because the substrate (ATP) deliverance to the adenylate cyclase linked to the GLP-1 receptor in the beta cell, is dependent on PG, the effect of receptor activation is glucose-dependent.

GLP-1 AND INSULIN

As mentioned GLP-1 is highly insulinotropic at physiological PG levels[70,16] and that GLP-1 is accountable for a large part the insulin response after carbohydrate intake. This was shown in studies using the GLP-1 receptor antagonist, exendin 9-39, which resulted in remarkable reduction in the postprandial insulin response[71–73]. Physiological increases in GLP-1 concentrations can evoke small insulin increments even at fasting PG levels[74,16].

In patients with T2D, infusion of GLP-1 at high physiological and pharmacological levels did result in sufficient insulin responses to normalise fasting glycaemia[55]. GLP-1 infusion in combination with infusion of glucose or meal tests resulted in potentiated insulin secretion. These findings indicated that GLP-1 upholds a sufficient residual insulinotropic potency in patients with T2D—although reduced compared to healthy subjects[23,75]. In 2002, Zander et al. performed the first clinical trial with longer duration GLP-1 infusion, using a continuous subcutaneous infusion of pharmacological doses of the peptide during six weeks. This resulted in markedly increased insulin secretion and improved glycaemic control already after one week, and this was maintained after six weeks of treatment[76] providing proof-of-concept of GLP-1 as a treatment for patients with T2D. This study paved the way for further development of incretin-based treatment.

GLP-1 AND GLUCAGON

Apart from the well-described stimulatory effect of GLP-1 on insulin secretion, a suppressive effect of GLP-1 on glucagon secretion has been known since the late 1980's[70,77]. This suppressive effect was first specifically investigated in studies using the isolated perfused porcine pancreas where small amounts of GLP-1 was added to the infusate, and this caused a dose-dependent suppression of glucagon secretion[77]. In a study involving infusions of GIP and GLP-1 during a hyperglycaemic clamp in humans by Vilsbøll et al. (mentioned above), only GLP-1 infusion was associated with inhibition of glucagon secretion beyond that caused by the glucose clamp itself [16]. Thus, based on these studies, it is reasonable to assume that meal-induced elevations of GLP-1 would inhibit glucagon secretion. Accordingly, studies using the GLP-1R antagonist exendin 9-39 showed considerable elevations in the glucagon response to carbohydrate-rich lemonade intake, illustrating the importance of endogenous GLP-1 secretion for regulation of glucagon[71]. The potential of GLP-1-induced glucagon suppression for glucose metabolism was first evaluated by Hvidberg et al.[74] using exogenous GLP-1, which was infused at low and high physiological plasma concentrations measuring PG and endogenous glucose production (EGP) in fasting, healthy volunteers[74]. GLP-1, at both doses, inhibited glucagon secretion and lowered PG due to a 25% reduction of hepatic glucose output.

The suppression of glucagon secretion appeared to be the most prominent effect of the hormone, although the individual contributions of glucagon suppression and insulin secretion on PG could not be distinguished. Kielgast et al.[78] evaluated the glucagon responses during a meal in healthy volunteers and in patients with type 1 diabetes (T1D) with or without residual beta cell function. In these studies, iv infusion of GLP-1 inhibited glucagon secretion in all three groups, whereas the antagonist exendin 9-39 elevated glucagon levels both in the fasting state and postprandial. The intra-islet hypothesis[79], which states that inhibition of glucagon secretion is secondary to stimulation of beta cells, is incompatible with the observation that GLP-1-induced inhibition of both basal and stimulated glucagon secretion is observed also in patients with no residual beta cells[80,81]. The specific mechanism by which GLP-1 inhibits glucagon secretion is not fully clarified. Direct inhibition of the alpha cell cannot be ruled out, since expression of the GLP-1 receptor has been reported in human alpha cells[82], however, attempts to visualise the receptor immunohistochemically or by ligand binding has failed so far[83,84]. Interestingly, GLP-1 stimulates somatostatin secretion from the pancreatic delta cells in pigs [77]. And from the isolated perfused rat pancreas, we have learned that GLP-1-induced suppression of glucagon secretion seems to involve paracrine inhibition of the alpha cells[85]. In the perfusate used in the rats, glucose levels were kept very low in order to disable any beta cell response. Perfusion of somatostatin antibodies as well as a somatostatin receptor antagonist, reduced or eliminated the inhibitory effect of GLP-1 on glucagon secretion[85]. Those results indicate that the inhibitory effect of GLP-1 on glucagon secretion is likely to involve paracrine somatostatin signalling.

Glucagon secretion may also be regulated by neuronal mechanisms to a greater extent than generally assumed[15,86]. Plamboeck et al. demonstrated the impact of vagal innervation in human individuals by characterising the effect of exogenous GLP-1 in truncally vagotomised individuals and matched healthy controls. It was demonstrated that the potency of GLP-1 is lower in respect to both insulinotropic and glucagonostatic actions if the vagus nerve is not intact[87] indicating that an intact vagal innervation of the islets of Langerhans is essential for the effect of GLP-1 on glucagon secretion. This supports the theory that at least some of the effects of GLP-1 may not be exerted in a classical endocrine manner, but possibly through the afferent neurons of the vagus[88]. The vagal nerve endings in the lamina propria of the intestine are interspaced between the secreting L cells and the capillary bed, and their cell bodies are found in the nodose ganglion[15,88]. Because of the rapid and intensive degradation (minutes) of GLP-1 by the DPP-4 enzyme, the levels of active GLP-1 'available' for these nerve endings in the immediate proximity of the L-cells are likely to be enormous as opposed to any other target cell outside the intestinal tract.

Studies involving pancreatic clamping indicate that approximately 50% of the glucose-lowering effect of GLP-1 is caused by inhibition of glucagon secretion, whereas the rest results from the well characterised effect on insulin secretion[89]. The glucagonostatic effect of GLP-1 seems to be preserved in patients with T2D[51]. The same has been found employing the GLP-1 analogue liraglutide in patients with T2D treated for one week[90]. The fasting and postprandial glucagon responses were significantly reduced, and, at least during the fasting state, this resulted in lower endogenous glucose production (EGP) and reduced PG levels. Interestingly, no significant changes in the insulin responses or gastric emptying were observed[90]. Comparable results were obtained

using the GLP-1 receptor agonist exendin-4, although these results are confounded by a markedly decrease in gastric emptying[91]. Nonetheless, during the overnight fast, the exendin-4 treatment inhibited glucagon secretion in this study[91]. Furthermore, in clinical studies using DPP-4 inhibitors[92,93], which have also been shown to enhance glucose-induced insulin secretion, insulin concentrations are usually unchanged whereas plasma glucagon levels are reduced[92,93]. These results underscore the importance of GLP-1 and glucagon interaction on glucose homeostasis.

The inhibitory effect of GLP-1 on glucagon secretion in vivo is glucose-dependent and only observed at PG levels at or above fasting levels[16,94]. In studies involving graded hypoglycaemic clamping in humans, the inhibitory effect of GLP-1 on glucagon secretion was lost at PG levels just below normal fasting levels[94]. This is important, in the sense that GLP-1 infusions do not inhibit the counter regulatory glucagon response to hypoglycaemia.

GLP-1 AND EFFECTS ON APPETITE

Infusion of GLP-1 in lean and obese healthy human individuals causes dose-dependent reductions in satiety measures and ad libitum food intake[95–98]. Animal studies using exendin 9-39 have demonstrated that GLP-1 receptor activation is important for the regulation of appetite and food intake[99,100]. On the other hand GLP-1 receptor knockout mice are not obese[101] indicating that GLP-1 receptor activation is not a prerequisite for body weight regulation. The mechanisms behind the anorectic actions of GLP-1 are believed to be mediated through both central and peripheral mechanisms[61,102–104]. as reviewed previously[10].

GLUCAGON

Glucagon, secreted from pancreatic alpha cells in response to low PG concentrations, plays a central role in the maintenance of fasting glycaemic levels through its stimulatory effect on EGP securing sufficient energy supply to the central nervous system (CNS) and muscles. After carbohydrate ingestion in healthy individuals, glucagon secretion is suppressed, removing a stimulus for EGP. The mechanisms behind postprandial glucagon suppression have been proposed to include the known inhibitory effect of a rise in both PG and insulin concentrations[105,106]. Glucagon is a 29 amino acid peptide hormone produced from proglucagon in pancreatic alpha cells. Posttranslational processing of proglucagon in the pancreas results in the formation of glucagon, glicentin-related polypeptide (GRPP), and the so-called "major proglucagon fragment"[107], all of which are released simultaneously upon alpha cell stimulation. Processing of proglucagon in the pancreatic alpha cells is catalysed by the locally expressed PC2 as opposed to the processing of proglucagon in the intestine by PC1/3 as described above[37]. Apart from hypoglycaemia, which is an important secretory stimulus, alpha cell secretion is also stimulated by other factors such as activity in the autonomic nervous system and by circulating amino acids [108].

In essence, low PG alters the activity of specific ATP-sensitive potassium (KATP) channels in both the brain[109] and on the pancreatic alpha cell surface resulting in increasing electrical activity and release of glucagon[110,111]. Whether the alpha cells are primarily reacting directly to changes in PG concentrations, or whether intra-islet paracrine interactions are essential (the intra islet hypothesis) is currently debated. Nevertheless, alpha cell secretion is also influenced by the incretin hormones (as will be the

main focus for the following); for example, the inhibition of glucagon secretion by GLP-1 as described previously. The effect of GIP on glucagon secretion seems to depend on the PG concentration, as studies in the perfused rat pancreas indicated that GIP stimulates glucagon secretion during hypoglycaemia[112]. In humans, administration of physiological doses of GIP during euglycaemia is associated with dose-dependent increase in glucagon secretion in healthy individuals[52,113,114]. Interestingly, Christensen et al. confirmed the findings from the perfused rat pancreas in humans. These studies showed that infusions of GIP resulted in increased plasma levels of glucagon when PG levels were clamped at euglycaemia and hypoglycaemia, respectively, but not during hyperglycaemia, where GIP was highly insulinotropic (as expected)[52]. Interestingly, in patients with T2D, the glucagonotropic effect of GIP is preserved[53].

GLUCAGON AND TYPE 2 DIABETES

As mentioned, the pathophysiology of T2D also include hyperglucagonaemia in the fasting state, lack of glucagon suppression following oral glucose, and exaggerated glucagon responses to mixed meals[79]. During fasting conditions and postprandial, hyperglucagonaemia results in increased EGP in patients with T2D, contributing significantly to the high fasting PG levels in these patients[115]. Thus, abnormal regulation of glucagon secretion plays a key role in the development of fasting and postprandial hyperglycaemia in patients with T2D. It has therefore been suggested to antagonise glucagon signalling for the treatment of diabetes as reviewed previously[116].

The glucagon response to OGTT in T2D is ambiguous. For the first 30–60 min, glucagon levels generally rise in response to an OGTT, in contrast to the evident suppression seen in the same patients when glucose is administered iv to reach identical PG concentrations (isoglycaemic clamping)[9,20,117]. Because of the isoglycaemia a role for postprandial glucose was ruled out. Furthermore, the increase occurs in the face of increased insulin secretion (compared to IIGI) (Fig 1.).

The regulation of this phenomenon in patients with T2D, compared to healthy individuals was the major question in study 2. Currently it is thought that the abnormal response is caused by stimulatory gastrointestinal factors[20]. Such a factor could be GIP as suggested by Lund et al.[51], who reproduced the amplified glucagon response elicited by an OGTT during an IIGI combined with physiological doses of GIP infused in patients with T2D[51]. GIP came out as a positive secretory stimulus, whereas GLP-2 revealed no effect, and GLP-1 was suppressive as expected[51]. These findings are in line with the clamp studies by Christensen et al. as mentioned above[53].

GLUCAGON EFFECTS ON APPETITE

In healthy individuals glucagon levels rise after ingestion of a mixed meal[118]. The postprandial rise in glucagon is most likely caused by amino acids and has been proposed to counterbalance meal-induced insulin secretion[79]. However, in 1977 Martin & Novin proposed that glucagon could also be considered a physiological satiety signal[119]. They showed that intraportal injections of glucagon in rats reduce food intake[119] and since then an inhibitory effect on food intake has been confirmed across many species[120]. In addition, spontaneous meal size was augmented in rats by glucagon immunoneutralisation[121], indicating that the postprandial glucagon increments represent a significant physiological satiety signal. Further studies by the same group suggested that the liver acts as the target organ and that hepatic

vagal fibres may be essential for transduction of the signal[122]. First, they showed that portal injections of glucagon were more effective with regards to reduced food intake compared to injections in vena cava. Secondly, it was demonstrated that the potency of glucagon was lost using portal injections in hepatically vagotomised rats, and thirdly, that immunoneutralisation of glucagon fails to increase food intake after hepatic vagotomy[122]. Glucagon receptor knock-out mice, on the other hand, are lean, but have severe alpha cell hyperplasia and hyperglucagonaemia[123]. The high levels of glucagon may activate other receptor systems, for example the GLP-1 receptors, which may explain the knock-out phenotype.

For decades, glucagon administration in humans has been known to reduce food intake and cause weight loss. In 1957, ethically questionable studies in "healthy individuals" in a mental institution included ten individuals admitted to the maximum security division, who were injected intramuscularly with high doses of glucagon (1 mg) prior to every meal for two weeks in a double-blinded crossover design. Significant weight loss of 0.45 pounds was observed during active treatment, as opposed to a weight gain of 3–4 pounds during placebo injections[124]. These findings were confirmed a few years later in voluntary medical students, along with a negative nitrogen balance and moderate glycosuria[125], and recently in a study using supraphysiological iv glucagon infusions (50 ng/kg/min for 45 min), which showed increased energy expenditure[126]. However, these experiments were carried out using very high doses of glucagon resulting in supraphysiological plasma levels, which in turn causes considerable elevations of PG as well as insulin levels[126,127]. As glucagon is able to stimulate the GLP-1 receptor, albeit at lower potency than GLP-1 (in vitro EC50 of glucagon on the GLP-1 receptor is about 100 times less compared to GLP-1)[128], using supraphysiological doses of glucagon makes it difficult to distinguish physiological effects from pharmacological effects, which most likely include other related pathways. Hence, the above-mentioned studies should be interpreted with caution. Nonetheless, studies aiming at physiological levels of glucagon have shown an effect as well; small, short term infusions of glucagon mimicking postprandial levels (3 ng/kg/min for 10 min) decreased ad libitum meals by 20% without altering hunger scores by visual analogue scale (VAS)[129]. This suggests that glucagon may constitute a physiological satiety signal, and might also offer an explanation for the superior satiating effect of protein rich meals as opposed to carbohydrate rich meals[130]. In addition, this positions glucagon as a potential therapeutic target for obesity.

OXYNTOMODULIN

In the early 80ties, the structure of oxyntomodulin elucidated[131,132], Oxyntomodulin is – as alluded to above – a product of proglucagon processing which has affinity for both the glucagon receptor and the GLP-1 receptor[133–135] – in other words a dual glucagon-GLP-1 receptor agonist. Oxyntomodulin binds to and activates the GLP-1 receptor with a somewhat lower affinity compared to GLP-1 itself [133]. Furthermore the peptide binds to and activates the glucagon receptor, but with a 10–100-fold lower affinity than glucagon[134]. Like GLP-1, oxyntomodulin is released from intestinal L cells in response to meal ingestion, with plasma concentrations being closely related to the caloric intake[134]. The amino acid sequence of oxyntomodulin correspond to the entire 29-amino acid sequence of the glucagon molecule but with a C-terminal extension of eight amino acids[132], identical to those

of glicentin. Together, the two peptides were designated “enteroglucagon” because of their cross-reaction with antibodies against a mid-region of the glucagon molecule. Like glucagon, it may be degraded by DPP-4 *in vitro*, but is unlikely to serve as a substrate *in vivo*[136]. It may be a substrate for the enzyme neutral endopeptidase 24.11 (half-life ~12 minutes[137,138]).

Oxyntomodulin effects on appetite

The acute effects of administration of oxyntomodulin in humans include inhibition of gastric emptying, gastric and pancreatic exocrine secretion, and food intake[137,139]. The latter effect is in line with the observation that repeated subcutaneous (sc) administration causes body weight loss in obese individuals[140]. Interestingly, oxyntomodulin-induced weight loss has been claimed to be caused by reduced food intake combined with increased activity-related energy expenditure[141]. An oxyntomodulin receptor analogue with an increased affinity for the murine glucagon receptor, demonstrated increased potency with regards to inhibition of food intake and body weight reduction compared to native oxyntomodulin[12]. This suggests that the appetite and body weight regulating effects of oxyntomodulin are not only mediated through activation of the GLP-1 receptor but also via the glucagon receptor. Nevertheless, the central effects of native oxyntomodulin seems mainly to be mediated through the GLP-1 receptor as the food intake-reducing effect of oxyntomodulin infused in the rat brain is blocked with exendin 9-39[139]. Furthermore, the effect of oxyntomodulin is abolished in GLP-1 receptor knock-out mice[142]. The impact of either pathway in human weight loss is still unknown (the major question of study 3).

STUDY 1: IMPAIRED REGULATION OF THE INCRETIN EFFECT IN PATIENTS WITH TYPE 2 DIABETES

BACKGROUND AND AIMS

This study was conducted to investigate the ability of patients with T2D to regulate the incretin effect. Nauck and colleagues have previously estimated the incretin effect in healthy individuals during increasing glucose loads and showed that the incretin effect was amplified with increased amounts of oral glucose[18], thus maintaining glucose levels at almost identical levels regardless of the increasing glucose loads. However, it has remained unclear whether patients with T2D are able to increase their incretin effect with increasing doses of oral glucose. Therefore, we aimed to quantify the incretin effect, incretin hormone responses (both

GIP and GLP-1, since GLP-1 had not been studied before) and gastric emptying in patients with T2D and matched healthy individuals using the isoglycaemic clamp technique with increasing glucose loads (25g, 75g and 125g-OGTTs and three corresponding IIGIs) in eight patients with T2D and in eight matched healthy individuals.

SUMMARY OF RESULTS AND DISCUSSION

We found a markedly reduced capability to amplify the incretin effect in response to increasing oral glucose loads among patients with T2D. However, we showed progressively prolonged responses of GIP and GLP-1 with increasing oral glucose load in both patients with T2D and healthy individuals with no difference between the groups. Gastric emptying was progressively delayed in response to increasing oral glucose in both groups (no between-group differences) presumably contributing importantly to preventing increases in postprandial PG excursions in both patients with T2D and healthy individuals. In addition, it is clear that both hormone responses, but in particular that of GIP, are closely related to the emptying rate of the gastric contents into the small intestine. With all three doses, a similar peak GIP response is reached rapidly after ingestion, and the increasing doses merely result in a prolongation of this level of secretion. Thus, it is clearly the gastric emptying rate of stimulatory nutrients (in this case glucose) that governs the rate of secretion of the incretin hormones. In healthy individuals, the amplification of the incretin effect in response to ingestion of increasing glucose loads constitutes the most likely explanation for their ability to limit the PG excursions [18]. As illustrated in Fig. 2, similar peak PG values in response to 25g, 75g and 125g-OGTTs were observed in our healthy individuals. In contrast patients with T2D displayed increasing PG excursions with increasing glucose load and, accordingly, increasing AUC and peak PG values (Fig. 2).

In the healthy individuals, a considerable difference in insulin responses between OGTT and IIGI days was observed. Only minor differences between the responses to OGTT and IIGI were found in patients with T2D. During the 125g-OGTT, patients with T2D only managed to reach an incretin effect similar to what healthy individuals exhibited with a fifth of the amount of glucose (25g) as depicted in Fig. 3

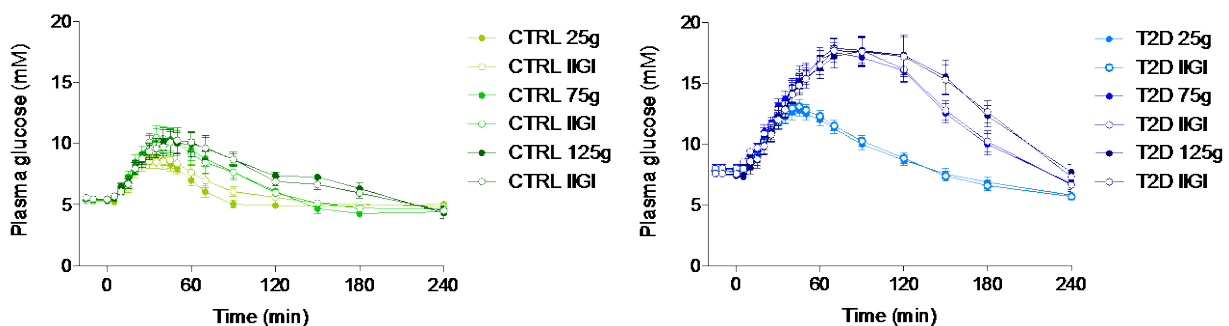


Figure 2

Plasma glucose. Glucose profiles are mean values (\pm SEM) following OGTTs (25g, 75g and 125g) (closed symbols) and corresponding IIGIs (open symbols). The blue lines represent the patients with T2D; the green lines represent the control individuals. The colour intensity represents the doses; light colour =25g, intermediate =75g and dark = 125g of oral glucose.

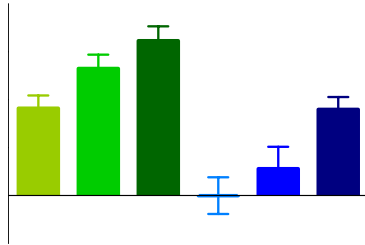


Figure 3

Incretin effect. Bars are mean values of the incretin effect (%) ± SEM following 25g, 75g and 125g OGTTs. The blue bars represent the patients with T2D; the green bars represent the control individuals. The colour intensity represents the doses; light colour =25g, intermediate =75g and dark = 125g of oral glucose.

We found no differences in GIP or GLP-1 responses during the OGTT between healthy individuals and patients with T2D[143,144] (Fig. 4). The similar incretin hormone responses in patients with T2D and control individuals in the current study supports that reduced insulinotropic potencies of the incretin hormones represent the major mechanism explaining the reduced incretin effect in patients with T2D[25].

The protracted PG profiles following larger oral glucose loads are probably due to decelerated gastric emptying in response to the glucose loads as indicated by the paracetamol results (Fig. 5). The regulation of gastric emptying may well be the main regulator of the postprandial PG in patients with T2D. This mechanism obviously serves to limit the amount of glucose emptied into small intestine and thereby limit the PG excursions. Importantly, this mechanism was preserved in the patients with T2D. By further analysis of the data in collaboration with Mari et al., we were able to show that the glucose-dependent insulin secretion rates in the patients reached the same levels as the control group although at much higher PG levels[145]. Only data from the IIGs were used in this particular model and are therefore ruling out any additional effect from the incretin hormones. Some intrinsic glucose-dependent regulatory effect therefore still exists alongside the impaired incretin effect and intact gastric emptying.

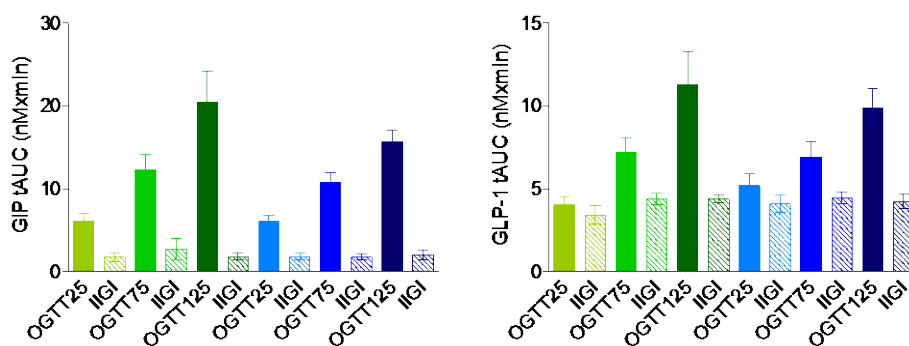


Figure 4

GIP and GLP-1. Bars are mean tAUC ± SEM following OGTTs (i.e. 25g, 75g and 125g) (filled bars) and corresponding IIGs (crossed bars) in both groups. The blue bars represents the patients with T2D, the green bars represents the control individuals. The colour intensity represents the doses; light colour =25g, intermediate =75g and dark = 125g of glucose.

STUDY 2: GLUCAGON RESPONSES TO INCREASING ORAL LOADS OF GLUCOSE AND CORRESPONDING ISOGLYCAEMIC IV GLUCOSE INFUSIONS IN PATIENTS WITH TYPE 2 DIABETES AND HEALTHY SUBJECTS

BACKGROUND AND AIMS

In healthy subjects, glucagon responses during 50g OGTT and a IIGI are suppressed equally whereas patients with T2D exhibit glucagon hypersecretion during oral glucose and normal suppression during IIGI - bypassing the gastrointestinal tract[9]. Meier et al. reported a similar tendency in healthy subjects challenged with 50% higher doses of oral glucose (i.e. 75g-OGTT) suggesting that gut-derived and/or gut-mediated secretion of glucagon may also occur in non-diabetic subjects[113].

In study 2 we investigated whether increasing orally administered glucose loads would elicit progressively inappropriate glucagon responses due to gut-mediated stimulation of glucagon secretion, and whether suppression of glucagon following corresponding IIGs (with no stimulation of the gastrointestinal tract) would be preserved; using plasma samples from study 1.

SUMMARY OF RESULTS AND DISCUSSION

With study 2 we confirmed that patients with T2D exhibit hypersecretion of glucagon in response to orally administered glucose whereas suppression was normal during IIGI. Interestingly, we show that a similar pattern can be observed in healthy subjects when more than 75g glucose is ingested orally, and that these differences in glucagon secretion between OGTT and IIGI increase with the amount of orally administered glucose. The 25g-OGTT and IIGI, respectively, resulted in clear and similar suppression of the glucagon levels in the control group (Fig. 6.). In contrast, patients with T2D exhibited delayed suppression in response to

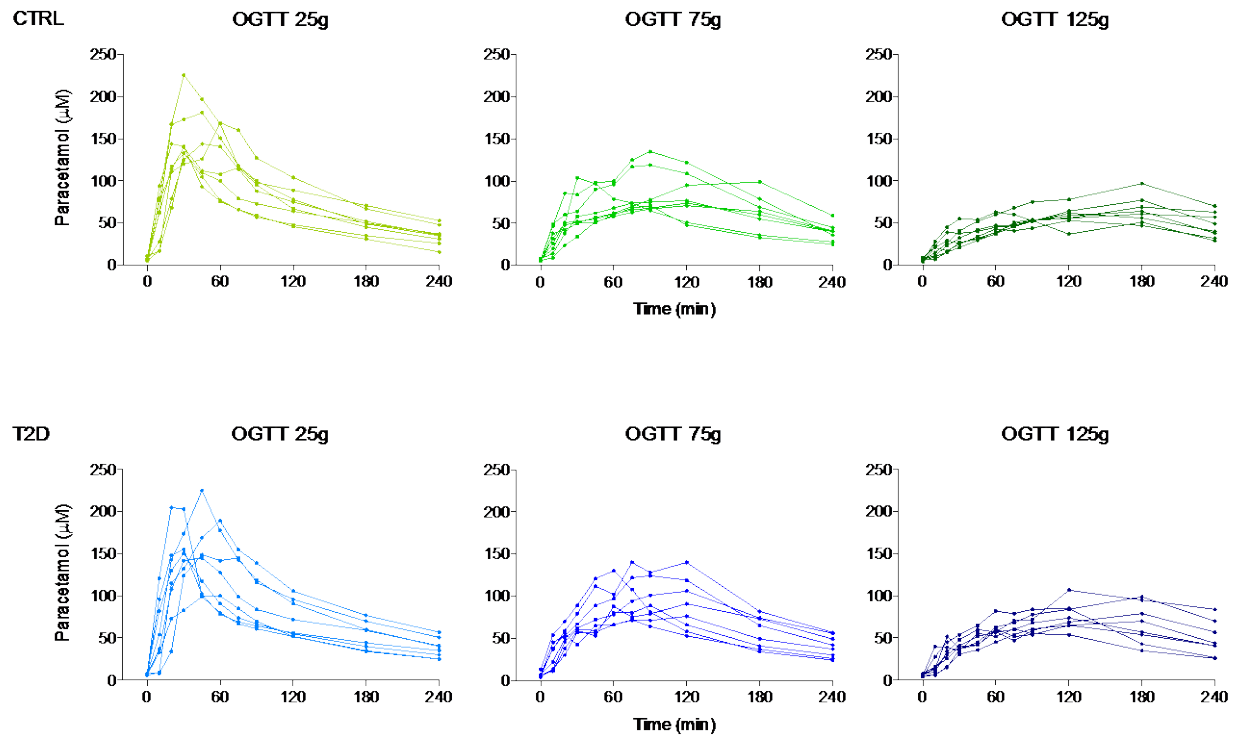


Figure 5
Paracetamol. Profiles are raw data following OGTTs (i.e. 25g, 75g and 125g) both groups. The blue lines (lower panel) represents the patients with T2D, the green lines (upper panel) healthy control individuals. The colour intensity represents the doses; light colour =25g, medium =75g and dark = 125g of glucose.

the 25g-OGTT, whereas the IIGI resulted in immediate suppression of glucagon (Fig. 6). A similar pattern was observed in response to the increasing OGTTs (i.e. 75g and 125g) and IIGIs resulted in suppression of glucagon in patients with T2D (Fig. 6). Interestingly, in the healthy subjects the glucagon responses to the increasing oral glucose loads showed progressively increasing differences to the respective IIGIs (Fig 6). The different glucagon responses to oral vs. iv glucose most likely arise as a consequence of direct stimulation of gut. It may be glucagon secreted from the gut or glucagonotropic factors released from the gut in response to oral glucose. One such factor may be GIP [51]. The differences in glucagon between healthy subjects and patients with T2D observed in this study can not be explained by differences in secretory patterns of GIP (Fig. 3). However, the glucagonotropic action of GIP may be different in the two groups as suggested by Christensen et al.[53]. Thus, the glucagonotropic effect of GIP may play a role in the postprandial hyperglucagonaemia characterising T2D.

Furthermore, glucagon produced and secreted from the enteroendocrine cells in the gut might also be the source of elevated OGTT-induced glucagon responses. In line with this, a case of human PC1/3-deficiency was characterised by elevated postprandial glucagon levels, indicating that proglucagon was processed by PC2[146]. This is supportive for the hypothesis that smaller fractions of proglucagon are being processed not only by PC1/3 but also by PC2. This may explain the previous observation that pancreatectomised subjects are able to secrete glucagon in response to a carbohydrate rich meal[147]. Overall our results imply that the hyperglucagonaemia observed in patients T2D after oral glucose might represent a pathologic variant of a gut-derived physiological phenomenon.

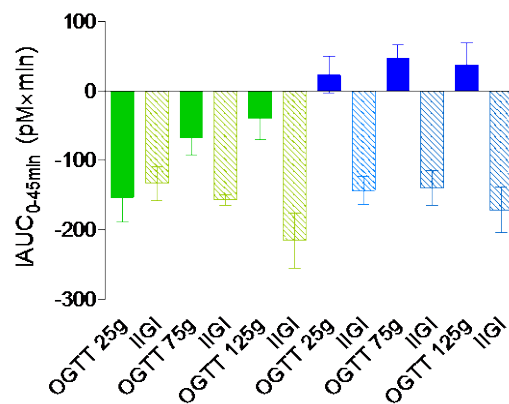


Figure 6
Glucagon incremental AUC. Bars represent mean + SEM values following OGTTs (i.e. 25g, 75g and 125g) (filled bars) and corresponding IIGIs (shaded bars) in both groups. The blue bars represents the patients with T2D, the green bars represents the healthy control subjects.

STUDY 3: EFFECT OF OXYNTOMODULIN, GLUCAGON, GLP-1 AND COMBINED GLUCAGON+GLP-1 INFUSION ON FOOD INTAKE, APETITE AND RESTING ENERGY EXPENDITURE

BACKGROUND AND AIMS

The mechanisms behind the body weight-lowering effect of the dual GLP-1/glucagon receptor agonist oxyntomodulin remain unclear. Furthermore, the role of glucagon as a physiological satiety signal in humans[129] has only been investigated sparsely. We

therefore aimed to evaluate the separate and combined effects of glucagon-receptor and GLP-1receptor activation on gastric emptying, composite appetite scores (CASs), resting energy expenditure (REE) (oxygen absorption ($\dot{V}O_2$)) and food intake in 15 young healthy men and to compare these to the effects of oxyntomodulin and saline infusions.

SUMMARY OF RESULTS AND DISCUSSION

Infusion of oxyntomodulin and the separate and combined infusion of GLP-1 and glucagon inhibited food intake similarly in healthy individuals, with no superior effect of combining GLP-1 and glucagon. We confirm the inhibitory effects of oxyntomodulin and GLP-1, respectively, on GE and appetite scores observed previously, but by adding glucagon to the infusion of GLP-1 we found no additive effects. Unexpectedly, glucagon alone had no effect on GE and appetite scores, but inhibited food intake to the same extent as oxyntomodulin, GLP-1 and GLP-1+glucagon.

Both the GLP-1, oxyntomodulin and GLP-1+glucagon infusions appeared to increase $\dot{V}O_2$ compared to saline but this observation is most likely confounded by a residual meal-induced thermogenesis[148] because the calorimetry was performed relatively soon after the paracetamol peak (Fig. 7) indicating that a considerable volume still resided in the stomach and a high rate of nutrient absorption probably was still going on compared to the saline infusion. Flint et al. previously concluded from a protocol very similar to ours using GLP-1 infusions, that the observed increases in energy expenditure most likely were linked to the meal[149].

In contrast we observed no significant changes in $\dot{V}O_2$ from baseline in any of the experiments in our study.

The lack of a clear effect on $\dot{V}O_2$ is in contrast to recently reported findings regarding infusions of glucagon and GLP-1[126]. But the dose of glucagon used in that particular study was more than 15 fold higher than ours and associated with large changes in glucose and insulin levels. Such levels are likely to influence REE and offer an explanation of the reported additive effect of combinations of GLP-1 and glucagon[126]. Our conclusion is consistent with recent findings showing no increases after short-term native GLP-1 infusions[150]. Long-term treatment with the GLP-1 analogue liraglutide using 24h chamber calorimetry has so far shown no differences in energy expenditure following the treatment[151,152].

Surprisingly, the infusion of glucagon did not change gastric emptying (Fig. 7). This finding is controversial since glucagon previously has been used to inhibit bowel motility[153,154]. However, the doses used to inhibit bowel motility were more than 3,000 fold higher than the dose used in the present study[153] and as mentioned above, such doses might activate the GLP-1 receptor pathway. Interestingly, the glucagon infusion did result in decreased food intake to the same extent as the other peptide infusions despite having no impact on gastric emptying and appetite scores.

We found a mean 180 kcal (120 g) difference in food intake following infusions of all the peptides compared to saline. This would roughly sum up to a body weight loss of 402 g of fat per week, which is in the range of what previously has been found in overweight and obese humans with the injection of oxyntomodulin[140].

CONCLUSIONS

In this thesis we demonstrated that the gut-regulated insulin and glucagon secretion is impaired in T2D despite a preserved GLP-1 and GIP secretion, and that glucagon and GLP-1 seem to inhibit food intake equally but without an additive effect from activating both receptors (with combined infusions or oxyntomodulin). From study 1 we learned that patients with T2D are characterised by impaired regulation of the incretin effect resulting in exaggerated post absorptive PG excursions during large glucose loads. Incretin hormone responses to the increasing oral glucose loads were clearly dose-dependent, and this apparently depended strongly on gastric emptying rates, but the impaired regulation of the incretin effect in T2D could not be explained by differences in the secretion of incretin hormones between the two groups. We found no difference in the gastric emptying between the groups, but we found a remarkable dose-dependent inhibition of gastric emptying, which might stand out as the main auxiliary regulator of postabsorptive PG in place of the incretin effect in patients with T2D.

In study 2 we demonstrate that in patients with T2D increasing amounts of oral glucose elicit hypersecretion of glucagon whereas corresponding IIGs result in significant glucagon suppression. Interestingly, we observed the same phenomenon in healthy subjects when larger glucose loads are ingested orally. This implies that type 2 diabetic hyperglucagonaemia observed after oral glucose may represent a pathologic version of a gut-derived physio-

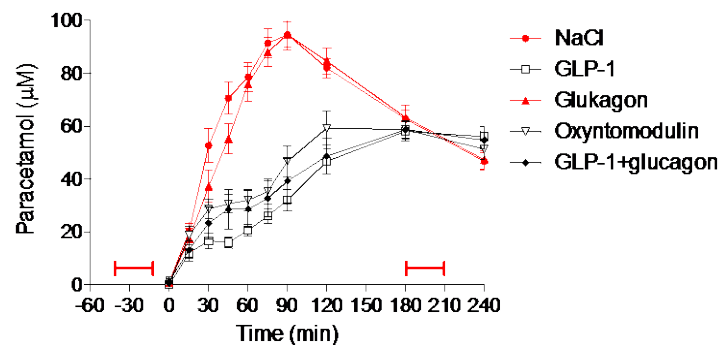


Figure 7

Plasma paracetamol. Profiles are mean values \pm SEM following meal tests during the respective infusions (i.e. NaCl, GLP-1, glucagon, oxyntomodulin and GLP-1+glucagon). The red lines represent the rapid gastric emptying; the black lines represent the infusions causing an inhibition of the gastric emptying. Red horizontal bars indicate timeframe of calorimetry.

logical phenomenon. As suggested, this could constitute a physiological signal contributing to inhibit food intake[129]. Importantly, our results do not support that abnormal alpha cell responses to glucose explain the hyperglucagonaemia of type 2 diabetes.

From study 3 we can confirm that glucagon has a potent inhibitory effect on food intake, although without effects on subjective appetite ratings and gastric emptying. Since this was elicited at levels of glucagon within the physiological range, these results support that glucagon is a physiological contributor to the regulation of food intake, although clearly not a regulator of subjective appetite sensing. Both GLP-1 and oxyntomodulin and the combination of GLP-1 and glucagon inhibited food intake similarly and inhibited gastric emptying similarly, however we found no additive effect of combining GLP-1 and glucagon on any measures. Clearly more studies are needed to clarify the physiological and pathophysiological role of glucagon and GLP-1, however from the results of this thesis both seem to be implicated in the regulation of food intake and in the pathophysiology of T2D.

PERSPECTIVES

In this thesis we followed a lead from the first protocol; inevitably new questions evolved along the way, some of which we have already addressed, however, we left important questions behind. The significance of the glucagon responses we observed in study 2 for PG has been investigated in pancreatic clamp studies, and similar elevations created using exogenous glucagon infusions have been shown to be of importance for the hyperglycaemic state of T2D [155]. However, the isoglycaemic clamp protocol makes it possible to investigate the influence of the endogenous glucagon response (present during OGTT, absent in IIGI) in relation to glucose metabolism, REE, food intake and appetite measures etc. in both patients with T2D and healthy subjects, particularly if combined with glucagon receptor antagonists. Such experiments would be of great interest and may actually be possible in the near future since several pharmaceutical companies are currently working on the development of glucose-lowering drugs based on antagonism of the glucagon receptor.

The origin of the glucagon response we measured following oral glucose stimulation is not clear. In light of the newly recognised potential of the enteroendocrine cells to produce a variety of potent peptides[43] and the apparent postprandial glucagon release observed in pancreatectomised subject[147,156] it would be interesting to address the tissue origin of secretion. There are several approaches to encircle this issue, for example to further investigate a group of pancreatectomised patients.

The mechanism of the potent inhibitory effect of GLP-1 on glucagon secretion is also not clarified. From the studies by Vilsbøll et al.[16] and Nauck et al.[94] we know that the effect of GLP-1 on glucagon secretion is dependent on PG levels. However, during the initial phase of the OGTTs with elevated GLP-1, GIP and insulin levels in plasma, and increasing PG we still find increasing glucagon levels. In parallel to the hypothesis of gut-derived glucagon and the hypothesis of GIP-induced glucagon secretion, one might propose an altered potency of the suppressive effect of GLP-1 with increasing PG.

The effect of glucagon on food intake found in study 3 would be interesting to investigate further. The long-term efficacy of this highly potent hormone is not known. Investigations not only concerning weight loss but also gluco-regulatory implications during longer term treatment in both healthy, obese and T2D subjects would be necessary with the prospect of treatment purposes.

The reason for the hyperglucagonaemia (both fasting and postprandial) in patients with T2D is not clarified and would also be very interesting study further. All attempts to interfere with glucagon signalling so far have resulted in (mild or severe) hyperglucagonaemia in both animal and human studies (as reviewed in [116]). It is therefore obvious to hypothesise that it is a matter of glucagon resistance. However, it could also be a matter of altered clearance similar to the hyperinsulinaemia in non-alcoholic fatty liver disease (NAFLD) patients[157].

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