

On the role of the gut in diabetic hyperglucagonaemia

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The thesis is based on the following three original papers

1. Lund A, Vilsbøll T, Bagger JI, Holst JJ, Knop FK. The separate and combined impact of the intestinal hormones, GIP, GLP-1, and GLP-2, on glucagon secretion in type 2 diabetes. *Am J Physiol Endocrinol Metab.* 2011 Jun;300(6):E1038-1046
2. Lund A, Bagger JI, Albrechtsen NJW, Christensen M, Grøndahl M, Hartmann B, et al. Evidence of Extrapancreatic Glucagon Secretion in Man. *Diabetes.* 2016 Mar 1;65(3):585-97
3. Lund A, Bagger JI, Christensen M, Grøndahl M, van Hall G, Holst JJ, et al. Higher Endogenous Glucose Production during OGTT vs Isoglycemic Intravenous Glucose Infusion. *The Journal of Clinical Endocrinology & Metabolism.* 2016 Aug 17;jc.2016-1948

INTRODUCTION

In normal physiology, glucagon from pancreatic alpha cells plays a central role in maintaining glucose homeostasis via its stimulatory effects on hepatic glucose production. When glucose concentrations are high, glucagon secretion is suppressed, and during low glucose concentrations, glucagon secretion is increased securing an essential supply of energy (i.e. glucose) to the central nervous system and muscles. In patients with diabetes, glucagon concentrations are elevated in the fasting state and fail to decrease appropriately or even increase during an oral glucose tolerance test (OGTT) or after ingestion of a mixed meal [1,2]. The inappropriately high glucagon concentrations result in increased endogenous glucose production (EGP) and, thereby, contribute significantly to the fasting and postprandial hyperglycaemia characterising patients with diabetes [3]. The importance of hyperglucagonaemia in diabetes was introduced 40 years ago when Unger and Orci proposed their "bihormonal hypothesis" stating that the combination of insulin deficiency and hyperglucagonaemia constitutes a central determinant for diabetic hyperglycaemia [4]. Since then, the importance of excess glucagon in diabetic

pathophysiology has been confirmed in several studies and was again recently highlighted by Unger's group when they showed that blocking of glucagon action in mice with chemically induced type 1 diabetes completely normalised fasting and postprandial glucose values [5]. Furthermore, over the years hyperglucagonaemia has increasingly been recognised as a therapeutic target and since the successful introduction of antidiabetic drugs with glucagon-lowering capabilities, the scientific and clinical interest in the role of glucagon in diabetic pathophysiology has attracted great attention [6,7]. Nevertheless, the mechanisms behind diabetic hyperglucagonaemia remain obscure. Most studies have focused on an increased secretion of glucagon from the pancreas, and, despite lack of solid evidence, the current dogma states that diabetic alpha cell resistance to the glucagon-suppressive effects of glucose and insulin, respectively, constitutes a decisive factor for the development of hyperglucagonaemia [8]. Interestingly, studies have shown that the hyperglucagonaemia in type 2 diabetes is aggravated by oral intake of glucose, but not during isoglycaemic iv glucose infusion (IIGI) [9,10]. Due to the isoglycaemic conditions in these studies, it is unlikely that impairment of glucose-sensing of the alpha cell plays a major role in the exaggerated glucagon responses after oral glucose ingestion in patients with type 2 diabetes. Neither does insulin resistance of the alpha cell seem to explain this phenomenon as insulin responses during oral administration of glucose are markedly higher than during IIGI. Thus, if anything, the larger insulin response to oral glucose would be expected to result in greater suppression of glucagon responses compared to IIGI; but the opposite occurs [11]. This difference in glucagon secretion between OGTT and IIGI is present not only in patients with diabetes, but also in non-diabetic control subjects when larger glucose loads are ingested [10,12]. Together, the above-mentioned findings strongly suggest that factors originating from nutrient-stimulation of the gastrointestinal tract play an important role in postprandial glucagon secretion.

The objective of the studies in this thesis was to investigate mechanisms behind the postprandial hyperglucagonaemia observed in patients with diabetes. In the following sections, physiological and pathophysiological regulation of glucagon secretion is described with emphasis on the role of the gut.

PANCREATIC ALPHA CELLS AND GLUCAGON SECRETION

Glucagon is a 29-amino acid peptide hormone produced and secreted from the alpha cells located in clusters of endocrine cells in the islets of Langerhans distributed throughout the pancreas. Besides alpha cells, the islets contain beta cells (producing insulin and amylin), delta cells (producing somatostatin) and PP cells (producing pancreatic polypeptide (PP)) [13]. The multicellular islets of Langerhans constitute the endocrine pancreas and the finely-tuned and balanced secretion of hormones from the approximately 1 million islets, with a total islet volume of approximately 0.5 to 1.3 cm³ in the adult human pancreas [14], is crucial for maintaining normal glucose homeostasis [15]. The

cytoarchitecture and composition of the islets of Langerhans differ between species. In rodents, beta cells are the most abundant accounting for 60-80% of the endocrine cells and are located primarily in the core of the islets with the other cell types scattered around the periphery (alpha cells: 15-20%; delta-cells < 10%; PP-cells < 1%) [16]. In humans islets, the proportion of delta and PP cells are similar to rodents but the proportion of beta cells is lower (48-59%) and the proportion of alpha cells is greater (33-46%), which may suggest a more prominent role of glucagon in humans. In human islets, the endocrine cells appear scattered in the islets allowing greater interaction among the different endocrine cell types [13]. The islets of Langerhans are richly vascularised, which ensures an effective sensing of the concentrations of nutrients and hormones allowing adaptive responses from the endocrine cells [13].

Normal regulation of glucagon secretion from the pancreatic alpha cells is complex and controlled by many factors including circulating nutrients [17,18], local control by other islet hormones [19-21], neural factors [22] and endocrine factors including gut-derived hormones (as discussed below) [23-25]. Glucose is probably the most important regulator of pancreatic glucagon secretion with hypoglycaemia being a powerful stimulator, and hyperglycaemia being a strong suppressor of glucagon secretion. Thus, glucagon concentrations increase when glucose concentrations fall below approximately 3.8 mmol/l [26], and decrease when glucose levels rise above normal fasting levels [12,27]. Several mechanisms have been suggested to account for the inhibition of glucagon secretion by hyperglycaemia. Insulin and somatostatin from the neighbouring beta and delta cells, respectively, are known to be powerful suppressors of glucagon secretion, and have been proposed to represent key-mediators of glucagon suppression during hyperglycaemia [28]. This 'auto-regulation' of islet hormone secretion is known as the "intra-islet hypothesis" and assumes that the islet cells of the pancreas are not independently functioning cells but that they mutually regulate secretion of one another at a basic feedback level in order to maintain glucose homeostasis [20]. Thus, the high insulin concentration during hyperglycaemia (as a result of a direct stimulatory effect of glucose on the beta cells) would inhibit glucagon secretion [29]. Glucose per se also seems to have a direct inhibitory effect on the alpha cells as suggested from *in vivo* human studies and studies of isolated islets from both rodents and humans [18,30,31]. More recent studies have suggested gamma-aminobutyric acid (GABA) also secreted from beta cells as a potential suppressor of glucagon secretion [32]. Glucagon secretion is stimulated by certain amino acids such as glutamine, alanine and arginine [33], with the latter being the most potent stimulatory amino acid and often used in tests to determine maximal alpha cell (and beta cell) secretion [34]. Proposed mechanisms involved in the vital glucagon secretion during hypoglycaemia encompass a sudden cessation of insulin secretion and thus, according to the intra-islet hypothesis, a loss of the paracrine inhibitory effect of insulin on the alpha cell [29]. A major argument for this hypothesis is the lack of a glucagon response to hypoglycaemia in insulin-deficient patients with type 1 diabetes [35]. Other mechanisms involve the influence of the autonomic nervous system and studies have shown that activation of sympathetic and parasympathetic nerves, both, potently stimulates glucagon secretion [36]. During a state of profound hypoglycaemia, other autonomic influences on the alpha cells, such as the stress hormone adrenaline may also contribute to glucagon secretion [37]. Studies have suggested that zinc from neighbouring beta cells may have a stimulatory effect on glucagon secretion

[38]. From the above it is clear that the regulation of glucagon secretion is complex and that normal glucagon regulation is important to maintain glucose homeostasis, avoid hypoglycaemia and to minimise postprandial glucose excursions.

As described above it has become clear that also the gastrointestinal tract may play a role in postprandial glucagon regulation. Gut hormones that are secreted in response to an OGTT include glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and GLP-2 which all previously have been shown to affect glucagon secretion [23,24,39,40]. An analysis of the effect of these hormones on glucagon regulation in normal physiology and in the pathogenesis of diabetes, is therefore of interest. Furthermore, a potential extrapancreatic source of glucagon secretion has been proposed from studies in totally pancreatectomised patients [41] and in the light of the differential glucagon responses to oral and *iv* glucose a thorough investigation of extrapancreatic glucagon secretion seems interesting.

GIP AND GLUCAGON SECRETION

The incretin hormone GIP is a 42-amino acid peptide hormone secreted from the enteroendocrine K cells dispersed throughout the small intestine. While the insulinotropic effect of GIP is well described, the effect of GIP on the pancreatic alpha cells and glucagon secretion has been more controversial. In 1978, preclinical studies proposed a glucagonotropic effect of GIP as studies in the perfused canine and rat pancreas demonstrated that GIP augmented glucagon secretion at low glucose concentrations [42,43]. In the following years, this glucagonotropic effect of porcine GIP could not be found in studies of non-diabetic subjects when glucose was held at fasting levels or during hyperglycaemic conditions [44]. In 1987, a study by Kreymann et al. described that infusion of synthetic human GIP significantly stimulated glucagon secretion in young healthy subjects, a finding that was not given much attention as it was overshadowed by the findings of substantial insulinotropic effects of GIP and GLP-1 and of a glucagonostatic effect of GLP-1 (as described below) [45]. The following years, studies showing a glucagonotropic effect of GIP accumulated [46,47]. Subsequent studies showed that the GIP receptor is expressed in pancreatic alpha cells [48], and that GIP, in the perfused rat pancreas, stimulates glucagon secretion by a direct mechanism through activation of protein kinase A [47,49]. In 2003 an investigation of the glucagonotropic effect of GIP was reported by Meier et al. showing that varying concentrations of GIP administered as bolus injections during fasting glycaemia dose-dependently increased plasma glucagon concentrations [24]. Interestingly, the effect of GIP on glucagon secretion has been shown to be dependent on the prevailing glucose concentrations, much like the well-known glucose-dependent effect of GIP on insulin secretion. Studies showed that a 'physiological' infusion of GIP in non-diabetic individuals elicited a glucagonotropic effect during hypoglycaemic and euglycaemic conditions, but that this glucagonotropic effect was lost during hyperglycaemia [50,51]. Several studies in non-diabetic individuals have shown that during hyperglycaemic conditions GIP does not seem to exert its glucagonotropic effects [52-56].

GLP-1 AND GLUCAGON SECRETION

The other incretin hormone, GLP-1, is a 30-amino acid peptide secreted from the enteroendocrine L cells distributed throughout the gastrointestinal tract with apparently increasing densities from the duodenum to the rectum. Since the discovery of GLP-1

in 1987 [45,57,58], this peptide hormone and its pleotropic effects in the body has been a topic of immense research [59]. The 1987 paper by Kreymann et al. [45] confirmed in humans the insulinotropic effects of GLP-1 previously demonstrated in rat and pig pancreata [57,58]. These authors also noted that an iv infusion of GLP-1, mimicking postprandial concentrations, significantly suppressed glucagon secretion. In 1988, a glucagonostatic effect of GLP-1 was reported in a preclinical study in the isolated perfused porcine pancreas, where GLP-1 added to the perfusate caused a dose-dependent suppression of glucagon secretion [60]. Since then, a bulk of evidence confirming the glucagonostatic effects of GLP-1 in normal physiology have accumulated [49,60–62]. Physiological doses of GLP-1 were shown to suppress glucagon concentrations in non-diabetic subjects during a three-stepped glucose clamp at 5.1 mmol/l (fasting concentration), 6 mmol/l and 7 mmol/l, implying that meal-induced elevations of GLP-1 inhibit glucagon secretion [63]. The role of postprandial GLP-1 elevations on glucagon secretion was confirmed in a study of non-diabetic subjects applying the GLP-1 receptor antagonist exendin 9-39 during a carbohydrate-rich drink. This study showed that blocking the effects of GLP-1 significantly increased postprandial glucagon concentrations, illustrating the importance of endogenous GLP-1 secretion for the regulation of glucagon [64]. Interestingly, the glucagonostatic effect of GLP-1 in vivo has been shown, like the insulinotropic effect of GLP-1, to be glucose-dependent and to be observed only at or above fasting plasma glucose levels [65]. From a therapeutic point of view, this represents a very important feature, as it implies that GLP-1 may not weaken the counter-regulatory glucagon response during hypoglycaemia [66].

The mechanisms behind the suppressive effects of GLP-1 on glucagon secretion are still not fully elucidated. It has been proposed that the suppressive effects are mediated through insulin which would be in-line with the intra-islet hypothesis and the fact that GLP-1 is considered one of the most insulinotropic substances known [20,67]. However, studies of patients with type 1 diabetes without residual beta cell function, and thus, without endogenous insulin production, have shown a preserved glucagonostatic effect of GLP-1 suggesting that other mechanisms must also be involved [61,68]. This was highlighted in a study by Kielgast et al. examining glucagon responses after meal ingestion in non-diabetic individuals and patients with type 1 diabetes, with or without residual beta cell function, during concomitant iv infusions of GLP-1 or exendin 9-39 [69]. The GLP-1 infusion potently suppressed postprandial glucagon concentrations in all three groups, whereas postprandial glucagon concentrations increased when the GLP-1 receptor was blocked by exendin 9-39. Preclinical studies have shown that a paracrine inhibition of the alpha cells by somatostatin might be involved [49,62]. Additionally, there is evidence that part of the effect of GLP-1 on insulin secretion (and maybe also on glucagon secretion) may be mediated via activation of parasympathetic nerves [36,70]. Plamboeck et al. aimed to elucidate this by characterising the effect of exogenous GLP-1 in truncally vagotomised subjects and demonstrated that the glucagonostatic effect of GLP-1 was compromised in these individuals, suggesting that an intact vagal innervation of the islets of Langerhans is important for GLP-1-mediated glucagon suppression [71].

GLP-2 AND GLUCAGON SECRETION

While the role of GLP-1 on islet hormone secretion has been thoroughly investigated in recent years, the role of the 33-amino acid sister-peptide, GLP-2, secreted from the same enteroendocrine L

cells, has gained less interest. This is likely because GLP-2, despite the structural relationship to GLP-1, has been shown to have no effect on insulin secretion [72]. Instead, GLP-2 has mainly been investigated with regards to its multiple biological effects on the gastrointestinal tract including stimulation of nutrient absorption and intestinal mucosal growth, and inhibition of gastrointestinal motility and gastric acid secretion [73,74]. Furthermore, a potential role of GLP-2 in the treatment of osteoporosis have been reported [75,76]. In 2003, a study from Sørensen et al. described a glucagonotropic effect of GLP-2 when infused to humans in physiological amounts. These findings were elaborated by Meier et al. who showed, in 2006, that a five-fold higher dose of GLP-2 than the one used in the study by Sørensen et al. increased glucagon concentrations in non-diabetic subjects both during fasting and postprandially [77]. Although not much is known about the mechanisms whereby GLP-2 regulates glucagon secretion, the expression of the GLP-2 receptor gene has been identified in alpha cells in studies of isolated rat islets suggesting that GLP-2 may have direct effects on the alpha cells. Furthermore, the GLP-2 receptor has been localised to alpha cells in both the rat and human pancreas using immunohistochemistry [39]. This study also confirmed a glucagonotropic effect of GLP-2 in the isolated perfused rat pancreas, and showed that the otherwise glucagonostatic effect of GLP-1 was reduced when GLP-2 was co-infused [39]. In contrast, a study from Bahrami et al. was not able to detect the GLP-2 receptor in mice islets, and did not find a rise in glucagon concentrations after GLP-2 administration to mice [78]. The glucagonotropic effect of GLP-2 was in 2010 shown to be independent of insulin secretion in a study of patients with type 1 diabetes (C-peptide-negative, and thus presumably without insulin production) [79]. A potential glucose-dependent effect of GLP-2 on glucagon secretion (as apparent for GIP and GLP-1) has not been investigated.

EXTRAPANCREATIC GLUCAGON SECRETION

Glucagon is generally thought of as a pancreas-specific hormone, secreted exclusively from the alpha cells in the islets of Langerhans. But a notion of possible extrapancreatic glucagon secretion was suggested as early as 1948 by Sutherland and deDube who showed that in rabbits and dogs a “glycogenolytic substance” could be extracted from the gastric mucosa [80]. At this time, however, a reliable method for the measurement of glucagon was not available. In 1960 the first radioimmunoassay (RIA) for insulin was introduced allowing for the first time reliable measurements of insulin concentrations [81]. Shortly after, the first glucagon RIA was developed [82]. In the following years, several investigations were conducted in totally pancreatectomised animals and humans in order to examine the possible existence of glucagon secreted from extrapancreatic tissues. Overall, these studies have reported considerable variation between species and provided conflicting conclusions regarding the presence or absence of extrapancreatic glucagon. Studies of totally pancreatectomised dogs have reported findings of “glucagon immunoreactivity” in plasma [83–86]. One of these studies found the gastric mucosa to be the place or origin [84] while another found the ileum to be responsible for the rise in glucagon immunoreactivity [85]. A study in ducks reported no trace of glucagon immunoreactivity following totally pancreatectomy [87], and several studies in totally pancreatectomised humans have contributed to the diverging conclusions with both confirmatory and non-confirmatory studies [41,88–99].

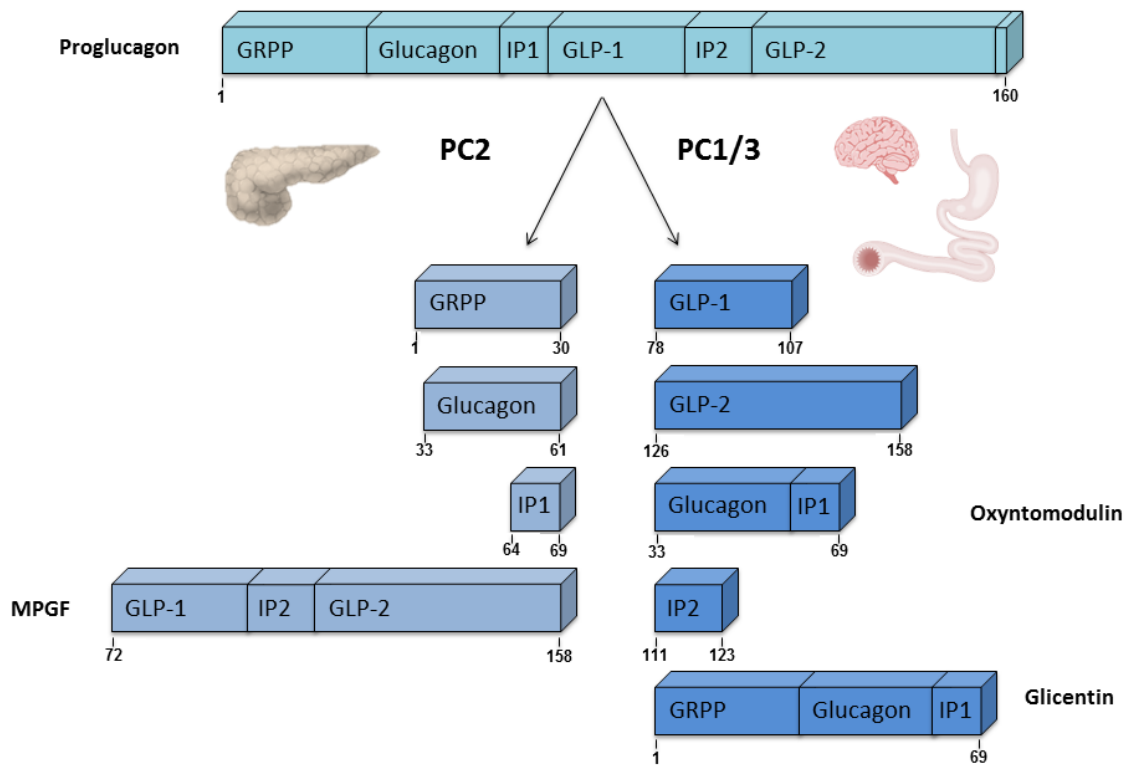


Figure 1

Differential processing of proglucagon by proenzyme convertase (PC) 1/3 (gut and brain) and PC2 (pancreas), respectively. GRPP: glicentin-related pancreatic polypeptide; GLP-1: glucagon-like peptide-1; GLP-2: glucagon-like peptide-2; IP-1: intervening peptide-1; IP-2: intervening peptide-2; MPGF: major proglucagon fragment. The numbers indicate amino acid positions in the 160-amino acid proglucagon sequence

One of the most important reasons for the controversy about the existence of extrapancreatic glucagon is that it has proved extremely difficult to measure fully processed 29-amino acid glucagon. For several reasons it has proved much more difficult to obtain sufficient sensitivity and specificity for the glucagon assay than for the insulin assay and still today this remains a limitation for many glucagon assays [82]. Firstly, circulating glucagon concentrations are relatively low (low picomolar range) which puts a high demand on the sensitivity of the assays. Secondly, many of the currently applied immunoassays are relying on either C-terminal or side-viewing antibodies that might react with some of the numerous glucagon-like peptides originating from the same precursor as glucagon, challenging the specificity of the assays. Lack of assay accuracy therefore, undoubtedly, explain many of the discrepancies in the literature regarding glucagon secretion. Glucagon is a product of the proglucagon gene (*GCG*), a gene expressed in pancreatic alpha cells, in specific enteroendocrine cells of the intestinal mucosa (L cells) and in a discrete set of neurons within the nucleus of the solitary tract [100–103].

GCG encodes proglucagon, a 160-amino acid peptide that is precursor to multiple biologically active peptides (Figure 1). The posttranslational processing of proglucagon is based on the presence and activity of the enzymes, proenzyme convertase (PC)1/3 and PC2 [104]. These enzymes are believed to be tissue-specific with PC2 being restricted to the pancreatic alpha cells, where it processes proglucagon into glucagon and three presumably biologically inactive fragments (glicentin-related pancreatic

polypeptide (GRPP), intervening peptide (IP)-1 and a major proglucagon fragment (MPGF) [104]. PC1/3 is expressed in the enteroendocrine L cells and the CNS, where it processes proglucagon into glicentin, oxyntomodulin, IP-2 and to the glucagon-like peptides: GLP-1 and GLP-2, which both, as described above, are known to regulate glucagon secretion from the alpha cells [105]. Besides these proglucagon-derived peptides, additional extended and truncated forms of the proglucagon products have been observed in pathological conditions that affect either the secretion or clearance of glucagon-like peptides [106,107]. Thus, antibodies directed against the C-terminal of glucagon will theoretically also detect N-terminally truncated (e.g. proglucagon 50-61 and 51-61) or extended forms of glucagon (e.g. proglucagon 1-61); side-viewing antibodies will also detect oxyntomodulin, glicentin and proglucagon 1-61 [108,109].

Based on these considerations and from the numerous discrepancies in the literature, it seems apparent that no clear-cut conclusion with regard to the existence of extrapancreatic glucagon can be drawn from the available studies in pancreatectomised subjects, and that more specific methods for measuring glucagon are essential to answer this question. In one study, a highly sensitive C-terminal assay was employed in combination with size-exclusion chromatography; in that study a component indistinguishable from glucagon was identified in plasma from totally pancreatectomised individuals after meal stimulation [41], suggesting that glucagon might be formed in the gastrointestinal tract also in humans.

HYPERGLUCAGONAEMIA IN DIABETES

In patients with diabetes the normal regulation of glucagon secretion suffers from important defects [6]. Patients with type 1 diabetes show greatly increased concentrations of glucagon in the state of diabetic ketoacidosis (where insulin shortage results in release of free fatty acids, which subsequently are converted to ketone bodies in the liver) [110,111], and show a deficient glucagon response to hypoglycaemia and, thus, lack a key physiological defence against this morbid and potentially fatal situation [112]. Whether this defect also applies for patients with type 2 diabetes is more controversial, but seems to exist at least in patients with long-duration of type 2 diabetes [113]. In patients with type 2 diabetes, glucagon concentrations are generally elevated in the fasting state compared to non-diabetic control subjects [12,114–119], although some studies have not found this difference [120,121]. Nonetheless, it is evident that fasting glucagon concentrations are undesirably high in the context of hyperglycaemia (known to suppress glucagon secretion) [28] and contribute to the increased rate of hepatic glucose output characterising patients with type 2 diabetes [115,117]. In patients with diabetes (both type 1 and 2) a pathological glucagon response is observed following ingestion of nutrients. Thus, plasma glucagon concentrations fail to decrease appropriately or even increase in response to an OGTT and show exaggerated increases following ingestion of a mixed meal [10,122–125]. These elevated glucagon concentrations in a situation of nutrient excess seem obviously to be an inappropriate response, and has been shown to contribute significantly to the exaggerated postprandial glucose excursions characterising patients with type 2 diabetes. In fact, some studies have proposed that postprandial hyperglucagonaemia could be responsible for up to 50% of the pathological increment in plasma glucose excursions following oral glucose ingestion in patients with type 2 diabetes [126,127]. As mentioned above, the mechanisms underlying hyperglucagonaemia in patients with diabetes are not clear. It has been proposed that the diabetic alpha cell suffers from resistance to the glucagon-suppressive effects normally exerted by glucose and insulin, as described [8]. Thus, diminished suppression of glucagon by glucose and insulin could theoretically explain both the fasting hyperglucagonaemia and the exaggerated postprandial glucagon responses observed in patients with type 2 diabetes (i.e., increased glucagon secretion from unsuppressed alpha cells). To examine this hypothesis in more detail, studies applying OGTTs and IIGIs have been conducted. Interestingly, such studies of patients with type 1 or type 2 diabetes showed that while hyperglucagonaemia is aggravated by oral intake of glucose, this is not apparent during iv glucose infusion [9,10,123]. Because of the isoglycaemic conditions during oral and iv glucose in these studies, it seems unlikely that compromised glucose sensing of the alpha cell plays a major role in the exaggerated glucagon responses after oral glucose ingestion. Neither does insulin resistance of the alpha cell seem to fully explain the derangement. This is apparent from studies of C-peptide-negative patients with type 1 diabetes, where a difference in intra-islet insulin between the two glucose administrations is highly unlikely [123], and from the studies of patients with type 2 diabetes as insulin responses during OGTT are - due to the incretin effect - higher than during IIGI [128–130]. Thus, if anything, the larger insulin concentrations during OGTT would be expected to cause greater suppression of glucagon secretion compared to IIGI. However, the opposite occurs [11]. Interestingly, it was recently demonstrated that this phenomenon is present not only in patients with diabetes, but also in non-diabetic subjects when larger (75 g or more) glucose loads are ingested [10,12]. These findings imply that factors originating from

nutrient-stimulation of the gastrointestinal tract are involved in the inappropriate postprandial glucagon response in patients with diabetes.

It is well established that the insulinotropic effects of exogenous administration of physiological amounts of GIP and GLP-1, respectively are severely diminished in patients with type 2 diabetes when compared to non-diabetic control subjects - a pathophysiological trait that seems to go beyond the general impairment in beta cell function [131–134]. However, the glucose lowering effect of a supraphysiological infusion of GLP-1 appears to be preserved [135], while that of a supraphysiological infusions of GIP is more or less absent [52,136]. Several studies have evaluated the effect of the incretin hormones on glucagon secretion in patients with diabetes. A study of supraphysiological infusion of GIP during a mixed meal in patients with type 2 diabetes showed that secretion of glucagon (and lack of insulin secretion) in response to GIP infusion lead to increased postprandial glycaemic excursions [137], suggesting that GIP might worsen overall glucose homeostasis in patients with type 2 diabetes via a predominantly glucagonotropic effect. This same study showed, by immunofluorescence techniques, the presence of GIP receptors on human pancreatic alpha cells and it was proposed that the glucagonotropic effect of GIP were mediated via a direct effect on the pancreatic alpha cells. Physiological infusion of GIP during a hyperglycaemic clamp has been shown to further minimise the already impaired glucagon suppression in patients with type 2 diabetes, but not in non-diabetic control subjects suggesting that the stimulatory effect of GIP on glucagon secretion could be an important pathophysiological element in type 2 diabetes [134]. The glucagonostatic effect of GLP-1 seems to be preserved in patients with type 1 diabetes as shown by Kielgast et al. [69] (described earlier). Also in patients with type 2 diabetes the glucagonostatic effect of GLP-1 seems to be preserved. This was apparent from a study involving stepwise increasing infusions of GLP-1 while clamping glucose concentrations at fasting values resulting in a dose-dependent suppression of glucagon secretion in both patients with type 2 diabetes and non-diabetic subjects [138]. The role of GLP-2 on glucagon secretion in diabetes is less well described. As mentioned above, one human study has addressed the effect of GLP-2 in patients with type 1 diabetes showing a glucagonotropic effect [79].

It is clear that the gut hormones do affect glucagon secretion and a thorough investigation of the postprandial interplay of these hormones on the inappropriate postprandial glucagon response in patients with diabetes was therefore of interest. Furthermore, the role of potential extrapancreatic glucagon secretion in this phenomenon inquires further investigation.

THE STUDIES INCLUDED IN THIS PHD THESIS

HYPOTHESIS AND OBJECTIVES

In the light of the preserved suppression of glucagon concentrations during iv glucose, we hypothesised that the inappropriate glucagon response following oral ingestion of glucose in patients with type 2 diabetes might arise as a consequence of nutrient-induced release of one or more glucagonotropic signals or nutrient-induced release of extrapancreatic (gut-derived) glucagon. The objective of Study I was to examine the effect of the gut hormones GIP, GLP-1 and GLP-2 on glucagon secretion in patients with type 2 diabetes. The objective of Study II was to examine the possible existence of extrapancreatic glucagon in humans by examining patients without a pancreas and non-diabetic control subjects. Furthermore, we sought to explore whether a potential

extrapancreatic glucagon secretion could represent a gut-dependent phenomenon. The objective of Study III was to elucidate whether the different hormonal responses during oral and iv glucose administration, respectively, would translate into differences in EGP in patients with type 2 diabetes and/or in non-diabetic control subjects.

METHODS

OGTT AND ISOGLYCAEMIC IV GLUCOSE INFUSION (IIGI)

The IIGI technique was developed as a method to investigate the incretin effect (i.e. the different insulin responses between oral and iv glucose administration) [129,139]. During an IIGI, the plasma glucose profile obtained during an OGTT is copied by an adjustable iv infusion of glucose. Thus, on separate days, two glucose administrations are applied to the same individual, one stimulating the gastrointestinal tract (the OGTT) and one bypassing the gastrointestinal tract (the IIGI). The two interventions result in the same peripheral plasma glucose concentrations, and, in theory, the same glucose concentrations at the level of the pancreas (but not at the level of the liver as discussed in Study III). Practically, arterialised blood is drawn from a cubital vein at regular intervals and samples are centrifuged and plasma glucose concentrations are analysed bedside. On the IIGI day frequent plasma glucose measurements makes it possible to adjust the glucose infusion rate (infused in a contralateral cubital vein) to obtain the same plasma glucose concentrations as during the OGTT day. We used the IIGI technique in all three studies with the primary aim, not to evaluate the incretin effect, but rather the different glucagon responses between oral and iv glucose administration. When performing an OGTT and an IIGI it is possible to calculate the gastrointestinal-mediated glucose disposal (GIGD) which reflects the percentage of an individual's glucose disposal following OGTT which is caused by the oral route of glucose administration [123,140]: $GIGD (\%) = 100\% \times (AUC_{OGTT} - AUC_{IIGI}) / AUC_{OGTT}$. GIGD describes not only the impact of the incretin effect but includes all factors affecting glucose disposal differently during oral vs. iv administration of glucose (including neural reflexes, activation of afferent nerves in the intestinal mucosa, differences in glucagon secretion, hepatic glucose production and first-pass hepatic uptake of glucose, and/or currently unknown factors [123,140].

Tracers

In the fasting state, EGP is the only contributor of glucose to the blood (main circulation) and since a rather steady state in glucose concentration is apparent while fasting this also means that the rate at which glucose disappears from the circulation (uptake by the periphery) is approximately the same. This situation becomes more complex during ingestion of a meal or an OGTT when the glucose entering the circulation can originate from both glucose uptake from the gut and from EGP. Additionally, a rapid and relatively large increase in plasma glucose concentration occurs initially following ingestion of nutrients meaning that the glucose appearance (from gut uptake and EGP) is higher than disappearance to the periphery and, later when the glucose concentration decreases the opposite occurs (glucose appearance being lower than glucose disappearance). By using tracer dilution methodology it is possible to calculate glucose appearance and disappearance rate. However, during non-steady state condition the changes in glucose concentration have to be taken into account as Steele recognised in 1968 [141]. With the Steele equations the time and magnitude of the differences in glucose appearance and disappearance are estimated, making it possible to explain the

underlying mechanism behind the changes seen in the glucose concentration. In Study II and Study III we applied a double-tracer approach during OGTT with one ingested glucose tracer ([U-13C6]glucose) and one glucose tracer infused intravenously ([6,6-D2]glucose). The iv glucose tracer measures the total rate of glucose appearance and the ingested tracer is used to calculate the appearance rate of the ingested glucose (by multiplying the rate of appearance of the ingested tracer by the tracer-to-tracee ratio of the glucose solution administered orally). EGP is then calculated by subtracting the rate of appearance of the ingested glucose from total rate of glucose appearance. In the IIGI studies, we did not have a second tracer in our varying glucose infusion and here, the EGP was calculated by subtracting the glucose infusion rate from the total rate of glucose appearance. In Study II we also applied a glycerol tracer ([1,1,2,3,3-D5]glycerol) to get a measure of the lipolysis rate. Plasma enrichments of the tracers were determined using liquid chromatography tandem-mass spectrometry [142].

Hormone infusions

Following ingestion of nutrients the concentrations of endogenous GIP, GLP-1 and GLP-2 are several fold higher in the intestinal and portal circulation compared with the peripheral circulation because of rapid degradation, following secretion, of these hormones [143,144]. The infusions of GIP, GLP-1 and GLP-2 in Study I were designed to mimic the endogenous response during OGTT in patients with type 2 diabetes; i.e. to reach high enough plasma concentrations in the intestinal and portal circulation so that both endocrine and potential neural pathways (originating from the small intestinal wall or the portal circulation) regulating alpha cell secretion of glucagon would be activated. Synthetic GIP was synthesised by Pegasus Pharma (Hannover, Germany), synthetic GLP-1 (7-36) amide was synthesised by PolyPeptide Laboratories (Linham, Sweden) and synthetic human GLP-2 was synthesised by PolyPeptide Laboratories (Wolfenbüttel, Germany). The peptides were dissolved in sterilised water containing 2% human serum albumin and subjected to sterile filtration. The initial (from 0-20 min) hormone infusion rates amounted to 4.0 pmol \times kg $^{-1}$ \times min $^{-1}$ for GIP, 0.6 pmol \times kg $^{-1}$ \times min $^{-1}$ for GLP-1 and 1.0 pmol \times kg $^{-1}$ \times min $^{-1}$ for GLP-2. From time 20 to 50 min, these doses were halved based on experiences from previous studies [77,145] and then terminated. The glucagon infusion (GlucaGen[®], Novo Nordisk A/S, Bagsværd, Denmark) applied in Study III during the IIGI+glucagon day was designed to mimic the glucagon concentration in the portal vein as obtained following OGTT in patients with type 2 diabetes. This is important, as the liver is the primary target organ for glucagon. Previous studies in humans have showed that there is a ratio of approximately 2 between portal and peripheral glucagon concentrations [146,147]. The glucagon infusion rate of 0.8 ng \times kg $^{-1}$ \times min $^{-1}$ from time 0 to 25 min following initiation of the IIGI was based on this ratio and previous observations of glucagon responses following OGTT in patients with type 2 diabetes and on previous glucagon infusion studies [9,12,148]. Glucagon analysis As alluded to above, previous and current glucagon assays are associated with specificity and sensitivity challenges [109]. In Study I and Study III we used a validated in-house radioimmunoassay with antiserum 4305, which binds to the C-terminus [19-29] glucagon sequence for the measurement of plasma glucagon concentrations [149]. As described above, this antibody will detect fully processed 29-amino acid glucagon, but will also detect N-terminally extended or truncated forms of glucagon. This has proved not to be an issue when measuring glucagon in healthy individuals

or patients with type 2 diabetes [108], but when trying to answer the question of the existence of fully processed 29-amino acid extrapancreatic glucagon measuring methods with greater specificity is required. In Study II, we applied a sandwich enzyme-linked immunosorbent assay (ELISA) with a combination of antibodies directed against the C-terminal and the N-terminal, respectively. Furthermore, we applied a mass spectrometry-based analysis of glucagon, which is capable of detecting peptide sequences down to the level of the individual amino acid (primary sequence) making it a highly specific method [150–152].

SUMMARY OF STUDIES AND DISCUSSION

STUDY I

We investigated whether a glucagon response similar to the inappropriate glucagon response during OGTT in patients with type 2 diabetes could be elicited during an IIGI (which by itself results in suppression of glucagon secretion in patients with type 2 diabetes) and concomitant infusion of GIP, GLP-1 and/or GLP-2. We examined 10 patients with type 2 diabetes (age [mean±SD]: 50.8 ± 10.7 years; body mass index (BMI): 33.2 ± 4.9 kg/m²; HbA_{1c}: 47.5 ± 7.7 mmol/mol; duration of diabetes: 4.8 ± 3.9 years) during six different experimental days, each day preceded by a 1-week wash-out of blood glucose lowering medication and an overnight fast: 75 g-OGTT, IIGI, IIGI+GIP, IIGI+GLP-1, IIGI+GLP-2 and IIGI+GIP+GLP-1+GLP-2 to elucidate the individual and the combined role of the gut hormones. We confirmed previous findings of initial hyperglucagonaemia during OGTT and suppression of plasma glucagon during IIGI in patients with type 2 diabetes. We show that glucagon suppression during IIGI is potentiated by iv infusion of GLP-1, which is in accordance with previous studies showing a potent glucose-dependent glucagonostatic effect of GLP-1 in both non-diabetic control subjects and patients with type 2 diabetes (as described above) (Figure 2).

This finding, combined with the observation that patients with type 2 diabetes have similar or only mildly reduced GLP-1 secretion in response to OGTT compared to non-diabetic control subjects [153,154], makes it unlikely that the attenuated suppression of glucagon following OGTT is a result of disturbed GLP-1 secretion or effect. Iv infusion of GLP-2 had no (or little) effect on glucagon secretion during IIGI, which, together with previous findings of normal postprandial GLP-2 responses in patients with type

2 diabetes [155] makes it unlikely that a difference in GLP-2 secretion or effect contribute to the inappropriate glucagon response during OGTT. We show that glucagon suppression during IIGI is 'overruled' and reversed by iv infusion of GIP, suggesting that GIP might play a role in the inappropriate glucagon response during OGTT in patients with type 2 diabetes. Finally, we show that infusion of GIP+GLP-1+GLP-2 concomitantly with the IIGI resulted in the same degree of hyperglucagonaemia as observed during OGTT. Interestingly, GIP has been proposed to function as a bifunctional blood glucose stabiliser with a predominantly insulinotropic effect during hyperglycaemia and a predominantly glucagonotropic effect during hypoglycaemia in non-diabetic subjects [51]. However, while infusion of GIP during hyperglycaemia does not seem to affect glucagon secretion in non-diabetic subjects [51], GIP infusion has been shown to attenuate the already delayed glucagon suppression during hyperglycaemia in patients with type 2 diabetes [27]. The present observation of a glucagonotropic effect of GIP in a setting of rising plasma glucose concentrations in patients with type 2 diabetes is in line with previous results showing a glucagonotropic effect of GIP infused during a mixed meal in patients with type 2 diabetes [137]. This, together with the observation that postprandial plasma concentrations of GIP are not different between patients with type 2 diabetes and non-diabetic subjects [156], suggests that the glucagonotropic effect of GIP are more pronounced in patients with type 2 diabetes and non-diabetic control subjects. There are some important considerations to take into account when interpreting the results from this study. We found higher fasting concentrations of glucagon during the OGTT day compared to the rest of the experimental days (although this difference did not reach statistical significance). We do not have a good explanation for this as we instructed patients to behave in the same way on the day preceding all experimental days. Nevertheless, it may be the result of an undesired order effect as the OGTT day, by design in studies like this, has to be performed as the first experimental day (the other experimental days were performed in randomised order). To adjust for this, and because we were interested in the intervention-effect on glucagon secretion, we looked at baseline-subtracted glucagon responses. We focussed on the first 30 minutes of each intervention, as this was where we, in this study, found the most pronounced difference in glucagon concentrations between the OGTT and the IIGI day, and here

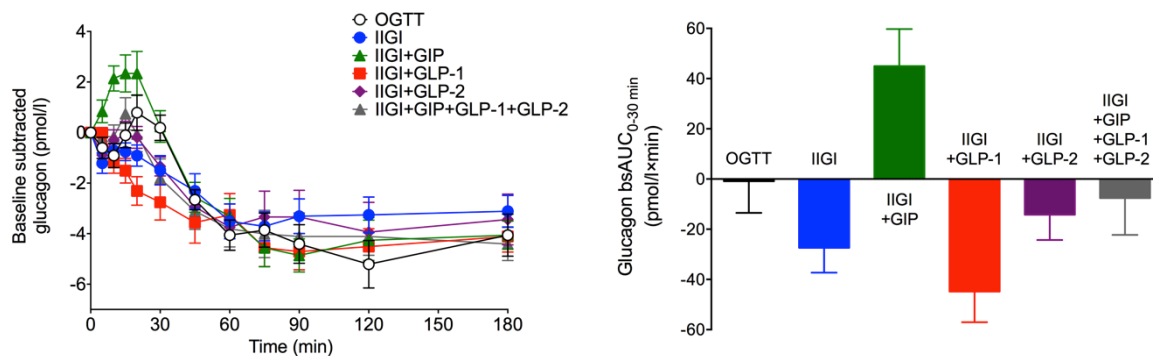


Figure 2

Baseline subtracted (bs) plasma glucagon concentrations (left) and initial glucagon responses expressed as bs area under curve (bsAUC) values (right) (means ± SEM) during 50 g-oral glucose tolerance test (OGTT) and isoglycaemic iv glucose infusion (IIGI) with concomitant infusions of saline, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide (GLP)-1, GLP-2, or a combination of the three hormones in patients with type 2 diabetes.

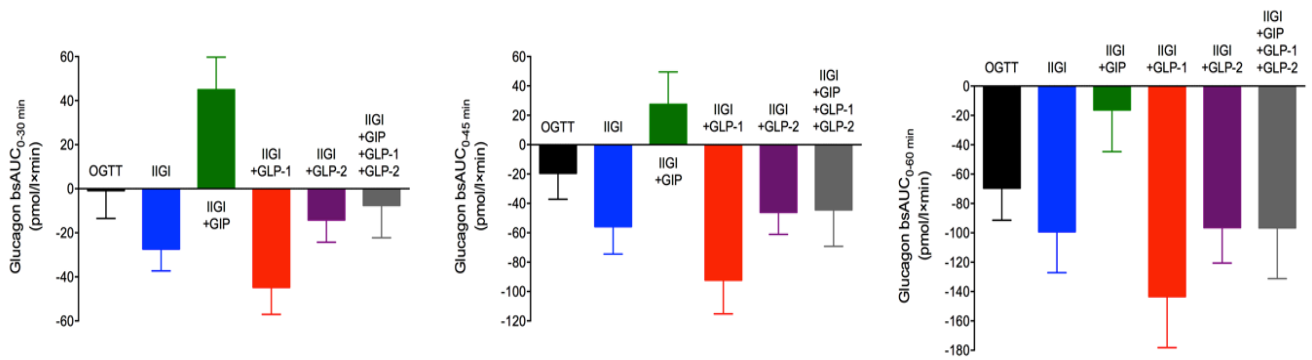


Figure 3

Initial glucagon responses expressed as baseline subtracted area under curve (bsAUC) values (0-30 min: left; 0-45 min: middle; 0-60 min: right) (means \pm SEM) during 50 g-oral glucose tolerance test (OGTT) and isoglycaemic iv glucose infusion (IIGI) with concomitant infusions of saline, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide (GLP)-1, GLP-2, or a combination of the three hormones in patients with type 2 diabetes.

the greatest impact of the hormone infusions occurred. Broadening the window to encompass the first 45 minutes, the picture is the same whereas differences levelled out when looking at the first 60 minutes or beyond (as illustrated in Figure 3).

The infusion of the gut hormones was, as described above, designed to reach high physiological plasma concentrations (measured peripherally). In this study, the endogenous responses of the gut hormones following OGTT were generally lower than expected from previous studies [157,158]. This was especially evident for the concentrations of GLP-1 and GLP-2, and the applied infusion rates of these hormones during the IIGIs resulted in plasma concentrations exceeding the endogenous responses more than expected. The GIP infusions resulted in higher concentrations compared to the endogenous GIP production on the OGTT day, but can be regarded to be in the physiological range as similar GIP responses have been observed following OGTT in patients with type 2 diabetes [137,157]. The fact that it especially was the infusions of GLP-1 (which shows a glucagonostatic effect during IIGI) and GLP-2 (which shows no or little effect on glucagon secretion during IIGI) that resulted in too high plasma concentrations, and, to a lesser extent, GIP (which shows a glucagonotropic effect) is not unimportant.

Thus, on the IIGI+GIP+GLP-1+GLP-2 day (designed to mimic the endogenous gut hormone responses during an OGTT) we must suspect a relatively 'too powerful' glucagonostatic effect from GLP-1 compared to the glucagonotropic effect of GIP, which ultimately may underestimate the reversal of glucagon suppression during IIGI on this day. Despite this, the glucagon response during the IIGI+GIP+GLP-1+GLP-2 day mimicked the glucagon response during OGTT, underlining a glucagonotropic role of GIP in these patients with type 2 diabetes.

STUDY II

We investigated the possible existence of extrapancreatic glucagon in humans and utilised oral and iv glucose challenges to explore whether a potential extrapancreatic glucagon secretion could represent a gut-dependent phenomenon. We subjected 10 totally pancreatectomised patients (age [mean \pm SD]: 59.8 \pm 9.9 years; BMI: 21.5 \pm 4.3 kg/m²; HbA_{1c}: 67.3 \pm 11.0 mmol/mol; time since operation: 4.6 \pm 4.5 years) and 10 age, sex and BMI-

matched non-diabetic control subjects (age: 58.4 \pm 5.0 years; BMI: 22.9 \pm 2.4 kg/m²; HbA_{1c}: 34.6 \pm 6.2 mmol/mol) to a 75 g-OGTT and a corresponding IIGI proceeded by an overnight fast and separated by minimum 72 hours. The majority of the patients (n = 7) had been operated because of adenocarcinomas, one because of a neuroendocrine tumour, one because of a mucinous cystadenoma and one because of severe chronic pancreatitis (Figure 4).

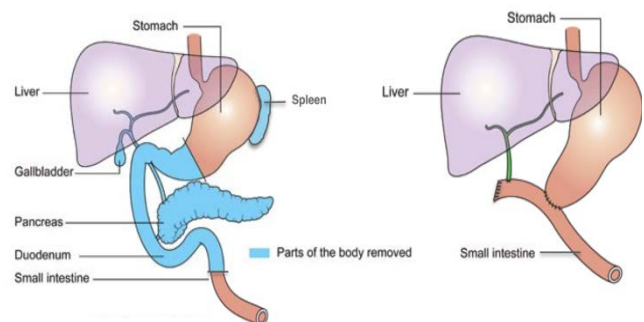


Figure 4

Illustration of the changed anatomy after total pancreatectomy operation. Left: The anatomy before operation, with the light blue-coloured organs indicating organs being removed during surgery. Right: The anatomy following surgery.

Patients were instructed to follow their normal daily insulin regimen the day before the experimental day but not to take any insulin in the morning of the experimental day. Applying sandwich ELISA and mass spectrometry-based proteomics, we demonstrate that glucagon is circulating in patients without a pancreas and that these patients respond with a significant secretion of glucagon during oral glucose ingestion whereas iv glucose infusion results in suppression of glucagon concentrations (Figure 5).

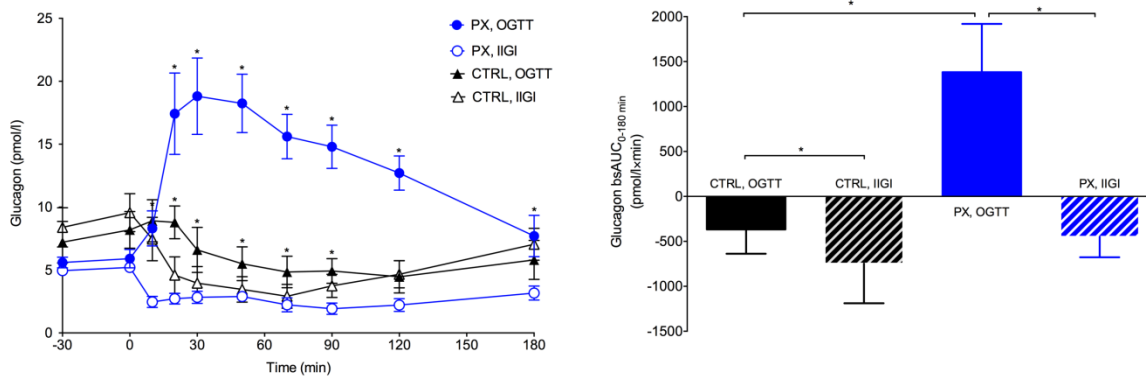


Figure 5

Plasma responses of glucagon (left) and glucagon expressed as baseline subtracted area under curve (bsAUC) (right) (means \pm SEM) during 75 g-oral glucose tolerance test (OGTT) and isoglycaemic iv glucose infusion (IIGI), in totally pancreatectomised patients (PX) and non-diabetic control subjects. Asterisks (*) indicate significantly ($p < 0.05$) differences.

As described above, a large number of studies in the field of glucagon secretion have paved the way for the scientific rationale of this study. Some previous studies in totally pancreatectomised animals and humans have suggested the existence of immunoreactive glucagon following total pancreatectomy [41,83,84,92], whereas other studies published in the same era have reached different conclusions: 1) that pancreatic glucagon is not present after total pancreatectomy [89,90,94,159], 2) that a rise in immunoreactive glucagon following total pancreatectomy is due to increments in plasma concentrations of glucagon precursors and not fully processed 'pancreatic glucagon' [96,99] or 3) to uncertain conclusions questioning the specificity of the applied glucagon assays [91,160]. Conclusions like these illustrate the importance of the specificity of the assays applied when measuring glucagon. One of the important strengths of the present study is the application of a sandwich ELISA and mass spectrometry-based proteomics, which enabled us to conclude that extrapancreatic 29-amino acid glucagon indeed is circulating in totally pancreatectomised patients and that oral glucose seems to stimulate its secretion whereas iv glucose seems to reduce plasma concentrations. With regard to the latter, a high degree of sensitivity of the employed assay is required - as shown for the sandwich ELISA used in the present study [108].

We found that the totally pancreatectomised patients had an absence of C-peptide and PP secretion during both glucose stimuli, which confirms the completeness of pancreatectomy and, thus, eliminates the possibility of the observed glucagon responses originating from remnant pancreatic tissue. Although we cannot, with this study, determine the exact source of the extrapancreatic glucagon secretion, the fact that oral glucose administration elicits a hyperglucagonaemic response, while iv glucose administration results in glucagon suppression, should direct the attention to the gut. Here, the proglucagon-producing enteroendocrine L cells are obvious candidates. In humans, the density of L cells increases from the duodenum to the large intestine [130]. Following a total pancreatectomy the anatomy of the gut is changed quite dramatically including the removal of the pyloric sphincter and the duodenum (as illustrated in Figure 4). Thus, during an OGTT, the glucose is delivered directly from the stomach to the jejunum where the L cells are more abundant. This likely results in a rapid and large stimulation of the L cells, which correlates with the significantly increased responses of the 'normally' PC1/3-processed L cell products (GLP-1 and oxyntomodulin)

that we also see in the totally pancreatectomised patients following OGTT in this study. We speculate that the L cells, when exposed to a large glucose load, respond not only with increased secretion of PC1/3 cleavage products but also with secretion of PC2 cleavage products, resulting in significant glucagon responses. In line with this recent studies have shown that PC2 may also be present in enteroendocrine cells of the human gastrointestinal tract [161,162]. To get an impression of whether a 'general' upregulation of PC2 was present in the totally pancreatectomised patients, we measured another PC2 cleavage product, namely GIP(1-30). However, we did not see increased concentrations of GIP(1-30) in the totally pancreatectomised patients. This, nevertheless, does not rule out a selective upregulation and/or activity of PC2 in the L cells.

We found that EGP (assessed by the use of stable glucose-isotopes) was higher in the totally pancreatectomised patients during OGTT compared to during IIGI, which might reflect the differential glucagon responses during the two different glucose administrations. In line with this, we also saw a negative GIGD in the pancreatectomy group ($-11.0 \pm 5.0\%$ [mean \pm SEM]) (i.e. we infused more glucose intravenously than we administered orally in the patients) compared to a GIGD of $62.0 \pm 2.4\%$ in the control group. The negative GIGD is likely a result of the increased rate of disappearance of glucose during the IIGI day (driven by higher glucosuria during this day) combined with the increased EGP during the OGTT day. In conclusion, using highly specific and sensitive analytical methods we provide evidence for the existence of extrapancreatic glucagon in man and suggest that it may play a role in diabetes secondary to total pancreatectomy, and possibly, although speculative, also in other conditions with postprandial hyperglucagonaemia, including type 2 diabetes.

STUDY III

We investigated whether the differential glucose administration routes, and thus, the differential hormonal responses translates into effects on glucose disappearance and EGP in 10 patients with type 2 diabetes (age [mean \pm SD]: 57.1 ± 6.7 years; BMI: 29.0 ± 4.3 kg/m²; HbA_{1c}: 53.8 ± 11.0 mmol/mol; duration of diabetes: 9.2 ± 5.0 years) and 10 matched non-diabetic control subjects (age: 56.0 ± 10.7 years; BMI: 29.8 ± 2.9 kg/m²; HbA_{1c}: 33.8 ± 5.5 mmol/mol) by applying glucose tracer methodology during OGTT and IIGI. We included a third experimental day: an IIGI in which glucagon suppression was prevented by an infusion of glucagon

designed to mimic glucagon concentration in the portal vein during OGTT in patients with type 2 diabetes (as described above). We corroborate the previous findings of inappropriate glucagon secretion in patients with type 2 diabetes during OGTT as opposed to during IIGI (Figure 6). We show that the disappearance rate of glucose is significantly greater during OGTT compared to during IIGI in non-diabetic control subjects, but similar between OGTT and IIGI in patients with type 2 diabetes. And finally, we show that EGP is higher during OGTT compared to during IIGI in both patients with type 2 diabetes and non-diabetic control subjects.

Since the first studies applying insulin assays and IIGIs in humans [139] it has been possible to quantitate the incretin effect and, subsequently, it has been demonstrated several times that the incretin effect is reduced in patients with type 2 diabetes [50,128,129,163]. As expected, the incretin effect in the diabetic group was reduced compared to the control group (31.0 ± 3.5 vs. $51.6 \pm 6.2\%$ [mean \pm SEM], $p = 0.011$). Accordingly, a greater glucose disappearance was observed during OGTT compared to during IIGI in non-diabetic control subjects, but, interestingly, not in patients with type 2 diabetes. The observation of less suppressed EGP during OGTT compared to during IIGI in both patients with type 2 diabetes and non-diabetic control subjects was surprising. It occurred despite the significantly larger insulin concentrations (known to suppress EGP [164]) during OGTT compared to IIGI. Furthermore, glucose per se is also known to suppress EGP [165] and as glucose concentrations must be higher in the hepato-portal system during OGTT compared to during IIGI (where it is the peripheral glucose concentrations from the OGTT that are copied) this would also be expected to suppress EGP more than during IIGI. We tried to evaluate the role of glucagon in these paradoxical results by applying an IIGI+glucagon day. The results from this day, however, did not seem to explain the difference in EPG between OGTT and IIGI as the prevention of glucagon suppression by exogenous glucagon infusion during IIGI did not restore EGP to OGTT levels in either the patient nor in the control group.

This finding is in contrast to previous studies concluding that lack of postprandial glucagon suppression contributes significantly to the postprandial hyperglycaemia in patients with type 2 (and type 1) diabetes [126,127]. Methodological differences between these studies and our study may explain this discrepancy. Both of the above-mentioned studies applied a pancreatic clamp technique by infusing somatostatin to shut down endogenous

hormone production from the pancreas (and many other organs in the body) while replacing basal insulin concentrations and concentrations of glucagon in a non-suppressed and a suppressed fashion, respectively. Although somatostatin has proven to constitute an important tool in the exploration of the physiological and pathophysiological roles of glucagon [28,166,167], pancreatic clamps are associated with some limitations. Jaspan et al. have shown that iv infusion of somatostatin results in reduction of portal vein blood flow [168], which might affect the hepatic glucose output. Other studies have shown that somatostatin significantly inhibits both glucagon-stimulated glycogenolysis and gluconeogenesis [169,170], although others have disputed these findings [171–173]. Furthermore, the inhibitory effects of somatostatin is not limited to glucagon and insulin, but also several other hormones (i.e. growth hormone, gastrin, cholecystokinin, secretin, GIP, GLP-1) [174] some of which might affect glucose homeostasis. Finally, these studies are by design flawed, as replacement of insulin concentrations to peripheral concentrations (where insulin has its most prominent effect), will greatly underestimate the portal insulin concentrations, and thus the effect of insulin on EGP. This is also apparent with regard to glucagon replacement, but presumably to a lesser degree as concentrations of glucagon (which exerts its main effect in the liver in humans) can be replaced to reach portal venous concentrations (in theory) without inducing error.

We chose to evaluate the impact of lack of glucagon suppression on EGP without the use of somatostatin. Our design, however, also suffer from limitations. First, we do not know the actual portal glucagon concentrations in our patients. The assumption of a two-fold elevation compared to peripheral levels is based on direct measurements in patients with cirrhosis of the liver [146], and the porto-peripheral gradient may be substantially greater in subjects without liver disease as suggested by Jaspan et al. [175]. This means that we might underestimate the effect of glucagon on EGP as our infusion rate of glucagon might simply be too low to replace actual portal concentrations. Secondly, we do not know whether endogenous glucagon secreted from the pancreas (or from extrapancreatic tissue [176]) affects EGP in other ways than the classical endocrine pathway. If endogenous glucagon secretion exerts some of its hepatic effect via neural pathways emanating in close relation to secretion site, the design of replacing portal glucagon concentrations by a peripheral iv infusion of glucagon is inherently wrong, and might underestimate the impact

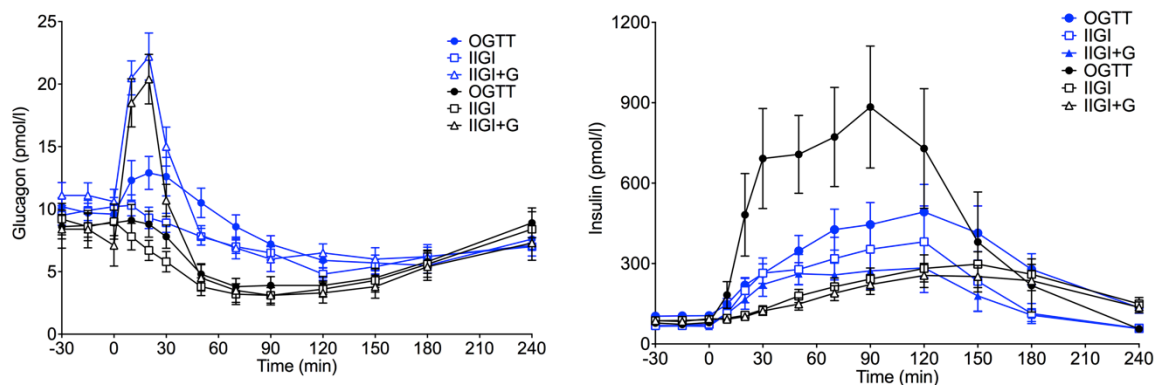


Figure 6

Plasma/serum responses of glucagon (left) and insulin (right) (mean \pm SEM) during 75 g-oral glucose tolerance test (OGTT) and isoglycaemic iv glucose infusion (IIGI) and IIGI+glucagon infusion (IIGI+G), respectively, in patients with type 2 diabetes (blue) and non-diabetic control subjects (black).

of the glucagon abundance during OGTT. Lastly, the large abrupt changes in glucose infusion rates during the IIGIs (needed to copy the rapidly rising glucose concentrations registered during the OGTT day) might have violated the assumptions of total and instantaneous tracer distribution in the glucose pool and stable distribution volume. Arguably, we could have added a tracer to our 'cold-glucose infusion' to minimise the change in tracer-to-tracee ratio and/or applied a triple tracer approach as suggest by others [177].

CONCLUSIONS

In this thesis, we aimed to elucidate mechanisms behind the inappropriate glucagon response observed in patients with diabetes following oral intake of nutrients. The studies were based on the observation that oral intake of glucose results in inappropriately high plasma concentrations of glucagon while iv infusion of glucose does not. This lead to the hypothesis that the gut plays a role for the inappropriate glucagon response observed in diabetes. We show that the gut hormones GIP, GLP-1 and GLP-2, secreted following oral intake of glucose, play a role in the regulation of glucagon secretion with especially GIP acting to increase glucagon secretion in patients with type 2 diabetes. We also show (using sandwich ELISA and mass spectrometry-based proteomics) that totally pancreatectomised patients respond with elevated plasma glucagon concentrations during oral intake of glucose whereas iv glucose supresses plasma glucagon in these patients. These findings challenge the classical conception of glucagon as a pancreas-specific hormone in man and constitute the basis for a novel explanation of the postprandial hyperglucagonaemia observed in patients with diabetes. Lastly, we show that oral glucose administration results in higher EGP compared to iv glucose administration in both non-diabetic subjects and patients with type 2 diabetes indicating a role of the gut in this differential response. In conclusion, this thesis provides insight into the role of the gut in diabetic hyperglucagonaemia and show that the gut may play a hitherto overlooked role in the inappropriate postprandial glucagon response in patients with diabetes.

PERSPECTIVES

Accumulating evidence demonstrates that hyperglucagonaemia plays an important role in diabetic hyperglycaemia and that targeting glucagon represents a valuable strategy in the treatment of diabetes [178,179]. Thus, several treatment modalities targeting glucagon have been and are currently investigated for the treatment of type 2 diabetes - including drugs that antagonise the glucagon receptor and drugs that suppress glucagon secretion. In light of this, these findings are intriguing as the gut might be a new target for postprandial glucagon reduction in patients with diabetes. We still face an exciting challenge in describing the relatively 'uncharted' regulation and physiological implications of gut-derived glucagon secretion and its role in diabetic pathophysiology.

One important question to answer is whether the 'bioactivity' of the extrapancreatic glucagon corresponds to that of pancreatic alpha cell glucagon. The results from Study II gives an indication of this (first of all the molecular identity of the two molecules, and secondly, the negative GIGD and higher EGP during the OGTT day compared to the IIGI day in the totally pancreatectomised patients), but to specifically address this question a study blocking glucagon receptor activity in totally pancreatectomised subjects would be highly interesting. We currently have the opportunity to employ a selective glucagon receptor antagonist (GRA) and have

recently initiated a study evaluating EGP and postprandial glucose excursions in totally pancreatectomised patients with and without administration of a GRA (approved by the local ethics committee in October 2015) and the first patients are currently being recruited.

In contrast to the regulation of glucagon secretion from the pancreatic alpha cell, the regulation of extrapancreatic glucagon secretion is completely unknown. It could therefore be interesting to illuminate whether extrapancreatic glucagon secretion is a physiological response that can be regulated like alpha cell glucagon secretion. Currently we are examining whether extrapancreatic glucagon secretion can be suppressed, like alpha cell glucagon secretion, by a GLP-1 receptor agonist administered to totally pancreatectomised patients prior to ingestion of a liquid meal.

The findings in Study II do not definitely establish the source of extrapancreatic glucagon in humans (although the two different glucose administration routes applied in the present study yield incremental (oral) and decremental (iv) glucagon responses and, thus, suggest that the gastrointestinal tract is the source). To unambiguously conclude this, we are planning a clinical study in which gastric and duodenal biopsies from totally pancreatectomised subjects and patients with type 2 diabetes will be collected and examined for expression of proglucagon and PC2 and potential co-localisation of proglucagon products and PC2 in enteroendocrine cells (including enteroendocrine cells isolated by laser capture microdissection).

The observation in Study III of higher EGP during OGTT compared to during IIGI in both patients with type 2 diabetes and non-diabetic subjects was, to us, surprising, and merits further investigation. Basu et al. employed a triple-tracer approach to assess postprandial glucose metabolism [177], which should minimise the change in tracer-to-tracee ratio, and thus potentially result in more precise tracer data. To achieve a better understanding of the isolated effect of the differential glucagon responses between the two glucose administration routes, a design with an intraportal infusion of glucagon mimicking the portal glucagon concentrations during OGTT would be of interest. An intraportal access for glucagon infusion could be achieved by a catheter inserted into the obliterated umbilical vein as accomplished in previous studies [180]

The hyperglucagonaemia in patients with diabetes have been investigated primarily with regards to an increased secretion of glucagon (including the studies of this thesis). However, the plasma level of a given hormone is determined not only by its secretion but also by its rate of elimination from the circulation. Hitherto, no studies have investigated the elimination of glucagon specifically in patients with type 2 diabetes. And it would therefore be of interest to investigate whether patients with type 2 diabetes have an altered clearance of glucagon.

SUMMARY

Patients with type 2 diabetes are characterised not only by compromised insulin secretion and action, but also by elevated plasma concentrations of the 29-amino acid peptide hormone glucagon, which generally is thought of as a pancreas-derived hormone (produced in and secreted from alpha cells in the islet of Langerhans). In patients with diabetes, circulating glucagon concentrations are elevated in the fasting state and fail to decrease appropriately or even increase in response to ingestion of nutrients. Glucagon is known to be a potent stimulator of hepatic glucose production, and, thus, the elevated glucagon concentrations in diabetes contribute decisively to the predominating trait of patients with diabetes namely hyperglycaemia. Interestingly, studies

have shown that while oral intake of glucose results in inappropriately high plasma concentrations of glucagon in patients with diabetes, intravenous (iv) infusion of glucose does not. The mechanisms behind these differential glucagon responses to oral vs. iv glucose administration are currently unexplained. Three hypotheses were tested in the present thesis: 1) Could the inappropriate glucagon response to oral glucose ingestion in patients with diabetes be attributed to the release of glucagonotropic/glucagonostatic peptides secreted from the gut? 2) Could the inappropriate glucagon response to oral glucose ingestion in diabetes be a result of extrapancreatic glucagon secretion (possibly originating from the gut)? And 3) Does the differential glucagon responses between oral and iv glucose administration affect endogenous glucose production (EGP). The overall aim of this PhD thesis was, thus, to investigate the role of the gut in diabetic hyperglucagonaemia and hyperglycaemia. In Study I we examined the effect of the three gut-derived hormones glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) on glucagon secretion in patients with type 2 diabetes. We applied a 50 g-oral glucose tolerance test (OGTT), and five isoglycaemic iv glucose infusions (IIGIs) with either saline, GIP, GLP-1, GLP-2 or a combination of the three hormones. We show that these gut-derived hormones affect glucagon secretion differently and that OGTT-induced secretion of these hormones may play a role in the inappropriate glucagon response to orally ingested glucose in patients with type 2 diabetes with especially GIP acting to increase glucagon secretion. In Study II we examined totally pancreatectomised patients and non-diabetic control subjects during a 75 g-OGTT and an IIGI. We applied sandwich enzyme-linked immunosorbent assay (ELISA) and mass spectrometry-based proteomics for plasma glucagon analysis and show that 29-amino acid glucagon circulates in patients without a pancreas and that glucose stimulation of the gut results in significant hyperglucagonemia in these patients – ultimately confirming the existence of extrapancreatic glucagon secretion in humans. In Study III we examined whether the different responses of insulin and glucagon, respectively, between oral and iv glucose administration translate into differences in EGP and glucose disappearance in patients with type 2 diabetes and non-diabetic control subjects. We applied glucose tracer methodology during a 75 g-OGTT, IIGI and IIGI + iv glucagon (to isolate the effect of glucagon) and show that EGP is less suppressed during OGTT than during IIGI in both patients with type 2 diabetes and non-diabetic control subjects.

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