Role of GLP-1 induced glucagon suppression in type 2 diabetes mellitus

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INTRODUCTION

The pathophysiology leading to impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM) includes insulin resistance in muscle, liver and fat tissue, impaired beta cell function, decreased beta cell mass, elevated glucagon levels, increased hepatic glucose production (HGP), decreased incretin effect(1), and decreased incretin responses to meals (2). It is known that islet function is reciprocally related to peripheral insulin sensitivity, in that insulin secretion is adaptively increased in insulin resistance (3-7). This intimate relationship suggests that the beta cell secretory capacity in relation to the ambient insulin sensitivity is a critical factor for maintenance of normal glucose tolerance (NGT) and developing diabetes, rather than the absolute insulin secretory capacity in itself. By the time diabetic hyperglycaemia develop, reduction in both beta cell function and beta cell mass have occurred (4,8).

However, diabetes is also an alpha cell disease, as the alpha cell gradually looses its ability to decrease glucagon secretion in response to increasing plasma glucose (PG) levels (7,9,10). The resulting increased plasma glucagon level, hyperglucagonaemia, in relation to the prevailing PG level, contributes to an increased HGP and thereby to the diabetic hyperglycaemia (11).

The aim of this thesis is to gain further insight into some of the pathophysiology regarding the alpha cell and the impaired regulation of glucagon secretion. We wanted to explore whether the incretin hormone, glucagon-like peptide-1 (GLP-1), which is a known inhibitor of alpha cell secretion (12,13), plays a role in T2DM alpha cell pathophysiology. Firstly, we addressed whether the diabetic alpha cell has a decreased sensitivity towards GLP-1 in comparison with that of healthy controls. The dose-response relationship between GLP-1 and glucagon suppression was established in patients with T2DM as well as in healthy control subjects (Study I). Secondly, we aimed to quantify the glucagon-suppressive effect of GLP-1 compared to its insulinotropic properties with regard to the overall glucose-lowering effect in patients with T2DM (Study II). For this, a protocol involving pancreatic as well as glycaemic clamps was employed. Finally, diabetic hyperglucagonaemia was explored further, by examining patients with type 1 diabetes mellitus (T1DM) and no residual beta cell function, in order to determine the possible role of intra-islet beta cell function for the glucagon response to oral glucose (Study III). We used an oral glucose tolerance test (OGTT) and compared the glucagon responses to those obtained with an isoglycemic intravenous (iv) glucose infusion (IIIG) and to the responses in healthy subjects. Isoglycaemic clamp technique is a method employed earlier (14-16) in patients with T2DM, in which paradoxical hypersecretion of glucagon in response to OGTT was demonstrated. The results suggest that non-islet, presumably gastrointestinal (GI) tract factors rather than intra-islet factors may explain this phenomenon.

PROGLUCAGON AND IT’S SECRETED PRODUCTS

The proglucagon gene was sequenced in 1983 by Bell and co-workers. Besides containing the glucagon sequence, Bell also found the sequence of two glucagon-like peptides, GLP-1 and glucagon-like mpeptide-2 (GLP-2) (17). The proglucagon gene is expressed in the pancreatic alpha cells (18), in the intestinal L cells(19), and in certain neurons of the brainstem with projections to the hypothalamus(20). In the alpha cell, proglucagon (PrG) is
cleaved by prohormone convertase 2 (PC2) (21,22) to produce glicentin-related pancreatic polypeptide (GRP, PrG 1-30), glucagon (PrG 33-61), and major proglucagon fragment (MPGF, PrG 72-102) (18). In the intestinal endocrine L-cell, processing of PrG is catalysed by prohormone convertase 1/3 (PC1/3) resulting in the formation of glicentin (which may be further processed to oxyntomodulin, PrG 33-69) and the glucagon-like peptides, GLP-1(PrG 78-107NH₃) and GLP-2 (PrG 126-158) (23-26). The highest density of L cells are present in the mucosa of the distal ileum and the proximal colon, but are present throughout the small intestine (27). In the brainstem, PrG also appears to be processed by PG1/3 (20). Only glucagon, GLP-1 and GLP-2 have known significant physiological effects. Both glucagon and GLP-1 play important roles in the regulation of glucose metabolism as described in the following. The role of GLP-2 in glucose metabolism is less established, but will also be addressed. Both GLP-1 and GLP-2 are involved in the metabolic adaptation to food ingestion, although they exhibit distinct effects. GLP-2 has potent intestinotrophic properties; it induces mucosal proliferation and inhibits apoptosis. It may also increase the expression of mucosal digestive enzymes and nutrient transporters(28). Thereby, the absorptive capacity of the intestine increases. This effect is exploited pharmacologically in patients with short bowel syndrome(29,30). In addition GLP-2 reduces chemotherapy induced mucosal damage(31), an effect that might be exploited in the future. More recently GLP-2 has been shown to affect calcium metabolism and bone homeostasis as it inhibits bone resorption with no effects on bone formation (32,33). Much research is ongoing regarding the role of proglucagon products in relation to food intake and appetite; recently oxyntomodulin (PrG 33-69) was shown to induce satiety when injected subcutaneously three times daily in obese subjects over a 4-week period. The treatment resulted in a reduction in body weight of 2.3 kg compared to 0.5 kg in a group treated with placebo. Besides inducing satiety and thereby a reduced food intake, oxyntomodulin was associated with increased activity-related energy expenditure, making it a potentially interesting agent in treating obesity (34,35).

GLUCAGON
The physiological role of glucagon is to counteract the actions of insulin and maintain euglycaemia thereby avoiding cerebral glucose deficiency in the fasting state. This effect is carried out by stimulating HGP. In postabsorptive conditions HGP is regulated in a balance between insulin-induced inhibition and glucagon-induced stimulation and it has been shown that glucagon is responsible for approximately half of the glucose production in the fasting state (36,37). Hypoglycaemia is the primary stimulus to glucagon secretion, however, the alpha cell is also stimulated by the autonomic nervous system and amino acids (38). Glucagon binds to a G protein-coupled receptor member of the class II G protein-coupled receptor subfamily (39). Receptor binding activates G protein mediated signal transduction (Gₐ and Gₐₜ) and Gₐₜ activates adenylat cyclase resulting in increasing cyclic adenosine mono-phosphate (cAMP) and thereby protein kinase A activity. Gₐ activation leads to an increase in intracellular Ca²⁺ through activation of phospholipase C (40). In hepatocytes, receptor activation leads to increased HGP by enhancing both glyco-genolysis and gluconeogenesis along with inhibition of glycogenesis (41-43). The glucagon receptor is also expressed in extrahepatic tissues, including the heart, intestinal smooth muscle, kidney, brain (brainstem and hypothalamus) and adipose tissue, but less is known about receptor activation in these tissues (44,45). The glucagon receptor has also been located to rodent pancreatic islet cells (alpha, beta and delta cells) by mRNA and immunoreactivity analysis (46). In the perfused pancreas and in isolated beta cells, glucagon stimulates insulin secretion, but the mechanism may involve stimulation of both the glucagon receptor and the GLP-1 receptor (46-48). By receptor binding to the alpha cell, glucagon may regulate its own secretion(49).

The “intra-islet” hypothesis regarding glucagon secretion, first published in 1972 (50,51), implies that a decrease in arterial glucose causes beta cell secretion to decrease, whereby a tonic alpha cell inhibition by insulin is diminished and glucagon secretion increases (50-52). Therefore, insulin and PG are considered strong regulators of the non-diabetic alpha cell; however, the diabetic alpha cell seems less influenced by the inhibitory actions of insulin and glucose (10,14,15,53). Other co-secreted beta cell products, such as zink and amylin, also appear to suppress alpha cell secretion, though the mechanisms are less well worked out (54,55). Also, the peptide product of the delta cell, somatostatin, potently inhibits glucagon secretion (56,57) and the human alpha cell expresses several of the five somatostatin receptor subtypes (58,59). Particular receptor subtype 2 transmits suppression of glucagon secretion (60,61). The inhibitory substances may reach the alpha cell via the microcirculation and/or the interstitial fluid (62,63). Similar to the rodent islet architecture, with a core consisting of beta cells surrounded by a mantle of non-beta cells (alpha and delta cells) (64-66), human islets are thought to have a (modified) core-mantle structure. According to this view, beta cells are contiguous and arranged either in smaller clusters surrounded by non-beta cells or along invaginations of the exterior surface of the islet (67,68). However, newer studies using two- and three dimensional techniques, suggest different compositions of human and rodent islets. These studies show that the human islet consists of a greater portion of alpha cells (human: 38%; mouse: 18%; P<0,05 (65)) and that the islet surface mainly consists of beta cells (64,66). No beta cell clustering was reported, instead beta cells were found to intermingle freely with other endocrine cells throughout the islet. Alpha, beta and delta cells had equivalent and random access to the blood vessels within the islet, ruling out the possibility that the different endocrine cells are organized in layers around the vessels (64-66). These results therefore question the concept of downstream regulation of alpha cells by beta cell secretion (51,69).

The alpha cell secretion is also influenced by gut hormones (12,70). The inhibitory effect of GLP-1 will be described in the following. The effects of GLP-2 and glucose-dependent insulino-tropic polypeptide (GIP) are less conclusive. The effect of GIP on glucagon secretion may depend on the PG level, as in vitro studies indicated that GIP stimulates glucagon secretion during hypoglycaemia (71), whereas no effect on glucagon secretion was reported during hyperglycaemia in patients with T1DM, T2DM or healthy subjects (72-74). However, GIP has been reported to stimulate the alpha cell in patients with liver cirrhosis and fasting hyperglycaemia (75). In healthy subjects, GIP was found to stimulate glucagon secretion dose dependently during euglycaemic conditions (76). Data regarding GLP-2 clearly indicates a stimulatory effect, independent of pancreatic insulin secretion. The effect is weak in physiological concentrations (77), however, a more pronounced stimulatory effect on glucagon secretion was reported in healthy subjects both in the fasting state and postprandially, when infusion rates of GLP-2 were increased to result in pharmacological levels (78). In addition, a stimulatory effect has been described in patients with T1DM during hyperglycaemia (74). The GLP-2 receptor has been localised immunohistochemi-
cally to alpha cells in both rat and human islets (74,77-80). It has also been shown that the glucagonostatic effect of GLP-1 can be outbalanced by the glucagonotropic effect of GLP-2, but GLP-2 does not affect insulin or somatostatin secretion (thought to mediate the GLP-1-induced inhibition) in the isolated perfused rat pancreas, where GLP-2 has been shown to be glucagonotropic (79,79).

**GLUCAGON AND TYPE 2 DIABETES**

Absolute or relative hyperglucagonaemia has been found in all forms of diabetes in which glucose production exceeds glucose disposal and thus causes "endogenous hyperglycaemia" (81,82). A defective alpha cell regulation seems to characterise subjects with IGT as well, presenting a defective glucagon suppression in response to increasing postprandial PG after ingestion of a meal or glucose alone even before T2DM develops (9,83). A similar reduction or delay in glucagon suppression is seen in established diabetes (14,84).

The glucagon response to oral glucose is puzzling. The early response has several times been shown to be positive or less negative compared to the expected suppression, seen in the same subjects when glucose is administered intravenously to reach identical plasma glucose concentrations (14,15). Most likely this paradoxical response is caused by stimulatory gastrointestinal(GI)-factors (14,76,78,84). Nevertheless, suppression of paradoxical response is caused by prior GIP administration, leading to the same PG excursion when glucose is administered iv compared to the response following intra-gastric administration (116).

**GLP-1**

GLP-1 is secreted from the L cell almost immediately(102) in response to ingestion of nutrients with lipids and carbohydrates as the most potent stimulating nutrients (90,103). Physiologically, GLP-1 is stimulated by mixed meal ingestion; however, secretion is also stimulated by individual nutrients, which besides glucose and lipids include other carbohydrates, fatty acids, amino acids and dietary fibres. The L cell is an open-type endocrine cell and is therefore believed to sense the arrival and passage of nutrients along the GI tract(104). In vitro studies on particularly the GLUTag cells (a L cell line model (105)) indicates that GLP-1 is stimulated by both non-metabolised and metabolised sugars (106). L cell secretion depends on a rise in cytoplasmic Ca++ from either intracellular stores or resulting from influxes across the plasma membrane. A glucose-sensing pathway has been identified in the GLUTag cells. Hereby, sugars trigger GLP-1 secretion by the electrogenic action of sodium-glucose transport protein 1 (SGLT1), depolarizing the membrane (107). Another secretory pathway may be through the sweet taste receptor pathway, which is also located to the L cell(108); this might explain an L cell secretory response to artificial sweeteners (109). The mechanism of protein-triggered GLP-1 release remains unclear. However, in the GLUTag cell line glutamine exerts a potent stimulus through the sodium-dependent amino acid transporter SLCL38A2 (110). Also G protein-coupled receptors are regarded as potentially important components of the signalling pathways in the L cell, allowing regulation by certain neurotransmitters and hormones (111-113), as well as luminal nutrients. Thus, lipids, fatty acids and bile acids might stimulate the GLP-1 release through specific G protein-coupled receptors including, GPR40, GPR120 and GPR119 (114-116).

The peak GLP-1 plasma concentration is reached 30-45 minutes after ingestion of e.g. glucose(14). After its release, the peptide is rapidly metabolized primarily by the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4)(117). DPP-4 is a serine peptidase. It cleaves the two N-terminal amino acids of peptides with a penultimate proline or alanine residue as in GLP-1. It is found abundantly in the renal and the intestinal brush borders, it is expressed in hepatocytes and luminal membranes of endothelial cells throughout the vascular bed, and it also appears in a soluble form in plasma (118). The metabolite, GLP-1 (9-36) NH2, has no activating effect on the beta cell, as an intact NH2-terminal is essential for receptor activation (119,120). Because of the wide distribution of DPP-4, GLP-1 undergoes rapid degradation; only about 25 % of the newly secreted hormone reaches the portal vein in its intact form, a further 40-50 % is cleared in the liver, so that only 10-15 % of the response to nutrients reaches the peripheral circulation as intact GLP-1 (121,122). The apparent plasma half life of GLP-1 is only 1-2 minutes (121).

GLP-1 stimulates the beta cell via a G-protein-coupled receptor. Thereby, an adenylyl cyclase is activated and intracellular cAMP increases, activating second messenger pathways such as the protein kinase A and Epac pathways (123,124). By increasing cAMP levels GLP-1 directly contributes to closure of the ATP-sensitive K+ channels (enhancing the effect of glucose), and the following elevation in Ca++ concentration through the L-type Ca++channel. This cascade leads to mobilization of insulin containing granules and insulin excocytosis (123,125). Insulin gene expression and several steps in the insulin biosynthesis are also stimulated by GLP-1 (126).

GLP-1 and GIP are both incretin hormones and both contribute to the so-called "incretin effect"; the increased insulin secretion seen after oral glucose compared to the response following intravenously administered glucose leading to the same PG excursion (116). During normal physiological circumstances, depending on the glucose load, the incretin effect ensures a two- to three-fold greater insulin response to oral versus intravenous isoglycaemic challenges (127). GLP-1 affects both alpha and beta cells in a glucose dependent manner (10,12,128). Regarding the insulinotropic effect, only glucose-induced insulin secretion is enhanced; the peptide has no effects on secretion at glucose levels below approximately 4 mmol/l (129). The glucagonostatic
GLUCAGONOSTATIC EFFECTS OF GLP-1

In healthy subjects, GLP-1 and GLP-1 receptor agonists suppress glucagon secretion during euglycaemia (130,155-157). However, the glucagon response to hypoglycaemia is unaffected as GLP-1 may modestly stimulate glucagon secretion induced by hypoglycaemia (156). The diabetic alpha cell is capable of responding to GLP-1 despite its defect in glucose sensing. This has been demonstrated in both T2DM and T1DM patients, indicating that the observed suppression is not dependent on endogenous insulin secretion (158). Independently of its administration (iv or subcutaneous), GLP-1 has proved efficient in preventing inappropriate meal-induced glucagon secretion and thereby reducing postprandial glucose excursions in patients with T1DM (159) and T2DM (158,160-162). Further, a dose related reduction in meal-stimulated glucagon release by subcutaneous injection of GLP-1 has been demonstrated in patients with T1DM (163). It may be that delay in gastric emptying can explain the decrease in postprandial glucagon in these patients (156). However, when examining patients in the fasting state, GLP-1 clearly inhibits alpha cell secretion. In addition GLP-1 induced during a hyperglycaemic clamp also inhibited arginine stimulated alpha cell secretion (164). In a study comparing glucagon responses to a hyperglycaemic clamp with and without GLP-1, absolute glucagon levels in patients were either equal to or lower than values observed in healthy subjects, indicating that GLP-1 improves glucose sensing in the alpha cell (10).

The first study of long-term GLP-1 treatment of T2DM patients was published by Zander et al. in 2002. Here patients were treated for 6-weeks with continuous subcutaneous infusions of supraphysiological doses of native GLP-1 using insulin pumps. A tendency towards a decrease in 8-hour daily glucagon profile after 1 and 6-weeks of treatment was reported. However, at the same time fasting and post-meal glucagon were reduced by 4-6 mmol/l, presumably illustrating an improved alpha cell sensitivity to prevailing PG levels (165). Similar results were obtained with the GLP-1 analogue liraglutide, where one week of treatment led to a decrease in 24 hour glucagon area under curve (AUC), but did not significantly affect fasting glucagon levels; however, glucagon responses to a 15 mmol/l glycaemic clamp and an arginine stimulation test were significantly decreased (166). Decreases in
plasma glucagon and glucose during a standardized meal have also been reported after 4-weeks of treatment with the DPP-4 inhibitor vildagliptin, a treatment that increases endogenous GLP-1 levels (167). A similar vildagliptin effect has been reported in T1DM, suggesting that this metabolic improvement does not require exogenous insulin (168).

The glucagon inhibitory effect of both glucose and GLP-1 may depend on the glycaemic control in patients with T2DM. This was investigated with combined hyperglycaemic-GLP-1/saline clamps before and after 4-weeks of super regulation (insulin treatment) in a group of T2DM patients characterised by poor glycaemic control prior to the intervention (85). The patients were clamped at PG 15 mmol/l and the GLP-1 infusion rate was designed to result in physiological plasma concentrations. However, differences in glucagon decrease (baseline minus nadir) between saline and GLP-1 infusions were neither observed before, nor after the intervention. The decrease was also comparable to the inhibition (baseline minus nadir) observed in the healthy subjects investigated with the same clamps. However, when comparing healthy subjects and patients, the inhibition of the diabetic alpha cell was clearly attenuated as the healthy subjects reached nadir within 25 minutes compared to a nadir time of 65 minutes in the patients (85). Still, the super-regulation may have resulted in an improvement in glucose sensing by the alpha cell as fasting glucagon levels decreased and thereby were normalised. However, in another study where pharmacologically levels of GLP-1 were obtained under the same clamp conditions, impaired glucagon response to hyperglycaemia was completely normalised (10).

Data regarding alpha cell expression of the GLP-1 receptor are inconsistent. Thus, the expression was detected by double immunofluorescent staining in a fraction (20%) of isolated rat alpha cells, and mRNA was unequivocally detected by single-cell reverse transcription-polymerase chain reaction (169). In other studies the presence of GLP-1 receptors in alpha cells could not be demonstrated by less sensitive Northern or Western blot analyses in rat alpha cells (47,125,169,170). Recently, robust data supporting GLP-1 induced glucagon inhibition to be mediated through delta cell receptor binding and thereby through somatostatin was published (60,171). As the beta cell is stimulated by GLP-1 directly the notion that GLP-1 induced glucagon suppression is conducted through the beta cell is obvious – according to the intra-islet theory described earlier; however, functional beta cells are not necessary for GLP-1 induced suppression of alpha cell secretion (70) and in such cases, suppression of glucagon carried out via somatostatin release seems more likely.

**GLUCAGON ASSAYS**

It is well known that existing pancreatic-glucagon-specific assays yield a rather wide range of ‘basal’ glucagon values in healthy subjects (156). Furthermore, as many pancreas-specific assays ‘read’ a large molecular weight interfering factor as glucagon (172,173). Dunning et al. have emphasized the importance of comparing values only within a single study and using a single assay system. Furthermore, for some assays changes from baseline may provide more reliable assessment of in vivo glucagon secretion than absolute basal fasting glucagon (156).

In our laboratory we use a C-terminal specific antibody, 4305 (174) for the glucagon assay, which reacts exclusively with the processed C-terminal of glucagon. In an attempt to characterize the diabetic hyperglucagonaemia, we employed detailed molecular strategies to characterize hypersecreted glucagon following OGTT in patients with T2DM (14). By purification with Sep-Pak and HPLC analysis we were able to eliminate possible cross-reacting molecules obtained in the plasma sample. Controls included analyses of samples spiked with exogenous glucagon. The following assay systems were then employed: a) an assay for the glucagon sequence 6-15 with antiserum no 4304 (174,175); b) an assay for the N-terminal of glucagon developed against a 1-10 glucagon fragment with antiserum no 4830, which also binds oxyntomodulin; and c) the C-terminal assay binding glucagon sequence 19-29 with antiserum no 4305 (22,176). Identical glucagon standards and 125I-glucagon were used in all assays. Antiseras 4304 and 4830 bind labelled and unlabelled glucagon and oxyntomodulin (PrG 33-69) with identical affinity (173,175,177), whereas antisera 4305 binds the glucagon (and the C-terminal fragment 19-29) and PrG 1-61 (glucagon 5000), but neither C-terminal truncated extended forms; e.g. it does not bind turkey glucagon which is modified in position 28. It also does not detect glicentin or other C-terminally extended products of the proglucagon gene (175). By submitting plasma samples to this set up, we confirmed that the glucagon detected by antisera 4305 in the diabetic subjects during OGTT does represent authentic, native glucagon (178).

As mentioned, the PrG gene is both expressed in intestinal L cells and pancreatic alpha cells with different post-translational processing and thereby different secretory products. Therefore glucagon is a difficult peptide hormone to identify as cross-reactions with other peptides containing identical sequences easily occur. The ideal assay would be a sandwich model, involving binding of both the N- and C-terminal sequences simultaneously. We tried to develop such an assay by combining the N-terminal antibody 4830 with either of the C-terminal antibodies 4316 or 4317. Unfortunately, we did not succeed, probably because of steric hindrance due to the limited size of glucagon (29 aa).

**STUDY I**

**PRESERVED INHIBITORY POTENCY OF GLP-1 ON GLUCAGON SECRETION IN T2DM**

As discussed, the decreased incretin effect (127) is due to both abolished insulinotropic effect of GIP (10), an impaired GLP-1 (and possibly GIP) response to a meal (90,179) and a decreased sensitivity of the beta cell to GLP-1 (152). However, little is known about alpha cell sensitivity to GLP-1. Kjems et al. investigated the dose-response relationship and potency of GLP-1 on glucose-mediated insulin secretion (152). This was done by exposing patients with T2DM and healthy subjects to a range of increasing glucose infusions, allowing determination of the beta cell responsiveness to glucose, based on deconvolution and C-peptide kinetics. In addition to the glucose clamps increasing doses of GLP-1 were infused, allowing calculations of the dose-response relationship between GLP-1 infusion rate and beta cell responsiveness to glucose in both T2DM and controls. The study confirmed that a slightly supraphysiological dose of GLP-1 (0.5 pmol/kg body weight/min) was capable of normalising the beta cell response to glucose. However, the GLP-1 induced improvement in beta cell responsiveness was decreased by a factor 3-5 in the patients, demonstrating decreased sensitivity of the diabetic beta cell to GLP-1.

We aimed to investigate the alpha cell sensitivity to GLP-1 in patients with T2DM and healthy subjects. The hypothesis was that the defective glucagon suppression in response to orally ingested glucose and meals seen in patients with T2DM might be
explained by a decreased sensitivity of the diabetic alpha cell to GLP-1 together with the decreased incretin level in these patients. It has been established that pharmacological doses of GLP-1 may normalise the alpha cell response to a hyperglycaemic clamp (10); however, the dose-response relationship between GLP-1 and glucagon inhibition has not been investigated, neither in healthy subjects nor in patients with T2DM. Further, as the defective beta cell response to both glucose and GLP-1 improves with improved glycaemic control(85,88) we hypothesised the same to be the case regarding the alpha cell, as the alpha cell also might be affected by acute glucotoxicity. These considerations led to the first protocol.

We examined ten patients (7 males, 3 females) with well treated T2DM (HbA1c: 7.1 %) and ten matched healthy subjects. All were studied with a glycaemic clamp at their individual fasting plasma glucose (FPG) level; patients had a 7-day wash-out period of their oral anti-diabetic medication prior to examination. Besides the glycaemic clamp, GLP-1 was infused in increasing doses resulting in plasma levels ranging from the physiological levels (0.25 pmol/kg body weight/min) to the maximal tolerable range (2.0 pmol/kg body weight/min). On day 2 saline was infused as control. As we also wanted to examine a possible de-sensitising effect of glucotoxicity in the patients, these were further examined with GLP-1 infusions during near-normal PG concentration achieved by using over-night adjustable insulin infusions. This resulted in PG levels of 5.9±0.3 mmol/l, but because it was decided to include an insulin wash-out period of 30 minutes before initiating the GLP-1 infusion, PG increased to 6.8±0.6 mmol/l. The main finding of this study was that these patients with T2DM had preserved sensitivity to GLP-1 with regard to glucagon suppression. GLP-1 inhibited alpha cell secretion potently and equally in both patients and healthy subjects with the only difference being a small delay in the patients. Significant glucagon suppression was detected at time 15 minutes in the healthy subjects and at time 30 minutes in T2DM. No difference was observed in regard to GLP-1 induced glucagon suppression during the two different PG clamp levels in the patients with T2DM; thus, glucotoxicity did not seem to play a role. Upon statistical analyses of the glucagon response using a two-way ANOVA, no differences were found between the three decreasing curves, indicating that the alpha cells responded equally to GLP-1 despite very different conditions; these being: a diabetic FPG, glucose tolerant euglycaemia or close to euglycaemia in the patients with T2DM. Our findings suggest that GLP-1 acts very potently on the alpha cell, but that the diabetic alpha cell is very deficient in glucose sensing as shown by the similar FPG concentrations and the similar response to GLP-1.

Our patients did not have hyperglucagonaemia in the fasting state; fasting glucagon levels were not significantly different from the levels of the healthy subjects though their FPG was very different. However, fasting glucagon levels in the patients were correlated to both their prevailing fasting PG and their insulin resistance (calculated as HOMA\(\beta\)). This was not the situation in the controls (who were also obese, BMI>30). In addition we found the initial glucagon response (glucagon AUC\(_{0-45}\), the response to the lowest infusion rate) to be correlated to their HOMA\(\beta\). All of this suggests that alpha cell function in these patients had some relation to the impaired insulin sensitivity and glucose tolerance. Another feature in these patients was that their beta cells responded remarkably well to GLP-1 stimulation when they were clamped at their diabetic FPG levels. When they were clamped at a ‘normalised’ PG (6.9±0.1 mmol/l), their beta cell responses were comparable to the response of the healthy subjects, who were clamped at euglycaemia (5.4±0.1 mmol/l). The similar insulin response was obtained in spite of different levels of glycaemia, indicating that the responses of the patients were still abnormal. In an attempt to quantify the GLP-1 mediated enhancement of insulin secretion, we calculated the C-peptide/PG-ratios i.e. the amplification of the glucose signal during the two GLP-1 infusion days; the slopes turned out to be parallel indicating a similar amplification of the glucose signal in the patients and healthy subjects. The absolute differences in the C-peptide/PG-ratios, however, were interpreted to reflect a decreased beta cell mass. With these results we concluded that our patients with T2DM had a preserved sensitivity of their alpha cells to GLP-1 induced glucagon inhibition, and we also concluded that glucagon was inhibited dose-dependently and that the inhibition was independent of the prevailing PG levels. The fact, that their beta cells seemed to retain full sensitivity to GLP-1, might be explained by the fact that these patients were metabolically well treated, in contrast to earlier investigated patients with T2DM.

**STUDY II**

**THE GLUCAGONOSTATIC AND INSULINOTROPIC EFFECTS OF GLP-1 CONTRIBUTE EQUALLY TO ITS GLUCOSE-LOWERING ACTION**

GLP-1 inhibits glucagon secretion, and this is thought to contribute to the improved glycaemic control reported in several clinical trials, whether the patients were treated with native hormone, GLP-1 mimetics (exenatide or liraglutide) or GLP-1 enhancers (DPP-4 inhibitors, e.g. sitagliptin or vildagliptin) (144,165-167,180,181). Hyperglucagonaemia, often found in patients with T2DM, has been discussed earlier. In experiments by Shah et al. similar hyperglucagonaemia brought about by exogenous glucagon resulted in accelerated glycoenolysis and hyperglycaemia. The authors concluded that glucagon antagonists would be beneficial in treatment of T2DM (182). The combined actions of GLP-1 to potentiate inhibit glucagon secretion together with its stimulatory effect on the beta cell and its glucose dependency may therefore be particularly expedient. The anti-diabetic and glucose-lowering action of GLP-1 is thus dual; 1) inhibition of glucagon secretion resulting in decreased stimulation of HGP(128), and 2) glucose-dependent stimulation of insulin secretion, resulting in increased peripheral glucose disposal and further inhibition of HGP (183).

In study II we aimed to quantify the contribution of glucagon inhibition in relation to the glucose-lowering effect seen when GLP-1 is administered to patients with T2DM. The rationale for the protocol was the following: When GLP-1 is infused during a glycaemic clamp, the amount of glucose required to maintain the clamp reflects both the increased glucose disposal mediated by GLP-1-induced insulin secretion, but also a decrease in HGP caused by GLP-1-induced glucagon inhibition. In this protocol we used glucose demand as endpoint measure during a combined glycaemic-pancreatic clamp. We investigated ten male patients with T2DM, all well-regulated according to HbA1c (6.9±0.8 %), plasma lipids and blood pressure. Oral anti-diabetic medication was paused one week prior to investigation, all were investigated with five glycaemic clamps combined with GLP-1 infusion alone or with somatostatin combined with one or two of the pancreatic hormones. All patients were clamped at their individual FPG, thereby eliminating both potential stimulatory and inhibitory effects of glucose on the islet cells. In addition to the glucose clamps, the protocol consisted of; day 1: GLP-1 (insulin stimulation and glucagon inhibition); day 2:
somatostatin together with GLP-1, basal insulin and basal (portal) glucagon (elimination of the pancreatic hormone response to GLP-1); day 3: somatostatin and basal insulin (isolated glucagon inhibition); day 4: somatostatin, stimulated insulin and basal (portal) glucagon (isolated insulin stimulation); and day 5: somatostatin, stimulated insulin (a control experiment evaluating the insulin and glucagon responses to GLP-1 as mimicked by somatostatin infusion versus infusions, as performed in protocols 3 and 4). On day 1, when GLP-1 was infused alone, we chose an infusion rate (1.0 pmol/kg body weight/min) investigated earlier and therefore known to stimulate insulin and to inhibit glucagon secretion (10). This dose was also defined as ED$_{50}$ in study I and results in “therapeutic” plasma concentrations in the investigated subjects. Insulin infusions on day 4 and day 5 mimicked the GLP-1-induced insulin increase exactly and insulin remained at basal level during day 2 and day 3, demonstrating the successful substitution of basal insulin levels. Somatostatin suppressed endogenous beta cell secretion efficiently as illustrated by suppressed C-peptide levels during all days with somatostatin administrations. Somatostatin also suppressed endogenous glucagon secretion efficiently, as seen during day 3 and 5. On day 1, GLP-1 efficiently suppressed glucagon secretion with plasma concentrations decreasing to just detectable levels during the 120 minutes clamp; furthermore, decremental and absolute AUC levels were alike on day 1, 3 and 5. During day 2 and day 4 glucagon was supplied by infusion aiming at reaching intrahepatic levels, which are almost twice the peripheral plasma levels (x 1.9)(184). However, the calculated infusion rate resulted in slightly increased PG levels (day 2). This suggested that either glucagon level were too high or (intraportal) insulin levels were to low. Also insulin levels are higher in the portal plasma and to mimic these higher levels would have been required. On the other hand, the peripheral insulin levels were perfectly matched by infusions. It was therefore decided to accept the slight increase in PG in the control examination on day 2 since it would be impossible to reproduce the higher portal insulin levels and the lower peripheral levels at the same time. The slight increase in PG was seen with the initial glucagon infusion rate of 1.25 ng/kg body weight/min (182) but also with the lower (1.0 ng/kg body weight/min (185)) and the lowest infusion rates (0.6 ng/kg body weight/min). All but two patients increased in PG; these two were the patients with the highest FPG; one was infused with 1.25 the other with 0.6 ng/kg body weight/min. The increase seen was presumably a result of HPG caused by an imbalance in the glucagon/insulin-ratio. Because of very similar responses to the different glucagon infusion rates, we chose to combine the different infusion rates. In addition, we concluded that the intra-hepatic glucagon concentrations were well matched as we aimed for a plasma plateau level of 2.6±1 pmol/l (fasting glucagon concentration x 1.9) and reached a plateau of 31±5 pmol/l on day 2 (P=0.23 compared to calculated level) and 31±5 pmol/l on day 4 (P=0.33 compared to calculated level). As hormone levels were satisfactorily matched we were able to use glucose demands as end-point measurements. These demands were; day 1: 24.5±4.1 g; day 2: 0.3±0.2 g; day 3: 10.6±1.1 g; day 4: 11.5±2.7 g and day 5: 24.5±2.6 g. The identical glucagon demands on day 1 and 5 reflects the accurately matched effects of the insulin and glucagon levels on the two days, i.e. the substitution clamp matched the GLP-1 clamp both with regards to hormone concentrations and glucose demand. On day 2 both insulin and glucagon levels were clamped at basal levels, therefore no glucose was needed to maintain PG at clamp level. Equal glucose infusions on day 3 (effect of glucagon inhibition) and day 4 (effect of insulin stimulation) lead to the conclusion that each contributes equally to the glucose-lowering effect of GLP-1 in patients with T2DM.

STUDY III

INAPPROPRIATE GLUCAGON RESPONSE AFTER ORAL AS COMPARED TO ISOGLYCAEMIC INTRAVENOUS GLUCOSE ADMINISTRATION IN PATIENTS WITH T1DM

Much is known about incretins in healthy subjects and T2DM patients, but investigations of patients with T1DM are few. Creutzfeldt et al. were among the first to report a glucose-lowering effect of GLP-1 in T1DM (70) associated with glucagon inhibition. Eleven patients were investigated, three of whom had no measurable C-peptide response to GLP-1 but all responded with an equal decrease in PG. Further, Greenbaum et al. described a population of patients with T1DM, but normal FPG, insulin and glucagon values (patients still had a functional beta cell response). Here, a significant glucagon level increase in response to an OGTT compared to healthy subjects was reported; an abnormal intra-islet insulin-mediated alpha cell supression was suggested as a cause (186). In a third study, Pörksen et al. investigated newly diagnosed T1DM adolescents. The patients were challenged with standardized meals containing 240 kcal. Pörksen et al. observed a decreasing beta cell function over the 12 month follow up period (meal test were given at 1, 6 and 12 months) associated with increasing postrandrial glucagon, GLP-1 and PG levels. The glucagon levels were positively associated with both PG and GLP-1 levels, but not with residual beta cell function (meal stimulated C-peptide) (187).

In study III, we investigated patients with T1DM, primarily in order to study glucagon secretion with no pancreatic insulin to influence the alpha cells, but also to evaluate gastrointestinal (GI) hormone responses in these patients. We investigated 9 patients with T1DM(one female); all were C-peptide negative (assessed with an iv arginine test) as lack of residual beta cell function was essential in this protocol rather than detection of specific auto-antibodies (only 4 were positive for GAD-65 and two for ICA (double positive)). These patients were matched with eight healthy male subjects; all were investigated with; day 1: 4-hour 50-g OGTT and day 2: 4-hour IIGI copying the PG excursion seen on day 1. In order to eliminate any effect of exogenous insulin, patients were instructed not to inject any insulin in the mornings of investigation. In addition, symptoms of hypoglycaemia or blood glucose measurements below 3 mmol/l postponed investigations in some of the patients in order to eliminate the possibility of reactive hyperglucagonaemia due to hypoglycaemia. As we were unable to calculate an incretin effect by the conventional method ((C-peptide AUC$_{OGTT}$ – C-peptide AUC$_{GI}$)/ C-peptide AUC$_{GI}$) because the patients were C-peptide negative, we measured their GI-mediated glucose tolerance (GITT), a measure of the efficiency of GI tract factors in eliminating the glucose and minimise glucose excursions during OGTT: (glucose$_{OGTT}$(g)−glucose$_{GI}$(g))/glucose$_{OGTT}$(g). We found that patient GITT was insignificantly different from zero (-6±9 %) while in healthy subjects (with a normal incretin effect of 65±4 %) we found a GITT of: 40±6 % (P<0.0001), illustrating that the GI tract factors play a major role in normal glucose tolerance.

We found pronounced glucagon inhibition in the T1DM patients during the IIGI, but paradoxical hyperglucagonaemia was observed in response to the 50-g OGTT. Furthermore, as also ob-
served in T2DM, the subsequent glucagon suppression in the T1DM was attenuated and at least 40 minutes delayed both compared to the glucagon suppression during the IIGI and compared to the response in the healthy subjects. As mentioned, the defective glucagon suppression reported by both Greenbaum et al. (186) and Pörksen et al. (186,187) was explained by the impaired beta cell function, and the paradoxical glucagon response to oral glucose in T2DM has been ascribed to defective insulin secretion and insulin resistance. Here we examined completely insulin deficient, but insulin sensitive T1DM patients and found, that they were able to suppress alpha cell secretion in response to iv glucose but not to similar glucose elevations brought about by oral administration. We also measured incretin hormones during the two days of investigation in both groups, but found no differences regarding GLP-1, GLP-2 or GIP secretory responses between patients and healthy subjects. With this study we are not able to explain the paradoxical glucagon secretion in response to oral glucose. Both GIP and GLP-2 have been reported to be glucagonotropic (74,76,78,79) under normoglycaemic conditions and GLP-2 also during hyperglycaemia. An abnormal sensitivity to these hormones in patients with T1DM could explain the abnormal inhibition of glucagon secretion, but further investigations of this are clearly needed.

DISCUSSION AND CONCLUSION
Our studies have provided new knowledge about the diabetic alpha cell and its regulation. T2DM is increasing rapidly worldwide and will have increasing socioeconomic consequences in the future (188). To combat this, more knowledge of pathophysiology and improved therapies are needed. Incretin based therapies improve glycaemic control by improving the beta cell response to glucose and by inhibiting glucagon secretion (189). Focus on the alpha cell in diabetes treatment is relatively new. Traditionally treatment of patients with T2DM has been directed towards beta cell secretion, insulin sensitivity, absorption of glucose or insulin replacement. Currently, glucagon antagonists are investigated as new interesting treatment of patients with T2DM (44,190,191), attacking hyperglycaemia and elevated endogenous glucose production leading to improved glycaemic control (44).

We investigated GLP-1-induced glucagon inhibition. The inhibition is shown to normalise plasma glucagon during hyperglycaemic conditions in diabetes (12) and is probably not associated with hypoglycaemia, as the incidence of hypoglycaemia is very low in reports on GLP-1-based treatment. Hypoglycaemia is mainly seen in combination therapy with sulphonylureas or insulin (192).

In study I we found that the diabetic alpha cell has preserved sensitivity to GLP-1 induced inhibition. This was not what we had expected. Earlier studies, concerning the diabetic beta cell and its response to GLP-1, show the opposite. Both Kjems et al. (152) and Højberg et al. (85) reported decreased beta cell sensitivity to both glucose and GLP-1. On the other hand, Højberg reported an improved response to both glucose (hyperglycaemic clamp at 15 mmol/l) and GLP-1 (combined hyperglycaemic-GLP-1 clamp) after 4-weeks of intensive treatment with multiple dosing of insulin given to patients with T2DM. In this study, only physiological GLP-1 plasma concentrations were investigated (0.5 pmol/kg body weight/min). Previously, Vilsbøll et al. (10) investigated patients with T2DM during hyperglycaemic clamps (15mmol/l), with or without GLP-1 in a pharmacological dose (1.0 pmol/kg body weight/min). The beta cell response to the clamp + GLP-1 in this group of patients was comparable to responses for the clamp alone in the healthy subjects. The two groups of patients also responded differently regarding glucagon secretion. Højberg et al. reported that physiological GLP-1 concentrations were unable to enhance glucose induced glucagon suppression further. Vilsbøll et al. found impaired glucose induced glucagon suppression to be completely normalised by pharmacological GLP-1-infusions. Looking at these conflicting results attained in two different groups of patients with T2DM, the explanation seems to be the investigated patients. The question regarding our results (study I) - preserved inhibitory potency of GLP-2 on the diabetic alpha cell - is whether we would find similar results in a group of patients matching the Højberg group? A common conclusion from all three studies must be that the sensitivity of the alpha cell or whatever target GLP-1 may interact with to reduce glucagon secretion depends on the patients, and is most likely influenced by duration of disease, glycaemic and metabolic control, and possibly duration of washout of anti-diabetic treatment prior to investigation. Thus metformin has recently been found to increase secretion of GLP-1, a clearly confounding factor(193,194). However, a major difference between studies by the Højberg and Vilsbøll protocols and our present study is the clamp level; both of the previous protocols investigated pancreatic hormone responses during hyperglycaemia, whereas we investigated the patients at their individual fasting PG or at a normalised PG level to eliminate effects of glucose per se on the alpha and beta cells.

Study II supports a very potent inhibitory effect of GLP-1 on glucagon secretion in patients with T2DM. Here we examined the glucagon inhibitory effect on glucose turn-over with a ‘pharmacological’ dose of GLP-1. We estimated this effect to match the effect mediated by the insulinotropic action of GLP-1. All peripheral hormone concentrations were matched during the pancreatic clamp; furthermore we matched successfully the effect of GLP-1-induced glucose turn-over with this technique (day 1 vs. day 5). Portal glucagon concentrations were matched (184), but we were unable to match the portal insulin concentrations, as judged by the slight PG increase on day 2, which investigated the glucose turn-over during basal insulin and glucagon concentrations, as supplied by infusion. As discussed earlier the insulin dose was probably insufficient to inhibit HGP during these circumstances. An increased insulin infusion would increase glucose disposal, and therefore we accepted this small PG increase. The consequence of this under-matched portal insulin is that our estimated glucagon effect on glucose turn-over might be underestimated. Since a higher insulin infusion rate on this day (basal portal glucagon and basal peripheral insulin) would have increased the amount of glucose required to maintain the clamp. On day 3 glucagon was inhibited and basal (peripheral) insulin was supplied, isolating the effect of glucagon inhibition alone on glucose turn-over. Also on this day elevation of insulin infusion rates would lead to further inhibition of HGP and a larger glucose demand. Consequently our estimated effect of glucagon inhibition would have been greater. Thus, if anything we have underestimated the effects of glucagon inhibition.

With study III we supply strong evidence against the intra-islet theory as an explanation of the abnormal glucagon response to OGTT. The examined T1DM patients had no functional beta cell mass, but were still able to suppress glucagon during IIGI, and this suppression cannot be due to intra-islet insulin. Further this study emphasises the importance of GI tract factors in normal glucose tolerance, as illustrated with a GIGT of 40 % in the healthy subjects compared to zero in the patients with T1DM. The protocol was carried out to investigate glucagon responses to OGTT compared to IIGI in these C-peptide negative patients. We saw a similar positive secretory glucagon response during the first 40
minutes of the OGTT, compared to suppression during the IIGI as previous seen in T2DM(14). Our glucagon assay detects the C-terminal sequence of the glucagon peptide; however, glucagon is difficult to measure as discussed earlier. It is possible that this hyper-response represents proglucagon 1-61/gut-glucagon/glucagon 5000 secreted from the intestinal L cells (173). With the present assay we are unable to eliminate this possibility. However, more thorough chromatography analyses of this hyper-response in patients with T2DM lead to the conclusion that the response consisted mainly of authentic glucagon(178). Pancreatic glucagon results from PC2 mediated processing of proglucagon. An explanation for the glucagon secretory response to oral glucose might involve unexpected proglucagon products secreted from the small intestine, e.g. in the form of PrG 1-61. Alternatively, a PC2 mediated processing of proglucagon in the L cell might occur in the diabetic state. Secretion of authentic glucagon has previously been reported in pancreatectomized humans in response to a fat and carbohydrate rich meal, indicating that the gut under certain circumstances is able to secrete glucagon(195). Whether diabetic hyperglycaemia might induce expression of PC2 in the L cell is currently under investigation(196,197).

**PERSPECTIVE**

These studies support the notion, that GLP-1-induced inhibition of alpha cell secretion is potent (study I) and effective (study II). Currently, glucagon receptor antagonists are under development to treat diabetes. Our studies prove GLP-1 to be a potent glucagon antagonist also in T2DM supporting GLP-1-based treatment of T2DM and possibly T1DM as well. However, these treatments have their limitations; the GLP-1 mimetics induce nausea because of their effects on gastric emptying and the DPP-4 inhibitors only increase endogenous GLP-1 levels slightly. The optimal GLP-1 treatment would be oral as opposed to subcutaneous administration as required today; much research is ongoing with a view to develop such orally active analogues. Further, approaches designed to stimulate the endogenous L cell secretion could lead to a more physiological treatment - possibly with fewer side-effects. Research aiming to elucidate the regulation of the alpha cell is ongoing. A mechanism for the glucagon inhibitory mechanism of GLP-1 in humans is still lacking: is there a direct receptor-mediated inhibition – supported by the finding of GLP-1 receptors on 20% of the alpha cells(169), or is the suppression indirect and mediated through somatostatin secreting delta cells, a theory supported by solid data in rodents(60)and in humans with T1DM(72)?

**SUMMARY**

This project consisted of two parts: a biochemical part and clinical studies. The overall aim was to elucidate the defective regulation of glucagon secretion in type 2 diabetes (T2DM). The aim in the biochemical part was to develop a glucagon ELISA by using C- and N-terminal antibodies generated in the laboratory. Much effort was put into this attempt; however, we were unsuccessful and had to use an alternative method in our attempt to characterize the paradoxical diabetic glucagon response further. By using Sep-Pac and HPLC separation methods, plasma from patients with T2DM known to have a defective suppression of glucagon was analyzed using three antibodies and RIA. In this way the hyperglucagonemia was found to consist mainly of authentic glucagon, rather than abnormally processed forms. The first clinical study included ten healthy controls matched to ten patients with T2DM. The aim was to investigate if GLP-1-induced glucagon inhibition was dose dependent and if suppression was equally potent in healthy controls and T2DM patients. Further, we investigated if the potency of the inhibition depended on the prevailing plasma glucose (PG) level. All participants were investigated with increasing doses of GLP-1 administered as iv-infusions and saline (control) during a glycaemic clamp at fasting plasma glucose (PPG) levels. Patients were investigated on a third occasion with GLP-1 infusions after an over-night normalisation of PG using adjustable insulin infusions. From these experiments we were able to conclude that GLP-1-induced glucagon inhibition is dose-dependent, but surprisingly GLP-1 suppressed the alpha cell equally potently in patients and controls – and the suppression was independent of PG level. Therefore we concluded that the paradoxical glucagon response to orally ingested glucose is not caused by decreased potency of GLP-1 with respect to glucagon suppression. It may be due to the decreased secretion of this hormone reported in earlier studies.

My second protocol aimed towards quantifying the glucose-lowering effect of GLP-1-induced glucagon inhibition seen in patients with T2DM. The glucose-lowering effect of GLP-1 is due to both insulin stimulation leading to peripheral glucose disposal and glucagon inhibition resulting in decreased stimulation of hepatic glucose production. With a five-day protocol including both glycaemic and pancreatic clamps in ten patients with T2DM we were able to isolate the contribution of glucagon suppression to the increased glucose turnover seen during a GLP-1 glycaemic clamp, and interestingly it was equal to the known insulinotropic effect of GLP-1. Finally, we investigated patients with type 1 diabetes (T1DM) and no residual beta cell function with oral glucose tolerance test (OGTT) and isoglycaemic intravenous glucose infusion (IIGI) in order to evaluate any differences in glucagon response to glucose +/- gastrointestinal (GI)-stimulation. Here we found that despite a perfectly normal inhibition of glucagon during the IIGI in the T1DM, they had a defective glucagon suppression in response to orally ingested glucose and a paradoxical secretion of glucagon was seen as in T2DM. Hereby, we proved that glucagon suppression in response to hyperglycaemia does not entirely depend on intra-islet insulin effects as has been suggested. Therefore we conclude that GI-tract factors rather than intra-islet dysregulation explain the paradoxical glucagon response in patients with diabetes.

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