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

Kristine Juul Hare

This review has been accepted as a thesis together with three previously published papers by University of Copenhagen 24th of February 2010 and defended on April 9th 2010.

Toturs: Jens Juul Holst, Tina Vilsbøll & Steen Larsen.

Official opponents: Jan Erik Henriksen, Juris J. Meier & Jørgen Vinten.

Correspondence: Department of Biomedical Sciences, The Panum Institute, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark.

E-mail: kjhare@sund.ku.dk

Dan Med Bull 2010;57: [B4181]

The three original papers are:

1. Hare,KJ, Knop,FK, Asmar,M, Madsbad,S, Deacon,CF, Holst,JJ, Vilsboll,T: Preserved inhibitory potency of GLP-1 on glucagon secretion in type 2 diabetes mellitus. *J Clin Endocrinol Metab* 94:4679-4687, 2009

2. Hare,KJ, Vilsboll,T, Asmar,M, Deacon,CF, Knop,FK, Holst,JJ: The glucagonostatic and insulinotropic effects of glucagon-like peptide 1 contribute equally to its glucose-lowering action. *Diabetes* 59:1765-1770, 2010

3. Hare,KJ, Vilsboll,T, Holst,JJ, Knop,FK: Inappropriate glucagon response after oral compared with isoglycemic intravenous glucose administration in patients with type 1 diabetes. *Am J Physiol Endocrinol Metab* 298:E832-E837, 2010

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 isoglycaemic intravenous (*iv*) glucose infusion (IIGI) 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 coworkers. 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 (GRPP, 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 78-107NH_2) 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 bowl 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 ($G_s\alpha$ and G_q), and $G_s\alpha$ activates adenylat cyclase resulting in increasing cyclic adenosine mono-phosphate (cAMP) and thereby protein kinase A activity. G_q activation leads to an increase in intracellular Ca^{++} through activation of phospholipase C (40). In hepatocytes, receptor activation leads to increased HGP by enhancing both glycogenolysis 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 insulinotropic 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 characterize 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 glucagon induced by *iv* glucose has also been shown to be impaired in patients with T2DM. This was demonstrated during a hyperglycaemic clamp of 15 mmol/l in which suppressed glucagon levels in obese patients with T2DM compared to matched healthy subjects were observed (10). The suppressive potency of glucose on the alpha cell might depend on the glycaemic control (85). Thus, the glucagonostatic effect of hyperglycaemia turned out to be unaltered after 4-weeks of super-regulation with multiple dosing of insulin in a group of patients with T2DM (85).

Hyperglucagonaemia in the fasting state has been reported in some studies (14,85-90), whereas others find no significant differences between patients with T2DM and healthy subjects (10,91-93). However, it is clear that in T2DM, the plasma glucagon levels are inappropriately high in the context of hyperglycaemia and hyperinsulinaemia (when present) – as both of these are thought to suppress glucagon sufficiently in the glucose tolerant subject (94,95). Therefore, it has been suggested that the diabetic alpha cell exhibits a reduced glucose sensitivity and/or insulin resistance (96,97). At any rate the increased glucagon levels in patients with T2DM clearly contribute significantly to the diabetic hyperglycaemia (86,87).

Loss of a glucagon secretory response to falling PG concentrations is a key feature of the pathophysiology of glucose counter-regulation in the most advanced stages of T2DM (52,98) and also characterizes the clinical syndrome of defective glucose counter regulation(99) in patients with T1DM (100,101).

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 SLC38A2 (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) NH_2, has no activating effect on the beta cell, as an intact NH_2-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 cleaved 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^{++}channels. This cascade leads to mobilization of insulin containing granules and insulin exocytosis (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 (IIGI) (127). 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

effect of GLP-1 is clearly seen in diabetes (T2DM and T1DM), where GLP-1 markedly lowers plasma glucagon concentrations while PG normalises (12,70); still, the PG will not decrease below 3-4 mmol/l. In healthy subjects, GLP-1 will also decrease glucagon, but this is most easily seen in clamp studies, where glucagon concentrations decrease to lower levels (130). However, examining healthy subjects during hyperglycaemia, glucagon levels will decrease by means of glucose alone(85). In *in vitro* studies GLP-1 has been shown to preserve and may even increase beta cell mass in isolated human islet (131). Furthermore, GLP-1 inhibits GI secretion and motility, including gastric emptying (132,133); it also induces satiety and reduces food intake in T2DM as well as healthy subjects (134,135). Recent experiments with a GLP-1 receptor antagonist, exendin-9, has demonstrated that it is one of the physiological regulators of food intake (136). The GLP-1 receptor has also been localized in the heart (137) and on endothelial cells (138). Studies in rodents have shown GLP-1-induced protection of the ischaemic and reperfused myocardium, administered both as pre- and post-ischaemia infusions. Surprisingly, positive effects were also seen in GLP-1R-/- animals and during post-ischaemic treatment with GLP-1 (9-39), indicating that an unknown receptor might be involved (138,139). In patients with heart failure receiving GLP-1 as add on to the conventional medication, GLP-1 improved ejection fraction, myocardial oxygen uptake, 6-min walking distance and quality of life (140). In addition, GLP-1 proved to have beneficial effects when infused during coronary artery bypass grafting. The infusion was initiated 12 hours before the operation and continued for 48 hours in total. The treatment resulted in less frequent arrythmias, and a decreased need for inotropic and vasoactive substances. In addition, the treated group had an improved glycaemic control and a lesser need for insulin treatment (141). Improvement in endothelial dysfunction has been reported in T2DM with coronary heart disease (142), and a significant decrease in blood pressure has been observed in patients with T2DM during clinical trials investigating GLP-1 analogues (143,144). This might result from endothelium dependent-vasodilation, as GLP-1 per se has been shown to have such beneficial effects in healthy subjects (145).

A reduced incretin effect(1) is a pathophysiological trait in T2DM and is found in both lean (1,14) and obese patients (16,146). A reduced incretin effect has also been found in diabetes secondary to chronic pancreatitis (15), in reversible forms (gestational diabetes(147) and during steroid induced insulin resistance and glucose intolerance (148,149)) indicating the decrease to be a consequence of - rather than a cause of - diabetes. Studies including patients with T2DM have revealed that these patients have impaired GLP-1-responses to a mixed meal; the decreased secretion is related to both body mass index (BMI) and to the actual diabetic state, but is independent of e.g. the presence of neuropathy (90,146). The individual GLP-1 response to a meal seems to decrease with increase in BMI, insulin resistance and duration of disease and with slowing of gastric emptying (90,146). The decreased meal response is independent of the degradation of GLP-1, as the hormone is metabolized similarly in patients and age- and weight-matched healthy subjects (150). All of this suggests that the loss of the incretin effect is secondary to insulin resistance and/or glucose intolerance. The decreased incretin response to a mixed meal has been shown to be related to the degree of glucose tolerance; subjects with IGT respond poorly to a mixed meal stimuli, however postprandial response was not as impaired as seen in patients with T2DM(90). Furthermore, the reduction in meal response has only been confirmed in the diabetic sibling in investigations of identical twins (151). Again indicating these changes to be consequences of the diabetic condition rather than causing it.

Beta cell sensitivity to GLP-1 is clearly reduced in patients with T2DM (88,152). However, at supraphysiological levels GLP-1 is cable of completely restore the beta cell sensitivity to glucose (10,152,153). The loss of potency of GLP-1 was confirmed in studies where GLP-1 was infused to reach physiological levels (postprandial levels) and plasma glucose was clamped at 15 mmol/l. During these conditions GLP-1 failed to affect insulin secretion at all (85). The impaired beta cell effect of GLP-1, together with the almost abolished insulinotropic effect of GIP, is likely to be the major cause of the decreased incretin effect (10). A normal incretin effect has been seen in patients with chronic pancreatitis and normal glucose tolerance, in contrast to patients with the same diagnose and secondary diabetes (15). First-degree relatives to T2DM patients, also exhibit a conserved incretin effect (154). Further, a reversible impairment of the incretin effect can be seen during steroid induced insulin resistance in both first degree relatives (149) and in unrelated healthy subjects(148).

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 of 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 infused 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 glucose 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 endogenous 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 ^{125}I-glucagon were used in all assays. Antisera 4304 and 4830 bind labelled and unlabeled glucagon and oxyntomodulin (PrG 33-69) with identical affinity (173,175,177), whereas antiserum 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 $HOMA_{IR}$). This was not the situation in the controls (who were also obese, BMI>30). In addition we found the initial glucagon response (glucagon $AUC_{0\text{-}45}$, the response to the lowest infusion rate) to be correlated to their $HOMA_{IR}$. 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 glycogenolysis and hyperglycaemia. The authors concluded that glucagon antagonists would be beneficial in treatment of T2DM (182). The combined actions of GLP-1 to potently 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 HbA_{1c} (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 26±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 glucose 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 rapport 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 mass). Here, a significant impairment in suppression of glucagon levels in response to an OGTT compared to healthy subjects was reported; an abnormal intra-islet insulin-mediated alpha cell suppression 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 postprandial 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 autoantibodies (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_{IIGI})/ C-peptide AUC_{OGTT}) because the patients were C-peptide negative, we measured their GI-mediated glucose tolerance (GIGT), a measure of the efficiency of GI tract factors in eliminating the glucose and minimise glucose excursions during OGTT: ($glucose_{OGTT}$(g)-$glucose_{IIGI}$(g))/$glucose_{OGTT}$ (g). We found that patient GIGT was insignificantly different from zero (-6±9 %) while in healthy subjects (with a normal incretin effect of 65±4 %) we found a GIGT 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 hyperglucagonaemia 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-1 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 inhibiters 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 hyperglucagonaemia 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 (FPG) 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 turn-over 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 +/- gastri-intestinal (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 intraislet dysregulation explain the paradoxical glucagon response in patients with diabetes.

Reference

1. Nauck,M, Stockmann,F, Ebert,R, Creutzfeldt,W: Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 29:46-52, 1986
2. Faerch,K, Borch-Johnsen,K, Holst,JJ, Vaag,A: Pathophysiology and aetiology of impaired fasting glycaemia and impaired glucose tolerance: does it matter for prevention and treatment of type 2 diabetes? *Diabetologia* 52:1714-1723, 2009
3. Bergman,RN: Lilly lecture 1989. Toward physiological understanding of glucose tolerance. Minimal-model approach. *Diabetes* 38:1512-1527, 1989
4. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* 46:3-19, 2003
5. Larsson,H, Ahren,B: Failure to adequately adapt reduced insulin sensitivity with increased insulin secretion in women with impaired glucose tolerance. *Diabetologia* 39:1099-1107, 1996

6. Larsson,H, Ahren,B: Islet dysfunction in obese women with impaired glucose tolerance. *Metabolism* 45:502-509, 1996
7. Larsson,H, Ahren,B: Insulin resistant subjects lack islet adaptation to short-term dexamethasone-induced reduction in insulin sensitivity. *Diabetologia* 42:936-943, 1999
8. Turner,RC, Cull,CA, Stratton,IM, Manley,SE, Kohner,EM, Matthews,DR, Neil,HAW, Levy,JC, Holman,RR: Uk Prospective Diabetes Study 16 - Overview of 6 Years Therapy of Type-Ii Diabetes - A Progressive Disease. *Diabetes* 44:1249-1258, 1995
9. Ahren,B, Larsson,H: Impaired glucose tolerance (IGT) is associated with reduced insulin-induced suppression of glucagon concentrations. *Diabetologia* 44:1998-2003, 2001
10. Vilsboll,T, Krarup,T, Madsbad,S, Holst,JJ: Defective amplification of the late phase insulin response to glucose by GIP in obese Type II diabetic patients. *Diabetologia* 45:1111-1119, 2002
11. Shah,P, Basu,A, Basu,R, Rizza,R: Impact of lack of suppression of glucagon on glucose tolerance in humans. *Am J Physiol* 277:E283-E290, 1999
12. Nauck,MA, Kleine,N, Orskov,C, Holst,JJ, Willms,B, Creutzfeldt,W: Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide 1 (7-36 amide) in type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 36:741-744, 1993
13. Orskov,C, Holst,JJ, Nielsen,OV: Effect of truncated glucagon-like peptide-1 [proglucagon-(78-107) amide] on endocrine secretion from pig pancreas, antrum, and nonantral stomach. *Endocrinology* 123:2009-2013, 1988
14. Knop,FK, Vilsboll,T, Madsbad,S, Holst,JJ, Krarup,T: Inappropriate suppression of glucagon during OGTT but not during isoglycaemic i.v. glucose infusion contributes to the reduced incretin effect in type 2 diabetes mellitus. *Diabetologia* 50:797-805, 2007
15. Knop,FK, Vilsboll,T, Hojberg,PV, Larsen,S, Madsbad,S, Volund,A, Holst,JJ, Krarup,T: Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? *Diabetes* 56:1951-1959, 2007
16. Knop,FK, Aaboe,K, Vilsboll,T, Madsbad,S, Holst,JJ, Krarup,T: Redcuced incretin effect in obese subjects with normal glucose tolerance as compared to lean control subjects. *Diabetes* 57:A410, 2008
17. Bell,GI, Santerre,RF, Mullenbach,GT: Hamster preproglucagon contains the sequence of glucagon and two related peptides. *Nature* 302:716-718, 1983
18. Holst,JJ, Bersani,M, Johnsen,AH, Kofod,H, Hartmann,B, Orskov,C: Proglucagon processing in porcine and human pancreas. *J Biol Chem* 269:18827-18833, 1994
19. Novak,U, Wilks,A, Buell,G, McEwen,S: Identical mRNA for preproglucagon in pancreas and gut. *Eur J Biochem* 164:553-558, 1987
20. Larsen,PJ, Tang-Christensen,M, Holst,JJ, Orskov,C: Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience* 77:257-270, 1997
21. Mojsov,S, Heinrich,G, Wilson,IB, Ravazzola,M, Orci,L, Habener,JF: Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J Biol Chem* 261:11880-11889, 1986
22. Orskov,C, Holst,JJ, Poulsen,SS, Kirkegaard,P: Pancreatic and intestinal processing of proglucagon in man. *Diabetologia* 30:874-881, 1987
23. Buhl,T, Thim,L, Kofod,H, Orskov,C, Harling,H, Holst,JJ: Naturally occurring products of proglucagon 111-160 in the porcine and human small intestine. *J Biol Chem* 263:8621-8624, 1988
24. Orskov,C, Holst,JJ, Knuhtsen,S, Baldissera,FG, Poulsen,SS, Nielsen,OV: Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. *Endocrinology* 119:1467-1475, 1986
25. Orskov,C, Bersani,M, Johnsen,AH, Hojrup,P, Holst,JJ: Complete sequences of glucagon-like peptide-1 from human and pig small intestine. *J Biol Chem* 264:12826-12829, 1989
26. Thim,L, Moody,AJ: The primary structure of porcine glicentin (proglucagon). *Regul Pept* 2:139-150, 1981
27. Thulesen,J: Glucagon-like peptide 2 (GLP-2), an intestinotrophic mediator. *Curr Protein Pept Sci* 5:51-65, 2004
28. Cheeseman,CI: Upregulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion in vivo. *Am J Physiol* 273:R1965-R1971, 1997
29. Jeppesen,PB, Hartmann,B, Thulesen,J, Graff,J, Lohmann,J, Hansen,BS, Tofteng,F, Poulsen,SS, Madsen,JL, Holst,JJ, Mortensen,PB: Glucagon-like peptide 2 improves nutrient absorption and nutritional status in short-bowel patients with no colon. *Gastroenterology* 120:806-815, 2001
30. Martin,GR, Beck,PL, Sigalet,DL: Gut hormones, and short bowel syndrome: the enigmatic role of glucagon-like peptide-2 in the regulation of intestinal adaptation. *World J Gastroenterol* 12:4117-4129, 2006
31. Hare,KJ, Hartmann,B, Kissow,H, Holst,JJ, Poulsen,SS: The intestinotrophic peptide, glp-2, counteracts intestinal atrophy in mice induced by the epidermal growth factor receptor inhibitor, gefitinib. *Clin Cancer Res* 13:5170-5175, 2007
32. Henriksen,DB, Alexandersen,P, Bjarnason,NH, Vilsboll,T, Hartmann,B, Henriksen,EE, Byrjalsen,I, Krarup,T, Holst,JJ, Christiansen,C: Role of gastrointestinal hormones in postprandial reduction of bone resorption. *J Bone Miner Res* 18:2180-2189, 2003
33. Henriksen,DB, Alexandersen,P, Hartmann,B, Adrian,CL, Byrjalsen,I, Bone,HG, Holst,JJ, Christiansen,C: Disassociation of bone resorption and formation by GLP-2: a 14-day study in healthy postmenopausal women. *Bone* 40:723-729, 2007
34. Wynne,K, Park,AJ, Small,CJ, Patterson,M, Ellis,SM, Murphy,KG, Wren,AM, Frost,GS, Meeran,K, Ghatei,MA, Bloom,SR: Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes* 54:2390-2395, 2005
35. Wynne,K, Park,AJ, Small,CJ, Meeran,K, Ghatei,MA, Frost,GS, Bloom,SR: Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes (Lond)* 30:1729-1736, 2006
36. Alford,FP, Bloom,SR, Nabarro,JD, Hall,R, Besser,GM, Coy,DH, Kastin,AJ, Schally,AV: Glucagon control of fasting glucose in man. *Lancet* 2:974-977, 1974

37. Cherrington,AD, Chiasson,JL, Liljenquist,JE, Lacy,WW, Park,CR: Control of hepatic glucose output by glucagon and insulin in the intact dog. *Biochem Soc Symp*31-45, 1978
38. Gromada,J, Franklin,I, Wollheim,CB: Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr Rev* 28:84-116, 2007
39. Jelinek,LJ, Lok,S, Rosenberg,GB, Smith,RA, Grant,FJ, Biggs,S, Bensch,PA, Kuijper,JL, Sheppard,PO, Sprecher,CA, .: Expression cloning and signaling properties of the rat glucagon receptor. *Science* 259:1614-1616, 1993
40. Jiang,G, Zhang,BB: Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab* 284:E671-E678, 2003
41. Consoli,A, Nurjhan,N, Capani,F, Gerich,J: Predominant role of gluconeogenesis in increased hepatic glucose production in NIDDM. *Diabetes* 38:550-557, 1989
42. Gastaldelli,A, Miyazaki,Y, Pettiti,M, Buzzigoli,E, Mahankali,S, Ferrannini,E, DeFronzo,RA: Separate contribution of diabetes, total fat mass, and fat topography to glucose production, gluconeogenesis, and glycogenolysis. *J Clin Endocrinol Metab* 89:3914-3921, 2004
43. Lefebvre,PJ: The intriguing diversity of the glucagon gene products. *Curr Diab Rep* 2:201-202, 2002
44. Ali,S, Drucker,DJ: Benefits and limitations of reducing glucagon action for the treatment of type 2 diabetes. *Am J Physiol Endocrinol Metab* 296:E415-E421, 2009
45. Hansen,LH, Abrahamsen,N, Nishimura,E: Glucagon receptor mRNA distribution in rat tissues. *Peptides* 16:1163-1166, 1995
46. Kieffer,TJ, Heller,RS, Unson,CG, Weir,GC, Habener,JF: Distribution of glucagon receptors on hormone-specific endocrine cells of rat pancreatic islets. *Endocrinology* 137:5119-5125, 1996
47. Moens,K, Heimberg,H, Flamez,D, Huypens,P, Quartier,E, Ling,Z, Pipeleers,D, Gremlich,S, Thorens,B, Schuit,F: Expression and functional activity of glucagon, glucagon-like peptide I, and glucose-dependent insulinotropic peptide receptors in rat pancreatic islet cells. *Diabetes* 45:257-261, 1996
48. Moens,K, Flamez,D, Van,SC, Ling,Z, Pipeleers,D, Schuit,F: Dual glucagon recognition by pancreatic beta-cells via glucagon and glucagon-like peptide 1 receptors. *Diabetes* 47:66-72, 1998
49. Ma,X, Zhang,Y, Gromada,J, Sewing,S, Berggren,PO, Buschard,K, Salehi,A, Vikman,J, Rorsman,P, Eliasson,L: Glucagon stimulates exocytosis in mouse and rat pancreatic alpha-cells by binding to glucagon receptors. *Mol Endocrinol* 19:198-212, 2005
50. Hope,KM, Tran,PO, Zhou,H, Oseid,E, Leroy,E, Robertson,RP: Regulation of alpha-cell function by the beta-cell in isolated human and rat islets deprived of glucose: the "switch-off" hypothesis. *Diabetes* 53:1488-1495, 2004
51. Samols,E, Stagner,JI, Ewart,RB, Marks,V: The order of islet microvascular cellular perfusion is B----A----D in the perfused rat pancreas. *J Clin Invest* 82:350-353, 1988
52. Raju,B, Cryer,PE: Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin-deficient diabetes: documentation of the intraislet insulin hypothesis in humans. *Diabetes* 54:757-764, 2005
53. Hare,KJ, Knop,FK, Asmar,M, Larsen,S, Madsbad,S, Holst,JJ, Vilsboll,T: Preserved Inhibitory Effect of Glucagon-Like Peptide-1 (GLP-1) on Glucagon Secretion in Type 2 Diabetes Mellitus. *Diabetes* 58:A372, 2009
54. Asmar,M, Knop,FK, Bache,M, Vilsboll,T, Madsbad,S, Holst,JJ: Human amylin, pramlintide and GLP-1: Influence on gastric emptying, appetite and food intake in humans. *Diabetes* 57:A406-A407, 2008
55. Ishihara,H, Maechler,P, Gjinovci,A, Herrera,PL, Wollheim,CB: Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nat Cell Biol* 5:330-335, 2003
56. Luft,R, Efendic,S, Hokfelt,T: Somatostatin--both hormone and neurotransmitter? *Diabetologia* 14:1-13, 1978
57. Sakurai,H, Dobbs,R, Unger,RH: Somatostatin-induced changes in insulin and glucagon secretion in normal and diabetic dogs. *J Clin Invest* 54:1395-1402, 1974
58. Ludvigsen,E, Olsson,R, Stridsberg,M, Janson,ET, Sandler,S: Expression and distribution of somatostatin receptor subtypes in the pancreatic islets of mice and rats. *J Histochem Cytochem* 52:391-400, 2004
59. Portela-Gomes,GM, Stridsberg,M, Grimelius,L, Oberg,K, Janson,ET: Expression of the five different somatostatin receptor subtypes in endocrine cells of the pancreas. *Appl Immunohistochem Mol Morphol* 8:126-132, 2000
60. de,HJ, Rasmussen,C, Coy,DH, Holst,JJ: Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. *Diabetologia* 51:2263-2270, 2008
61. Rossowski,WJ, Coy,DH: Specific inhibition of rat pancreatic insulin or glucagon release by receptor-selective somatostatin analogs. *Biochem Biophys Res Commun* 205:341-346, 1994
62. Charollais,A, Gjinovci,A, Huarte,J, Bauquis,J, Nadal,A, Martin,F, Andreu,E, Sanchez-Andres,JV, Calabrese,A, Bosco,D, Soria,B, Wollheim,CB, Herrera,PL, Meda,P: Junctional communication of pancreatic beta cells contributes to the control of insulin secretion and glucose tolerance. *J Clin Invest* 106:235-243, 2000
63. Kleinman,R, Gingerich,R, Ohning,G, Wong,H, Olthoff,K, Walsh,J, Brunicardi,FC: The influence of somatostatin on glucagon and pancreatic polypeptide secretion in the isolated perfused human pancreas. *Int J Pancreatol* 18:51-57, 1995
64. Brissova,M, Fowler,MJ, Nicholson,WE, Chu,A, Hirshberg,B, Harlan,DM, Powers,AC: Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* 53:1087-1097, 2005
65. Cabrera,O, Berman,DM, Kenyon,NS, Ricordi,C, Berggren,PO, Caicedo,A: The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc Natl Acad Sci U S A* 103:2334-2339, 2006
66. Kharouta,M, Miller,K, Kim,A, Wojcik,P, Kilimnik,G, Dey,A, Steiner,DF, Hara,M: No mantle formation in rodent islets -- the prototype of islet revisited. *Diabetes Res Clin Pract* 85:252-257, 2009
67. Brelje,TC, Scharp,DW, Sorenson,RL: Three-dimensional imaging of intact isolated islets of Langerhans with confocal microscopy. *Diabetes* 38:808-814, 1989

68. Weir,GC, Bonner-Weir,S: Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. *J Clin Invest* 85:983-987, 1990
69. Samols,E, Bonner-Weir,S, Weir,GC: Intra-islet insulin-glucagon-somatostatin relationships. *Clin Endocrinol Metab* 15:33-58, 1986
70. Creutzfeldt,WO, Kleine,N, Willms,B, Orskov,C, Holst,JJ, Nauck,MA: Glucagonostatic actions and reduction of fasting hyperglycemia by exogenous glucagon-like peptide I(7-36) amide in type I diabetic patients. *Diabetes Care* 19:580-586, 1996
71. Pederson,RA, Brown,JC: Interaction of gastric inhibitory polypeptide, glucose, and arginine on insulin and glucagon secretion from the perfused rat pancreas. *Endocrinology* 103:610-615, 1978
72. Creutzfeldt,W, Nauck,M: Gut hormones and diabetes mellitus. *Diabetes Metab Rev* 8:149-177, 1992
73. Meier,JJ, Hucking,K, Holst,JJ, Deacon,CF, Schmiegel,WH, Nauck,MA: Reduced insulinotropic effect of gastric inhibitory polypeptide in first-degree relatives of patients with type 2 diabetes. *Diabetes* 50:2497-2504, 2001
74. Christensen,M, Knop,FK, Vilsboll,T, Aaboe,K, Holst,JJ, Krarup,T, Madsbad,S: Glucagon-Like Peptide-2, but Not Glucose-Dependent Insulinotropic Polypeptide, Stimulates Glucagon Release in Patients with Type 1 Diabetes Mellitus. *Diabetes* 58:A368, 2009
75. Dupre,J, Caussignac,Y, McDonald,TJ, Van,VS: Stimulation of glucagon secretion by gastric inhibitory polypeptide in patients with hepatic cirrhosis and hyperglucagonemia. *J Clin Endocrinol Metab* 72:125-129, 1991
76. Meier,JJ, Gallwitz,B, Siepmann,N, Holst,JJ, Deacon,CF, Schmidt,WE, Nauck,MA: Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycaemia. *Diabetologia* 46:798-801, 2003
77. Sorensen,LB, Flint,A, Raben,A, Hartmann,B, Holst,JJ, Astrup,A: No effect of physiological concentrations of glucagon-like peptide-2 on appetite and energy intake in normal weight subjects. *Int J Obes Relat Metab Disord* 27:450-456, 2003
78. Meier,JJ, Nauck,MA, Pott,A, Heinze,K, Goetze,O, Bulut,K, Schmidt,WE, Gallwitz,B, Holst,JJ: Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans. *Gastroenterology* 130:44-54, 2006
79. de,HJ, Pedersen,J, Orskov,C, Holst,JJ: The alpha cell expresses glucagon-like peptide-2 receptors and glucagon-like peptide-2 stimulates glucagon secretion from the rat pancreas. *Diabetologia* 50:2135-2142, 2007
80. Schmidt,WE, Siegel,EG, Creutzfeldt,W: Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. *Diabetologia* 28:704-707, 1985
81. Unger,RH, Orci,L: Glucagon and the A cell: physiology and pathophysiology (second of two parts). *N Engl J Med* 304:1575-1580, 1981
82. Unger,RH, Orci,L: Glucagon and the A cell: physiology and pathophysiology (first two parts). *N Engl J Med* 304:1518-1524, 1981
83. Nauck,MA, Meier,JJ, Wolfersdorff,AV, Tillil,H, Creutzfeldt,W, Kobberling,J: A 25-year follow-up study of glucose tolerance in first-degree relatives of type 2 diabetic patients: association of impaired or diabetic glucose tolerance with other components of the metabolic syndrome. *Acta Diabetol* 40:163-172, 2003
84. Meier,JJ, Deacon,CF, Schmidt,WE, Holst,JJ, Nauck,MA: Suppression of glucagon secretion is lower after oral glucose administration than during intravenous glucose administration in human subjects. *Diabetologia* 50:806-813, 2007
85. Hojberg,PV, Vilsboll,T, Rabol,R, Knop,FK, Bache,M, Krarup,T, Holst,JJ, Madsbad,S: Four weeks of near-normalisation of blood glucose improves the insulin response to glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetologia* 52:199-207, 2009
86. Baron,AD, Schaeffer,L, Shragg,P, Kolterman,OG: Role of hyperglucagonemia in maintenance of increased rates of hepatic glucose output in type II diabetics. *Diabetes* 36:274-283, 1987
87. Basu,R, Schwenk,WF, Rizza,RA: Both fasting glucose production and disappearance are abnormal in people with "mild" and "severe" type 2 diabetes. *Am J Physiol Endocrinol Metab* 287:E55-E62, 2004
88. Hojberg,PV, Zander,M, Vilsboll,T, Knop,FK, Krarup,T, Volund,A, Holst,JJ, Madsbad,S: Near normalisation of blood glucose improves the potentiating effect of GLP-1 on glucose-induced insulin secretion in patients with type 2 diabetes. *Diabetologia* 51:632-640, 2008
89. Raskin,P, Aydin,I, Yamamoto,T, Unger,RH: Abnormal alpha cell function in human diabetes: the response to oral protein. *Am J Med* 64:988-997, 1978
90. Toft-Nielsen,MB, Damholt,MB, Madsbad,S, Hilsted,LM, Hughes,TE, Michelsen,BK, Holst,JJ: Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *J Clin Endocrinol Metab* 86:3717-3723, 2001
91. Ohneda,A, Watanabe,K, Horigome,K, Sakai,T, Kai,Y, Oikawa,S: Abnormal response of pancreatic glucagon to glycemic changes in diabetes mellitus. *J Clin Endocrinol Metab* 46:504-510, 1978
92. Unger,RH, guilar-Parada,E, Muller,WA, Eisentraut,AM: Studies of pancreatic alpha cell function in normal and diabetic subjects. *J Clin Invest* 49:837-848, 1970
93. Woerle,HJ, Szoke,E, Meyer,C, Dostou,JM, Wittlin,SD, Gosmanov,NR, Welle,SL, Gerich,JE: Mechanisms for abnormal postprandial glucose metabolism in type 2 diabetes. *Am J Physiol Endocrinol Metab* 290:E67-E77, 2006
94. Gerich,JE, Charles,MA, Grodsky,GM: Regulation of pancreatic insulin and glucagon secretion. *Annu Rev Physiol* 38:353-388, 1976
95. Stagner,JI, Samols,E: The vascular order of islet cellular perfusion in the human pancreas. *Diabetes* 41:93-97, 1992
96. Hamaguchi,T, Fukushima,H, Uehara,M, Wada,S, Shirotani,T, Kishikawa,H, Ichinose,K, Yamaguchi,K, Shichiri,M: Abnormal glucagon response to arginine and its normalization in obese hyperinsulinaemic patients with glucose intolerance: importance of insulin action on pancreatic alpha cells. *Diabetologia* 34:801-806, 1991
97. Ward,WK, Bolgiano,DC, McKnight,B, Halter,JB, Porte,D, Jr.: Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. *J Clin Invest* 74:1318-1328, 1984

98. Segel,SA, Paramore,DS, Cryer,PE: Hypoglycemia-associated autonomic failure in advanced type 2 diabetes. *Diabetes* 51:724-733, 2002
99. Cryer,PE: Diverse causes of hypoglycemia-associated autonomic failure in diabetes. *N Engl J Med* 350:2272-2279, 2004
100. Bolli,GB, De,FP, De,CS, Perriello,G, Ventura,MM, Benedetti,MM, Santeusanio,F, Gerich,JE, Brunetti,P: A reliable and reproducible test for adequate glucose counterregulation in type I diabetes mellitus. *Diabetes* 33:732-737, 1984
101. Gerich,JE, Langlois,M, Noacco,C, Karam,JH, Forsham,PH: Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science* 182:171-173, 1973
102. Ahren,B, Holst,JJ: The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes* 50:1030-1038, 2001
103. Dube,PE, Brubaker,PL: Nutrient, neural and endocrine control of glucagon-like peptide secretion. *Horm Metab Res* 36:755-760, 2004
104. Eissele,R, Goke,R, Willemer,S, Harthus,HP, Vermeer,H, Arnold,R, Goke,B: Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *Eur J Clin Invest* 22:283-291, 1992
105. Drucker,DJ, Jin,T, Asa,SL, Young,TA, Brubaker,PL: Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. *Mol Endocrinol* 8:1646-1655, 1994
106. Herrmann,C, Goke,R, Richter,G, Fehmann,HC, Arnold,R, Goke,B: Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. *Digestion* 56:117-126, 1995
107. Gribble,FM, Williams,L, Simpson,AK, Reimann,F: A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line. *Diabetes* 52:1147-1154, 2003
108. Rozengurt,E, Sternini,C: Taste receptor signaling in the mammalian gut. *Curr Opin Pharmacol* 7:557-562, 2007
109. Tolhurst,G, Reimann,F, Gribble,FM: Nutritional regulation of glucagon-like peptide-1 secretion. *J Physiol* 587:27-32, 2009
110. Reimann,F, Williams,L, da,S, X, Rutter,GA, Gribble,FM: Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells. *Diabetologia* 47:1592-1601, 2004
111. Abello,J, Ye,F, Bosshard,A, Bernard,C, Cuber,JC, Chayvialle,JA: Stimulation of glucagon-like peptide-1 secretion by muscarinic agonist in a murine intestinal endocrine cell line. *Endocrinology* 134:2011-2017, 1994
112. Balks,HJ, Holst,JJ, von zur,MA, Brabant,G: Rapid oscillations in plasma glucagon-like peptide-1 (GLP-1) in humans: cholinergic control of GLP-1 secretion via muscarinic receptors. *J Clin Endocrinol Metab* 82:786-790, 1997
113. Herrmann-Rinke,C, Voge,A, Hess,M, Goke,B: Regulation of glucagon-like peptide-1 secretion from rat ileum by neurotransmitters and peptides. *J Endocrinol* 147:25-31, 1995
114. Edfalk,S, Steneberg,P, Edlund,H: Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. *Diabetes* 57:2280-2287, 2008
115. Hirasawa,A, Tsumaya,K, Awaji,T, Katsuma,S, Adachi,T, Yamada,M, Sugimoto,Y, Miyazaki,S, Tsujimoto,G: Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 11:90-94, 2005
116. Thomas,C, Gioiello,A, Noriega,L, Strehle,A, Oury,J, Rizzo,G, Macchiarulo,A, Yamamoto,H, Mataki,C, Pruzanski,M, Pellicciari,R, Auwerx,J, Schoonjans,K: TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab* 10:167-177, 2009
117. Deacon,CF, Nauck,MA, Toft-Nielsen,M, Pridal,L, Willms,B, Holst,JJ: Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. *Diabetes* 44:1126-1131, 1995
118. Mentlein,R: Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul Pept* 85:9-24, 1999
119. Deacon,CF, Johnsen,AH, Holst,JJ: Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J Clin Endocrinol Metab* 80:952-957, 1995
120. Gefel,D, Hendrick,GK, Mojsov,S, Habener,J, Weir,GC: Glucagon-like peptide-I analogs: effects on insulin secretion and adenosine 3',5'-monophosphate formation. *Endocrinology* 126:2164-2168, 1990
121. Deacon,CF, Pridal,L, Klarskov,L, Olesen,M, Holst,JJ: Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig. *Am J Physiol* 271:E458-E464, 1996
122. Hansen,L, Deacon,CF, Orskov,C, Holst,JJ: Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* 140:5356-5363, 1999
123. Gromada,J, Holst,JJ, Rorsman,P: Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. *Pflugers Arch* 435:583-594, 1998
124. Holz,GG: Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell. *Diabetes* 53:5-13, 2004
125. Ding,WG, Renstrom,E, Rorsman,P, Buschard,K, Gromada,J: Glucagon-like peptide I and glucose-dependent insulinotropic polypeptide stimulate Ca2+-induced secretion in rat alpha-cells by a protein kinase A-mediated mechanism. *Diabetes* 46:792-800, 1997
126. Drucker,DJ, Philippe,J, Mojsov,S, Chick,WL, Habener,JF: Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci U S A* 84:3434-3438, 1987
127. Nauck,MA, Homberger,E, Siegel,EG, Allen,RC, Eaton,RP, Ebert,R, Creutzfeldt,W: Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. *J Clin Endocrinol Metab* 63:492-498, 1986
128. Hvidberg,A, Nielsen,MT, Hilsted,J, Orskov,C, Holst,JJ: Effect of glucagon-like peptide-1 (proglucagon 78-107amide) on hepatic glucose production in healthy man. *Metabolism* 43:104-108, 1994

129. Holst,JJ: On the physiology of GIP and GLP-1. *Horm Metab Res* 36:747-754, 2004
130. Hare,KJ, Knop,FK, Asmar,M, Madsbad,S, Deacon,CF, Holst,JJ, Vilsboll,T: Preserved Inhibitory Potency of GLP-1 on Glucagon Secretion in Type 2 Diabetes Mellitus. *J Clin Endocrinol Metab* 2009
131. Farilla,L, Bulotta,A, Hirshberg,B, Li,CS, Khoury,N, Noushmehr,H, Bertolotto,C, Di,MU, Harlan,DM, Perfetti,R: Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology* 144:5149-5158, 2003
132. Nauck,MA, Niedereichholz,U, Ettler,R, Holst,JJ, Orskov,C, Ritzel,R, Schmiegel,WH: Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. *Am J Physiol* 273:E981-E988, 1997
133. Wettergren,A, Schjoldager,B, Mortensen,PE, Myhre,J, Christiansen,J, Holst,JJ: Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man. *Dig Dis Sci* 38:665-673, 1993
134. Naslund,E, Barkeling,B, King,N, Gutniak,M, Blundell,JE, Holst,JJ, Rossner,S, Hellstrom,PM: Energy intake and appetite are suppressed by glucagon-like peptide-1 (GLP-1) in obese men. *Int J Obes Relat Metab Disord* 23:304-311, 1999
135. Verdich,C, Flint,A, Gutzwiller,JP, Naslund,E, Beglinger,C, Hellstrom,PM, Long,SJ, Morgan,LM, Holst,JJ, Astrup,A: A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. *J Clin Endocrinol Metab* 86:4382-4389, 2001
136. Holst,JJ: The physiology of glucagon-like peptide 1. *Physiol Rev* 87:1409-1439, 2007
137. Bullock,BP, Heller,RS, Habener,JF: Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. *Endocrinology* 137:2968-2978, 1996
138. Ban,K, Noyan-Ashraf,MH, Hoefer,J, Bolz,SS, Drucker,DJ, Husain,M: Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. *Circulation* 117:2340-2350, 2008
139. Sonne,DP, Engstrom,T, Treiman,M: Protective effects of GLP-1 analogues exendin-4 and GLP-1(9-36) amide against ischemia-reperfusion injury in rat heart. *Regul Pept* 146:243-249, 2008
140. Sokos,GG, Nikolaidis,LA, Mankad,S, Elahi,D, Shannon,RP: Glucagon-like peptide-1 infusion improves left ventricular ejection fraction and functional status in patients with chronic heart failure. *J Card Fail* 12:694-699, 2006
141. Sokos,GG, Bolukoglu,H, German,J, Hentosz,T, Magovern,GJ, Jr., Maher,TD, Dean,DA, Bailey,SH, Marrone,G, Benckart,DH, Elahi,D, Shannon,RP: Effect of glucagon-like peptide-1 (GLP-1) on glycemic control and left ventricular function in patients undergoing coronary artery bypass grafting. *Am J Cardiol* 100:824-829, 2007
142. Nystrom,T, Gutniak,MK, Zhang,Q, Zhang,F, Holst,JJ, Ahren,B, Sjoholm,A: Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease. *Am J Physiol Endocrinol Metab* 287:E1209-E1215, 2004
143. Klonoff,DC, Buse,JB, Nielsen,LL, Guan,X, Bowlus,CL, Holcombe,JH, Wintle,ME, Maggs,DG: Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. *Curr Med Res Opin* 24:275-286, 2008
144. Vilsboll,T, Zdravkovic,M, Le-Thi,T, Krarup,T, Schmitz,O, Courreges,JP, Verhoeven,R, Buganova,I, Madsbad,S: Liraglutide, a long-acting human glucagon-like peptide-1 analog, given as monotherapy significantly improves glycemic control and lowers body weight without risk of hypoglycemia in patients with type 2 diabetes. *Diabetes Care* 30:1608-1610, 2007
145. Basu,A, Charkoudian,N, Schrage,W, Rizza,RA, Basu,R, Joyner,MJ: Beneficial effects of GLP-1 on endothelial function in humans: dampening by glyburide but not by glimepiride. *Am J Physiol Endocrinol Metab* 293:E1289-E1295, 2007
146. Muscelli,E, Mari,A, Casolaro,A, Camastra,S, Seghieri,G, Gastaldelli,A, Holst,JJ, Ferrannini,E: Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 57:1340-1348, 2008
147. Vilsboll,T, Kosinski,M, Holst,JJ, Cypryk,K, Knop,F: The reduced incretin effect in diabetes mellitus is completely reversible. *Diabetes* 57:A411-A412, 2008
148. Hansen,KB, Vilsboll,T, Holst,JJ, Knop,FK: Impaired Incretin Effect Can Be Induced in Healthy Normal Glucose Tolerant Subject. *Diabetes* 58:A378, 2009
149. Jensen,DH, Aaboe,K, Henriksen,JE, Madsbad,S, Krarup,T: Reduced incretin effect in dexamethasone-induced insulin resistance and glucose intolerance in diabetic offspring. *Diabetologia* 52:S227-S228, 2009
150. Vilsboll,T, Agerso,H, Krarup,T, Holst,JJ: Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects. *J Clin Endocrinol Metab* 88:220-224, 2003
151. Vaag,AA, Holst,JJ, Volund,A, Beck-Nielsen,HB: Gut incretin hormones in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM)--evidence for decreased glucagon-like peptide 1 secretion during oral glucose ingestion in NIDDM twins. *Eur J Endocrinol* 135:425-432, 1996
152. Kjems,LL, Holst,JJ, Volund,A, Madsbad,S: The influence of GLP-1 on glucose-stimulated insulin secretion: effects on beta-cell sensitivity in type 2 and nondiabetic subjects. *Diabetes* 52:380-386, 2003
153. Nauck,MA, Heimesaat,MM, Orskov,C, Holst,JJ, Ebert,R, Creutzfeldt,W: Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 91:301-307, 1993
154. Meier,JJ: The contribution of incretin hormones to the pathogenesis of type 2 diabetes. *Best Pract Res Clin Endocrinol Metab* 23:433-441, 2009
155. Degn,KB, Brock,B, Juhl,CB, Djurhuus,CB, Grubert,J, Kim,D, Han,J, Taylor,K, Fineman,M, Schmitz,O: Effect of intravenous infusion of exenatide (synthetic exendin-4) on glucose-dependent insulin secretion and counterregulation during hypoglycemia. *Diabetes* 53:2397-2403, 2004

156. Dunning,BE, Foley,JE, Ahren,B: Alpha cell function in health and disease: influence of glucagon-like peptide-1. *Diabetologia* 48:1700-1713, 2005
157. Vilsboll,T, Krarup,T, Madsbad,S, Holst,JJ: Both GLP-1 and GIP are insulinotropic at basal and postprandial glucose levels and contribute nearly equally to the incretin effect of a meal in healthy subjects. *Regul Pept* 114:115-121, 2003
158. Gutniak,M, Orskov,C, Holst,JJ, Ahren,B, Efendic,S: Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus. *N Engl J Med* 326:1316-1322, 1992
159. Kielgast,U, Madsbad,S, Holst,J: Impact of endogenous and exogenous GLP-1 on glucagon secretion in type 1 diabetic patients with and without residual beta -cell function. *Diabetologia* 52:S58-S59, 2009
160. Gutniak,MK, Linde,B, Holst,JJ, Efendic,S: Subcutaneous injection of the incretin hormone glucagon-like peptide 1 abolishes postprandial glycemia in NIDDM. *Diabetes Care* 17:1039-1044, 1994
161. Gutniak,MK, Larsson,H, Sanders,SW, Juneskans,O, Holst,JJ, Ahren,B: GLP-1 tablet in type 2 diabetes in fasting and postprandial conditions. *Diabetes Care* 20:1874-1879, 1997
162. Meier,JJ, Gallwitz,B, Salmen,S, Goetze,O, Holst,JJ, Schmidt,WE, Nauck,MA: Normalization of glucose concentrations and deceleration of gastric emptying after solid meals during intravenous glucagon-like peptide 1 in patients with type 2 diabetes. *J Clin Endocrinol Metab* 88:2719-2725, 2003
163. Behme,MT, Dupre,J, McDonald,TJ: Glucagon-like peptide 1 improved glycemic control in type 1 diabetes. *BMC Endocr Disord* 3:3, 2003
164. Ahren,B, Larsson,H, Holst,JJ: Effects of glucagon-like peptide-1 on islet function and insulin sensitivity in non-insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 82:473-478, 1997
165. Zander,M, Madsbad,S, Madsen,JL, Holst,JJ: Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* 359:824-830, 2002
166. Degn,KB, Juhl,CB, Sturis,J, Jakobsen,G, Brock,B, Chandramouli,V, Rungby,J, Landau,BR, Schmitz,O: One week's treatment with the long-acting glucagon-like peptide 1 derivative liraglutide (NN2211) markedly improves 24-h glycemia and alpha- and beta-cell function and reduces endogenous glucose release in patients with type 2 diabetes. *Diabetes* 53:1187-1194, 2004
167. Ahren,B, Landin-Olsson,M, Jansson,PA, Svensson,M, Holmes,D, Schweizer,A: Inhibition of dipeptidyl peptidase-4 reduces glycemia, sustains insulin levels, and reduces glucagon levels in type 2 diabetes. *J Clin Endocrinol Metab* 89:2078-2084, 2004
168. Foley,JE, Ligueros-Saylan,M, He,YL, Holst,JJ, Deacon,CF, Dunning,BE, Leone-Jones,A, Yu,T, Kelley,DE: Effect of vildagliptin on glucagon concentration during meals in patients with type 1 diabetes. *Horm Metab Res* 40:727-730, 2008
169. Heller,RS, Kieffer,TJ, Habener,JF: Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas. *Diabetes* 46:785-791, 1997
170. Tornehave,D, Kristensen,P, Romer,J, Knudsen,LB, Heller,RS: Expression of the GLP-1 receptor in mouse, rat, and human pancreas. *J Histochem Cytochem* 56:841-851, 2008
171. Fehmann,HC, Habener,JF: Functional receptors for the insulinotropic hormone glucagon-like peptide-I(7-37) on a somatostatin secreting cell line. *FEBS Lett* 279:335-340, 1991
172. Ahren,B, Lundquist,I: Glucagon immunoreactivity in plasma from normal and dystrophic mice. *Diabetologia* 22:258-263, 1982
173. Baldissera,FG, Holst,JJ: Glicentin 1-61 probably represents a major fraction of glucagon-related peptides in plasma of anaesthetized uraemic pigs. *Diabetologia* 29:462-467, 1986
174. Holst,JJ: Evidence that glicentin contains the entire sequence of glucagon. *Biochem J* 187:337-343, 1980
175. Holst,JJ: Evidence that enteroglucagon (II) is identical with the C-terminal sequence (residues 33-69) of glicentin. *Biochem J* 207:381-388, 1982
176. Orskov,C, Jeppesen,J, Madsbad,S, Holst,JJ: Proglucagon products in plasma of noninsulin-dependent diabetics and nondiabetic controls in the fasting state and after oral glucose and intravenous arginine. *J Clin Invest* 87:415-423, 1991
177. Holst,JJ: Extraction, gel filtration pattern, and receptor binding of porcine gastrointestinal glucagon-like immunoreactivity. *Diabetologia* 13:159-169, 1977
178. Hare,KJ, Knop,FK, Torang,S, Madsbad,S, Krarup,T, Vilsboll,T, Holst,JJ: Hyperglucagonemia in patients with type 2 diabetes mellitus - Molecular analysis of postprandial glucagon responses. *Diabetes* 57:A407, 2008
179. Vilsboll,T, Krarup,T, Sonne,J, Madsbad,S, Volund,A, Juul,AG, Holst,JJ: Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. *J Clin Endocrinol Metab* 88:2706-2713, 2003
180. Buse,JB, Rosenstock,J, Sesti,G, Schmidt,WE, Montanya,E, Brett,JH, Zychma,M, Blonde,L: Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). *Lancet* 374:39-47, 2009
181. Drucker,DJ, Buse,JB, Taylor,K, Kendall,DM, Trautmann,M, Zhuang,D, Porter,L: Exenatide once weekly versus twice daily for the treatment of type 2 diabetes: a randomised, open-label, non-inferiority study. *Lancet* 372:1240-1250, 2008
182. Shah,P, Vella,A, Basu,A, Basu,R, Schwenk,WF, Rizza,RA: Lack of suppression of glucagon contributes to postprandial hyperglycemia in subjects with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 85:4053-4059, 2000
183. Ritzel,R, Orskov,C, Holst,JJ, Nauck,MA: Pharmacokinetic, insulinotropic, and glucagonostatic properties of GLP-1 [7-36 amide] after subcutaneous injection in healthy volunteers. Dose-response-relationships. *Diabetologia* 38:720-725, 1995
184. Holst,JJ, Burcharth,F, Kuhl,C: Pancreatic glucoregulatory hormones in cirrhosis of the liver: portal vein concentrations during intravenous glucose tolerance test and in response to a meal. *Diabete Metab* 6:117-127, 1980
185. Orskov,L, Holst,JJ, Moller,J, Orskov,C, Moller,N, Alberti,KG, Schmitz,O: GLP-1 does not not acutely affect

insulin sensitivity in healthy man. *Diabetologia* 39:1227-1232, 1996

186. Greenbaum,CJ, Prigeon,RL, D'Alessio,DA: Impaired beta-cell function, incretin effect, and glucagon suppression in patients with type 1 diabetes who have normal fasting glucose. *Diabetes* 51:951-957, 2002
187. Porksen,S, Nielsen,LB, Kaas,A, Kocova,M, Chiarelli,F, Orskov,C, Holst,JJ, Ploug,KB, Hougaard,P, Hansen,L, Mortensen,HB: Meal-stimulated glucagon release is associated with postprandial blood glucose level and does not interfere with glycemic control in children and adolescents with new-onset type 1 diabetes. *J Clin Endocrinol Metab* 92:2910-2916, 2007
188. King,H, Aubert,RE, Herman,WH: Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 21:1414-1431, 1998
189. Vilsboll,T, Holst,JJ, Knop,FK: The spectrum of antidiabetic actions of GLP-1 in patients with diabetes. *Best Pract Res Clin Endocrinol Metab* 23:453-462, 2009
190. Kodra,JT, Jorgensen,AS, Andersen,B, Behrens,C, Brand,CL, Christensen,IT, Guldbrandt,M, Jeppesen,CB, Knudsen,LB, Madsen,P, Nishimura,E, Sams,C, Sidelmann,UG, Pedersen,RA, Lynn,FC, Lau,J: Novel glucagon receptor antagonists with improved selectivity over the glucose-dependent insulinotropic polypeptide receptor. *J Med Chem* 51:5387-5396, 2008
191. Madsen,P, Kodra,JT, Behrens,C, Nishimura,E, Jeppesen,CB, Pridal,L, Andersen,B, Knudsen,LB, Valcarce-Aspegren,C, Guldbrandt,M, Christensen,IT, Jorgensen,AS, Ynddal,L, Brand,CL, Bagger,MA, Lau,J: Human glucagon receptor antagonists with thiazole cores. A novel series with superior pharmacokinetic properties. *J Med Chem* 52:2989-3000, 2009
192. Gilbert,MP, Pratley,RE: Efficacy and safety of incretin-based therapies in patients with type 2 diabetes mellitus. *Eur J Intern Med* 20 Suppl 2:S309-S318, 2009
193. Mannucci,E, Tesi,F, Bardini,G, Ognibene,A, Petracca,MG, Ciani,S, Pezzatini,A, Brogi,M, Dicembrini,I, Cremasco,F, Messeri,G, Rotella,CM: Effects of metformin on glucagon-like peptide-1 levels in obese patients with and without Type 2 diabetes. *Diabetes Nutr Metab* 17:336-342, 2004
194. Migoya,EM, Miller,J, Larson,P, Tanen,M, Hilliard,D, Deacon,C, Gutierrez,M, Stoch,A, Herman,GA, Stein,PP, Holst,JJ, Wagner,JA: Sitagliptin, a selective DPP-4 inhibitor, and metformin have complementary effects to increase active GLP-1 concentrations. *Diabetes* 56:A74, 2007
195. Holst,JJ, Pedersen,JH, Baldissera,F, Stadil,F: Circulating glucagon after total pancreatectomy in man. *Diabetologia* 25:396-399, 1983
196. Jackson,RS, Creemers,JW, Farooqi,IS, Raffin-Sanson,ML, Varro,A, Dockray,GJ, Holst,JJ, Brubaker,PL, Corvol,P, Polonsky,KS, Ostrega,D, Becker,KL, Bertagna,X, Hutton,JC, White,A, Dattani,MT, Hussain,K, Middleton,SJ, Nicole,TM, Milla,PJ, Lindley,KJ, O'Rahilly,S: Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *J Clin Invest* 112:1550-1560, 2003
197. Ugleholdt,R, Zhu,X, Deacon,CF, Orskov,C, Steiner,DF, Holst,JJ: Impaired intestinal proglucagon processing in mice lacking prohormone convertase 1. *Endocrinology* 145:1349-1355, 2004