New physiological effects of the incretin hormones GLP-1 and GIP

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2. Asmar M, Tangaa W, Madsbad S, Hare KJ, Astrup A, Flint A, Bülow J and Holst JJ: On the role of glucose-dependent insulinotropic polypeptide, GIP, in postprandial metabolism in humans. Am J Physiol Endocrinol Metab 2010 Mar; 298(3):E614-21

3. Asmar M, Simonsen L, Madsbad S, Stallknecht B, Holst JJ and Bülow J: GIP enhances fatty acid re-esterification in subcutaneous, abdominal adipose tissue in lean humans. Diabetes 2010, Sep;59(9):2160-3.

INTRODUCTION

The global obesity epidemic resulting from a combination of genetic susceptibility, increased availability of high-energy foods and decreased requirement for physical activity in modern society. Obesity and excess weight are major risk factors for chronic diseases, including type 2 diabetes (1), cardiovascular diseases (2), hypertension and stroke (3) , and certain forms of cancer (4-6). The health consequences range from increased risk of premature death, to serious chronic conditions that reduce the overall quality of life. Importantly, body weight reduction in the range of 10% is associated with significant improvements in a wide range of co-morbid conditions. Unfortunately, lifestyle interventions alone rarely result in long-term weight reduction, and hence, there is a major need to develop new anti-obesity drugs. The ideal anti-obesity agent resulting in large and sustained body weight losses without side-effects has not yet been found (7;8). An encouraging new approach for development of future anti-obesity and anti-diabetic drugs is based on analogues of the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (9;10). These hormones are nature's regulators of postprandial insulin secretion, forming the endocrine part of the so-called "entero-insular axis". The incretin hormones may account for up to 70% of meal-induced insulin release in man, and thereby they indirectly facilitate the uptake of glucose in muscle, liver and adipose tissue (11). Besides stimulating insulin release and glucose-uptake, GLP-1 and GIP also stimulate insulin gene-transcription, increase pancreatic β-cell mass and protect against β-cell apoptosis (12). Moreover, the potential of GLP-1 to slow gastric emptying, and to reduce appetite and food intake makes it an attractive tool in the fight against obesity, and several companies are developing weight-lowering drugs based on GLP-1 (10) . Interestingly, another peptide, amylin, co-secreted with insulin, exerts very similar effects on gastric emptying, appetite and food intake in humans (13). Currently, it is not known whether the inhibiting effects of GLP-1 on gastric emptying, appetite and food intake are directly mediated by GLP-1, or if they are secondary to the robust insulin responses, and thereby amylin responses, elicited by GLP-1. Hence this PhD thesis seeks to further elucidate the mechanisms of these effects in order to strengthen the development of anti-diabetic drugs with potential weight lowering capabilities.
GIP, too, has actions other than its physiological glucose-dependent insulinotropic actions. In vitro and animal studies indicate that GIP exerts direct effects on adipose tissue and lipid metabolism, promoting fat deposition. Due to the therapeutic potential in obesity treatment, a rapidly increasing number of functional studies are investigating effects of acute and chronic loss of GIP signaling in glucose and lipid homeostasis. However, the physiological significance of GIP as a regulator of lipid metabolism in humans remains unclear. In this thesis, there is a special emphasis on the role of GIP in the regulation of lipid metabolism in humans.

BACKGROUND

The incretin hormones and amylin

The following sections provide an overview of GLP-1, GIP and amylin history, metabolism and receptors. The effects of GLP-1 and amylin on glucagon, gastric emptying, food intake and appetite, and the effects of GIP on fat metabolism and adipose tissue blood flow will be described.

Glucose-dependent insulinotropic polypeptide

History; In 1971, a peptide hormone, named gastric inhibitory polypeptide (GIP) was isolated from porcine intestine (14), based on its ability to inhibit gastric acid secretion in dogs (15). Subsequent studies, however, demonstrated a glucose-dependent stimulation of insulin secretion by GIP in animals and humans, suggesting an incretin role for the peptide (16;17). Because a physiological action of GIP to inhibit gastric acid secretion in man could not be demonstrated as opposed to its incretin action, GIP was renamed glucose-dependent insulinotropic polypeptide, thus retaining the acronym.

Synthesis and secretion; GIP is synthesized within and released from K-cells, the majority of which are located in the duodenum and proximal jejunum, but with smaller numbers also occurring throughout the entire small intestine (18;19). GIP is secreted in response to nutrient ingestion, especially glucose or fat (20;21), where fat is the most potent stimulator of GIP secretion in humans (22). Postprandial responses of GIP to a meal are proportional to its caloric value and rate of nutrient delivery into the intestine. More specifically, it is the rate of nutrient absorption rather than the mere presence of nutrients in the intestine that stimulates GIP release (23). Thus, GIP secretion is reduced in individuals with intestinal malabsorption or after the administration of pharmacological agents that reduce nutrient absorption (24;25). Glucose, administered either orally or intraduodenally to humans results in rapid release of GIP, with levels reaching a peak within 15 to 30 minutes and returning to fasting values with a similar time course to circulating glucose and insulin (3h) (22;26). The GIP response to oral or intraduodenal fat is slower and more prolonged than with glucose, partly due to delayed gastric emptying (22;27).

Degradation and elimination; The half-life of intact biologically active GIP is approximately 7 minutes in healthy subjects (28). Rapidly after its release, GIP (1-42) is degraded by the enzyme dipeptidyl-peptidase IV (DPP IV), which cleaves two amino acids from the N terminus of the GIP molecule, resulting in the generation of the inactive metabolite GIP (3-42). The observations that GIP levels are increased in uremic patients or individuals with chronic renal failure, together with impaired GIP clearance in nephrectomized rats, points to the kidney as the major route of GIP clearance (29).

Receptor; The GIP receptor (GIPR) was initially cloned from a rat cerebral cortex cDNA library (30) and was followed by the cloning of the hamster (31) and human GIPRs (32). The GIPR gene is expressed in the pancreas, stomach, small intestine, adipose tissue, adrenal cortex, pituitary, heart, testis, endothelial cells, bone cells, trachea, spleen, thymus, lung, kidney, thyroid and several regions in the brain (30;33;34). GIPR is a member of the 7-transmembrane-spanning G-protein-coupled receptor (GPCR) family (30;35). Activation of GIPR signaling is coupled to increases in cAMP and intracellular Ca^{2+} levels, as well as activation of PI-3K, PKA, PKB, MAPK, and phospolipase A2 (36).

Glucagon like peptide-1

History; In 1983, the discovery of a second incretin hormone, glucagon like peptide-1 (GLP-1), followed the cloning and sequencing of mammalian proglucagon genes and complementary DNAs (cDNAs). In addition to glucagon, the proglucagon gene turned out to encode 2 peptides that are approximately 50% homologous to glucagon and thus were named glucagon-like peptide 1 and glucagon-like peptide 2 (GLP-2) (37). Based on their homology to glucagon, both peptides were tested for insulinotropic activity, but only GLP-1 was capable of stimulating insulin secretion (38).

Synthesis and secretion; GLP-1 is secreted from intestinal endocrine L-cells which are most frequent in the ileum and colon (39). However, immuno-reactive GLP-1 cells have been found throughout the entire small intestine, and co-localization of GLP-1 and GIP in cells in the mid-small intestine suggests that cells capable of releasing both GIP and GLP-1 also exist (19). The major physiological stimulus to L cells is nutrient ingestion, with circulating levels rising by two- or threefold in response to a mixed meal in humans (40). Simple carbohydrates, fats and whey proteins also stimulate GLP-1 secretion (41;42). That L cells respond to nutrients is not unexpected, as numerous immunohistochemical studies have demonstrated that they are “open” type epithelial cells that make contact with the luminal contents at their apical surfaces (43). The secretion profile of GLP-1 is sometimes biphasic starting with an early (within 10-15 min) phase that is followed by a longer (30-60 min) second phase, and it has been suggested that the stimulus for the early postprandial peak of GLP-1 is mediated indirectly through a neuroendocrine pathway. However, this was questioned in recent extensive studies employing both isolated perfused porcine ileum and intact pigs, and using a variety of active agents as well as electrical stimulation of the abdominal vagal trunks to investigate the importance of the neural regulation (44;45). It was demonstrated that the sympathetic innervation to the gut is inhibitory for GLP-1 secretion, whereas the extrinsic vagal innervations had no effect. Intrinsic, cholinergic activity may play a minor role. As already pointed out, there are L-cells in the proximal jejunum, and these may very well be responsible for the early response. Multiple forms of GLP-1 are secreted in vivo, including GLP-1 (1-37) and GLP-1 (1-36) amide, which are thought to be inactive and GLP-1 (7-37) and GLP-1 (7-36) amide, which are biologically active. In humans, GLP-1 (7-36) amide is the predominantly released product (46).

Degradation and elimination; The half-life of intact GLP-1 is less than 2 minutes in the circulation owing to rapid degradation. GLP-1 is extremely susceptible to the catalytic activity of DPP-4, which cleaves off the two NH_2 terminal amino acids (47). The metabolite thus generated, GLP-1 (9-37) or GLP-1 (9-36) amide is inactive with respect to insulin secretion. There is some evidence that the metabolite may have some activity in the cardiovascular system. A large part of the GLP-1 that leaves the gut has already been degraded to the metabolite, such that less than 25% of newly secreted GLP-1 enters the portal vein in intact, insulinotropic form (48). A similar degradation amounting to ~40-50% takes place in the liver, and it can be calculated that only ~10-15% of newly secreted GLP-1 reaches the systemic circulation in the intact form (49). In agreement with this, the concentrations of intact GLP-1 in plasma are very low (50). The metabolite is also cleared rapidly, mainly in the kidneys, with a half-life of 4-5 min (29).

Receptor; As for GIP, the GLP-1 receptor (GLP-1R) belongs to the family of 7-transmembrane-spanning G-protein-coupled receptors (51). GLP-1 receptors have been found in various organs including pancreatic islets, lung, heart, kidney, stomach, intestine, pituitary, skin, nodose ganglion neurons of the vagus nerve, and several regions of the CNS, including the hypothalamus, amygdala

and brainstem. The GLP-1R is capable of signaling through Gαs subunit as well as additional G protein subunits such as Gαq, Gαo and Gαi, leading to increases in intracellular cAMP and Ca2+ concentrations and activation of downstream pathways, including PKA, PKC, PI-3K, Epac2 and MAPK signaling pathway (51-53).

Amylin

History; In 1901, Opie described a "hyaline" appearance within the islets of Langerhans from diabetic patients (54). The hyaline material present in pancreata from diabetic patients was histologically identified as amyloid (55). However, the phenomenon was largely forgotten until the mid-1980s, when two groups of researchers, working independently, characterized the nature of the amyloid deposits in samples of pancreas taken post mortem from patients with type 2 diabetes, and identified the key component as the peptide amylin (56;57).

Synthesis and secretion; Amylin is a 37-amino acid peptide that has approximately 50% sequence identity to calcitonin gene-related peptide (CGRP). It is also structurally related to calcitonin, and belongs to the family of peptides which also includes calcitonin and adrenomedullin (58). Amylin is produced in the pancreatic β cells as a 89 amino acid preprohormone (59), which is processed within β-cell secretory granules to become the 37 amino acid hormone (60). Processing of amylin preprohormone includes cleavage of both C- and N-terminal flanking peptides (59) by the prohormone convertases PC2 (61) and PC3 (62). Posttranslational processing includes amidation at the C terminal (63) and formation of a disulphide loop between Cys-2 and Cys-7 (56). After this, the transformation from the precursor protein pro-amylin to the biologically active amylin is complete (60). The disulphide bridge between the cysteine residues at positions 2 and 7 is thought to be responsible for amyloid formation in humans (64). Study of amylin has been complicated by the fact that human amylin has an inherent tendency to self-aggregate in solutions, to form fibrils, with subsequent loss of activity, and to adhere to surfaces. This prompted Amylin Pharmaceuticals Inc to develop an analogue named Pramlintide, where the amino-acid residues at positions 25, 28 and 29 are replaced with proline, which promotes stability in solution and preservation of biological activity (65). Most of the biology of human amylin in humans has therefore been deduced from responses to pramlintide. Only one previous study in rats has documented that Pramlintide retained the full spectrum of biological actions and pharmacokinetic and pharmacodynamic properties of amylin (66).
Early descriptions of a concordance of secretory patterns of amylin and insulin in humans were interpreted as evidence of their co-secretion (67). It has been shown that factors that modulate insulin secretion also appear to cause an obligatory modulation of amylin secretion. This was true for stimulation of secretion by glucose, arginine and carbachol and was true for inhibition of secretion by somatostatin (68). Although there is notable similarity of plasma concentration profiles of amylin and insulin, amylin levels are only 10-15% those of insulin in the fasting state (69;70). The plasma clearance rates of amylin and insulin differ, with amylin being cleared more slowly than insulin and at a comparable rate to C-peptide. This is consistent with the fact that amylin, like C-peptide, is cleared through the kidney (71). This difference in clearance rates of amylin and insulin contributes to the fasting amylin:insulin ratio being higher than that observed shortly after the release of these peptides from the β cell. Thus, amylin plasma levels immediately after acute stimulation of β cell peptide release with glucose or nonglucose secretagogues are closer to 1% that of insulin (70;72;73).
The co-localization and co-secretion of insulin and amylin are consistent with the concordance of deficiencies in amylin and insulin secretion observed with the progression of diabetes mellitus. Like insulin, amylin is absent in patients with long-standing type 1 diabetes, while it is elevated in obese subjects with normal and impaired glucose tolerance and in patients with early, diet-controlled type 2 diabetes, but decreased in patients in the later stages of type 2 diabetes (74).

Degradation and elimination; Pharmacokinetic studies of amylin in humans either have been restricted to pramlintide (75;76) or have reported parameters derived for endogenous amylin by fitting pharmacokinetic models to concentration profiles (77). After subcutaneous administration of high doses of pramalintide, t½ ranged from 26±8 to 42±2 min. After intravenous bolus doses and continuous intravenous infusions, terminal half lives were 21-47 and 20-46 min, respectively and clearances were ~ 1 L/min (75). In one study in which human amylin was injected, modeled t½ was 9.5 min and volume distribution was 45 ml/kg (77). As mentioned, amylin is cleared mainly by proteolytic degradation at the kidney (71).

Receptor; The enigma of the amylin receptor was solved following the identification of receptor activity modifying proteins (RAMPs) (78). When associated with a calcitonin receptor, these single transmembrane spanning molecules alter their pharmacology from calcitonin-preferring to amylin-preferring (79). With at least two forms of the calcitonin receptor and three forms of RAMP, there is the potential for six subtypes of amylin receptor (79).

Physiological effects of GIP, GLP-1 and amylin

Effects of GLP-1 on glucagon, gastric emptying, food intake and appetite.

GLP-1's major physiological role lies in its strictly glucose-dependent stimulation of insulin secretion from pancreatic β cells. Furthermore, GLP-1 stimulates β cell proliferation and enhances differentiation of new β cells from progenitor cells in the pancreatic duct epithelium. It also increases insulin biosynthesis and suppresses glucagon secretion, inhibits gastric emptying, enhances satiety and reduces caloric intake (80).

Glucagon secretion; GLP-1 reduces plasma levels of glucagon in both healthy subjects as well as in subjects with type 1 and type 2 diabetes (81). This effect of GLP-1 contributes to lowering plasma glucose levels as a result of the reduction in hepatic glucose output. Indeed, administration of exogenous GLP-1 to patients with type 1 diabetes led to reductions in fasting hyperglycemia via its glucagonostatic action (82). Similar to the GLP-1 effects on insulin secretion, this glucagonostatic action of GLP-1 depends on glucose levels (83). Its inhibitory effect on glucagon secretion is normally lost below normal fasting glucose levels, thereby reducing the risk for developing hypoglycemia. By using the GLP-1 antagonist exendin(9-39), the role of endogenous GLP-1 in inhibition of glucagon secretion has been elucidated. Human studies showed that exendin(9-39) dose-dependently blocks the inhibition of glucagon by GLP-1 (42;84). This was accompanied by elevated blood glucose levels.
The exact mechanism of GLP-1-induced inhibition of glucagon secretion has not been completely elucidated. GLP-1 is able to

inhibit glucagon secretion and hereby lower glucose in type 1 diabetic patients without residual β-cell function (82), indicating that a paracrine inhibitory effect of insulin or other β-cell products is not essential. In contrast, GLP-1 has been reported to stimulate glucagon secretion in isolated rat pancreatic α-cells (85), making a direct inhibitory effect on the α-cells less likely. Instead, GLP-1 may act by stimulating the secretion of somatostatin, which in turn may inhibit glucagon secretion (86).

Gastric emptying; Postprandial glucose homeostasis is determined not only by the stimulation of insulin secretion and the suppression of hepatic glucose production, but also by the velocity of gastric emptying (87). The inhibitory function of GLP-1 on gastric emptying confirms the role of GLP-1 as an important enterogastrone (88). The effect of GLP-1 on gastric emptying is dose dependent and highly significant at physiological concentrations of approximately 25 pM (89). GLP-1 inhibits antro-pyloro-duodenal motility (90) as well as tone of the gastric fundus, while stimulating the pyloric tone (91), thus slowing the transfer of nutrients to the distal gut, the so called "ileal brake" effect. The mechanisms by which GLP-1 inhibits gastric emptying appear to be complex and to involve communication with the central and the peripheral nervous systems. GLP-1 receptors are expressed in the stomach on gastric parietal cells (92), but these are stimulatory, indicating that GLP-1 cannot inhibit gastric secretion via direct mechanisms. Central administration of GLP has also been shown to delay gastric emptying (93). However, the inhibition of GLP-1 receptor (GLP-1R) signaling with exendin(9-39) or vagal afferent denervation abolished the inhibitory effect of centrally and peripherally administered GLP-1 on gastric emptying, indicating that vagal afferent nerves mediate the inhibitory action of GLP-1 on gastric motility (93;94) In addition, although GLP-1 is capable of crossing the blood-brain barrier (bbb) and directly accessing the CNS, high-molecular-weight GLP-1R agonists, such as albumin-bound GLP-1 that presumably do not cross the blood-brain barrier, are still capable of inhibiting gastric emptying (95), thus underscoring the importance of ascending neural pathways for GLP-1R agonist-dependent control of gastric emptying. Taken together, these data suggest that the inhibitory effect of GLP-1 on gastric emptying is mediated by the vagus nerve and involves GLP-1 receptors located in the CNS and/or on vagal afferent fibers that relay sensory information to the brainstem.

Food intake and appetite; Flint et al reported a 21% reduction in food intake as well as an increase in satiety and fullness during GLP-1 administration in healthy human subjects (96), and similar results were obtained in patients with type 2 diabetes (97). The mean reduction in energy intake observed during GLP-1 infusion in different studies was 12% (98). In a long-term study with the subcutaneous infusion for over 6 weeks in patients with type 2 diabetes, GLP-1 treatment caused a progressive and sustained weight reduction of 1.9 kg (99). However, the mechanisms by which GLP-1 inhibits food intake and appetite remain unclear. Substantial evidence suggests that brain GLP-1 and GLP-1R play a role in the control of feeding, as do peripheral GLP-1 and GLP-1R. Different studies support the view that peripheral GLP-1 reduces food intake through an effect on peripheral GLP-1R. The albumin-bound GLP-1 does not cross the bbb, as mentioned, but still reduces feeding when administered systemically (95). Although GLP-1 can cross bbb, several lines of evidence suggest that peripheral GLP acts to reduce food intake primarily via vagal afferent activation. GLP-1R mRNA is expressed in the nodose ganglion and GLP-1R has been observed on vagal terminals innervating the hepatic portal vein (HPV). It has been demonstrated that either total subdiaphragmatic vagotomy (100) or selective vagal deafferentiation(101) prevents intraperitoneal (ip) injected GLP-1-induced anorexia. Recent pharmacological studies using exendin(9-39) provide further support for this view. Peripherally administered exendin(9-39) increases food intake when delivered to satiated rats and also blocks the satiety induced by nutrient preloads (102). These effects appear to be based on ip administered exendin(9-39)'s ability to block peripheral GLP-1R, because the same dose of ip exendin(9-39) that blocked the anorexic effect of systemic GLP-1 failed to blunt the anorexic effect of CNS GLP-1 administration. Conversely, intracerebroventricular (icv) exendin(9-39) blocked the feeding-inhibitory effect of icv GLP-1 but failed to attenuate peripheral GLP-1-induced anorexia (102). Taken together, these data support the idea that GLP-1 released by the intestine promotes satiety by activating peripheral GLP-1R, whereas neuronal GLP-1 affects feeding through GLP-1R in the brain. It is clear that brain GLP-1 is involved in food intake and appetite. Thus, early studies demonstrated that the icv administration of GLP-1 inhibited appetite and food intake (103;104), while gastric distension has been shown to increase the activity of neurons (expression of c-Fos) in the brainstem that produce GLP-1 (105). Recently, Shick and colleagues identified the lateral, dorsomedial and ventromedial hypothalamus to be involved in the mediation of satiety effects (106). In addition, destruction of the arcuate nucleas and other regional hypothalamic regions that are involved in the regulation of food intake (107) abolished the inhibitory effect of icv administered GLP-1 on food intake and appetite (108).

Effects of amylin on glucagon, gastric emptying, food intake and appetite.

Amylin may complement the effects of insulin on postprandial glucose excursions by several mechanisms. These include the inhibition of postprandial glucagon secretion, slowing the rate of gastric emptying and suppressing food intake and appetite.

Inhibition of glucagon; several lines of evidence support a physiological glucagonostatic effect. Amylin infusions dose-dependently inhibited arginine-stimulated glucagon secretion in rats with an EC50 that approached endogenous amylin concentrations (109). In studies in patients with diabetes, exaggerated postprandial glucagon profiles were ameliorated by pramlintide at doses resulting in physiological amylin concentration (110;111). Recently, Gedulin and colleagues demonstrated that amylin receptor blockade with AC187 in rats increased glucagon secretion, fitting with the notion that endogenous amylin plays a physiological role in postprandial glucagon regulation (112). However, during hypoglycemia the suppressive effect of amylin on glucagon was not observed (113;114).

Knowledge of the mechanism by which amylin inhibits nutrient-stimulated glucagon secretion is still incomplete. While in the isolated perfused rat pancreas, amylin failed to inhibit glucagon secretion (114;115), another study reported that amylin totally inhibits insulin, glucagon and somatostatin secretion following arginine stimulation (116). Alternatively, the effect of amylin on glucagon secretion could be mediated through the autonomic nervous system, in analogy with the vagally mediated effect of amylin on gastric emptying (117). In this context, it is of interest that binding of amylin has been demonstrated in the area postrema in the hindbrain of the rat, involving the dorsal motor nucleus of the vagus (118). Although it is presently unknown

whether the glucagonostatic effect of amylin is mediated through the vagus nerve, it is well known that autonomic innervation of alpha cells has an important role in the regulation of glucagon secretion (119).

Inhibition of gastric emptying; Amylin and/or pramlintide have been shown to inhibit gastric emptying in animals (120) and in humans, including non-diabetic subjects, as well as those with type 1 diabetes and type 2 diabetes (13;121). Several lines of evidence obtained in clinical and animal studies point to modulation of gastric emptying being a physiological action of amylin secreted in response to meals. The evidence includes 1. a dose response relationship where doses were associated with physiological concentration changes (13;121), 2. an accelerated gastric emptying in amylin-deficient models (122), and 3. an accelerated gastric emptying during amylin receptor blockade (112). The potential mechanisms by which pramlintide/amylin delay gastric emptying are either a direct effect on the stomach or an effect on the CNS. Because no amylin receptors have been identified in the stomach, a direct effect of amylin on the stomach seems unlikely, whereas several lines of evidence support the proposal that amylin's gastric effects involve the area postrema. Studies in rat brain have revealed the presence of amylin receptors in the area postrema (123), and amylin's gastric effects are abolished in rats in which this structure is lesioned (124). In addition, an intact vagus nerve is necessary for amylin to slow gastric emptying. In rats, that underwent total subdiaphragmatic vagotomy, amylin was no longer effective at inhibiting gastric emptying (117).

Inhibition of food intake and appetite; In addition to its effect on glucagon secretion and the rate of gastric emptying, amylin appears to affect appetite and food intake. The effects of acute administration of amylin on satiety were recently evaluated in patients with type 2 diabetes and in obese non-diabetic subjects (125). Subjects were fasted overnight, and given placebo or pramlintide subcutaneously, followed immediately by a liquid preload meal. They were then offered an ad libitum buffet meal 60 min later. Pramlintide reduced energy intake substantially in both diabetic (202 kcal; 23%) and obese (170 kcal; 16 %) subjects without affecting meal duration or inducing nausea. More recently, the same group assessed the acute effects of a more physiological dose of pramlintide in healthy subjects of normal weight (126). Using a comparable study design, treatment with pramlintide was shown to reduce total energy intake by 221 kcal, or 14%, supporting a physiological role of amylin in appetite control. Amylin has been shown to inhibit feeding by facilitating meal-ending satiety. Amylin injections in food-deprived rats reduced the size of the first post deprivation meal without affecting intra-meal feeding rate or the size or timing of subsequent meals (127). The observation that amylin does not cause a conditioned taste aversion indicates that the reduction in food intake was not secondary to malaise, but likely represented pleasurable meal-termination satiety (127).
The most important argument indicating that amylin is a physiological regulator of meal size is provided by studies showing that peripherally or centrally delivered amylin antagonists produce an effect opposite to that of amylin, i.e. an increase in eating, mainly via a meal size effect (128;129). In the latter study, the amylin antagonist AC 187 was infused directly into the area postrema (128). Several experimental studies support the idea that the satiating effect of peripheral amylin is mediated by direct humoral action on the area postrema (128;130;131). Behavioral studies showed that whereas amylin's effect is abolished in rats with lesions in the area postrema region, none of the techniques that block neural afferent information from the periphery to the brain was able to abolish the anorectic action of peripheral amylin (132).
Feeding studies have been complemented by electrophysiological and immunohistochemical studies (133;134). A direct, dose-dependent stimulating effect of amylin on area postrema neurons underlines their sensitivity to amylin. Further, peripheral amylin produced a strong expression of the immediate early gene product c-Fos protein as a marker of neuronal activation in area postrema neurons (133).

Effects of GIP on fat metabolism and adipose tissue blood flow.
In addition to GIP's strong insulinotropic effect, a role of GIP in the regulation of lipid homeostasis and in the development of obesity has been inferred from different animal studies.

Fat metabolism; It has been observed that GIP secretion is potently stimulated by lipids and that postprandial GIP levels seem to be higher in the obese subjects (50;135). Furthermore high-fat feeding in rodents has been demonstrated to induce K cell hyperplasia and enhance GIP gene expression and intestinal GIP content, resulting in elevated circulating GIP concentrations (136). Hence, it was hypothesized that GIP may be a mediator of delivery of fat to the adipose tissue, and thereby function as a link between food intake and obesity. In agreement with GIP as a regulator of fat metabolism, GIP infusion was found to increase chylomicron clearance from plasma in dogs (137) and lower plasma triglyceride levels after an intraduodenal fat infusion in rats, whereas immunoneutralization of endogenous GIP resulted in decreased clearance of triglycerides (138). However, a similar effect could not be demonstrated in response to an intravenous lipid infusion in dogs or humans (139;140). Jorde and colleagues studied the effect of porcine GIP on clearance of plasma triglycerides after a 2-min intravenous Intralipid® infusion for a period of 25 minutes in 6 subjects (139). In this study, GIP failed to modify plasma triglycerides levels.
Nevertheless, several studies investigating effects of GIP in adipocyte cell lines or explants have reported that GIP directly regulates adipocyte metabolism. In agreement with a direct effect of GIP, adipocytes have been reported to express GIP receptors (34). Furthermore, the effect of GIP in perfused isolated adipocytes could be blocked by a GIP receptor antagonist (141). Studies in isolated adipocytes, adipose tissue explants and the pre-adipocyte cell line, 3T3-L1, have reported that GIP mediates uptake of glucose and fatty acids (142-144), stimulates lipoprotein lipase activity (LPL) (145-147) and inhibits catecholamine and glucagon stimulated lipolysis (141;142). Some studies have found insulin independent effects whereas others investigating concomitant effects of GIP and insulin found that GIP mainly potentiated insulin mediated effects. In rats, GIP alone induced a limited increase in fatty acid incorporation into adipose tissue, while in the presence of insulin, the effect was significantly enhanced (144). Miyawaki et al demonstrated that GIP stimulates insulin induced glucose uptake in 3T3-L1 adipocyte cell lines, but GIP had no effect when given alone (148). Starich et al. reported that GIP potentiated insulin mediated glucose uptake in rat adipocytes and increased insulin receptor affinity (149). In recent studies, McIntosh and coworkers looked further into mechanisms by which GIP could promote uptake of triglycerides to adipose tissue (146). They showed that in the presence of constant insulin levels, GIP was able to stimulate LPL activity in a dose dependent manner in 3T3-L1 and human subcutaneous adipocytes, resulting in

increased intracellular triglyceride concentrations. In both experimental systems, a similar signaling pathway involving increased phosphorylation of protein kinase B (PKB) and reduced phosphorylation of LKB1 and AMP-activated protein kinase (AMPK) was involved.

Adipose tissue blood flow (ATBF); Blood flow in adipose tissue may be an important regulator of the metabolism in the tissue, and the regulation of blood flow in adipose tissue is tightly linked to local metabolic demands under varying conditions (150;151). In lean, healthy individuals, adipose tissue blood flow (ATBF) is responsive to nutrient ingestion (152-154). The ATBF response to nutrient intake may be of particular importance in the regulation of metabolism by facilitating transport and deposition of nutrients, as well as signaling between adipose tissue and other metabolically active tissues (155). The mechanisms by which ATBF is regulated in the postprandial period are not clear. There are many possible regulators of ATBF including neural and endocrine systems and ATBF may well be regulated by multiple factors at the same time. Stimulation of vascular β-adrenoreceptors in the subcutaneous adipose tissue is probably one of the mechanisms involved (156). It is controversial whether insulin per se regulates ATBF. Previous reports demonstrated that hyperinsulinemia under conditions of euglycemia (157;158) and hypoglycemia increased ATBF (159), but other studies could not reproduce effects during euglycemia (159;160). Recently, a study reported that ATBF was markedly higher during oral glucose than during the i.v. insulin-glucose infusions (161), indicating that gastrointestinal endocrine mediators could be involved in the induction of the postprandial vasodilatation in adipose tissue. GLP-1 and GIP are likely candidates for the postprandial increase in ATBF, but GLP-1 was recently demonstrated not to influence ATBF in humans (162). To our knowledge, the effect of GIP on ATBF has not been examined. The correlation between the postprandial vasodilatation in adipose tissue and the increase in circulating GIP concentrations makes GIP a likely candidate eliciting the vasodilatation.

STUDY 1

DO THE ACTIONS OF GLP-1 ON GASTRIC EMPTYING; APPETITE AND FOOD INTAKE INVOLVE RELEASE OF AMYLIN IN HUMANS?

Background and aim; It has been postulated that the effects of GLP-1 on gastrointestinal secretion and motility and on appetite and food intake are mediated by the insulinotropic properties of the hormone. As mentioned, during insulin secretion the peptide amylin is co-secreted from the pancreatic beta-cells (67). Amylin has, much like GLP-1, been shown to inhibit glucagon secretion (109), delay gastric emptying (13) and reduce appetite and food intake in humans. The aim of the first study was to investigate whether the effects of GLP-1 on gastric emptying, appetite and food intake are mediated directly or indirectly via release of amylin.

Method; We enrolled 12 healthy subjects (aged 31±6, body mass index (BMI) 23.5±1.3 kg/m2) and 11 patients with type 1 diabetes without residual beta-cell capacity (aged 31±7, BMI 23.2±1.1 kg/m2). None of the healthy controls had a family history of diabetes, all had normal oral glucose tolerance as assessed by 75 g oral glucose tolerance test, and none had islet cell or glutamate decarboxylase-65 autoantibodies. The patients were C-peptide negative, as assessed by glucagon test. Their HbA1C levels were 7.6±0.8% (mean±SD) and mean duration of disease was 9.5±3.4 years. None had overt diabetic complications or other somatic illness. All received long acting insulin supplemented with fast acting insulin. The patients continued their normal regimen of long acting insulin, but did not take fast acting insulin in the morning of the experiments.
The subjects were investigated in a randomized order on five different occasions during continuous infusion of two different doses of GLP-1 (1 pmol/kg/min and 0.4 pmol/kg/min, respectively), amylin (0.3 pmol/kg/min), pramlintide (0.35 pmol/kg/min) or saline and each infusion was maintained for a period of 270 min. The protocol included intake of a fixed meal to allow determination of islet hormone responses as well as gastric emptying rates (assessed by the paracetamol absorption method), followed by a postprandial period in which we measured appetite ratings by visual analogue scales (VAS), and finally an ad libitum meal from which food intake could be evaluated. Because the T1DM subjects had no beta cell secretion left, they depended on exogenous insulin, and in addition to their regular evening dose on the night before the experiments, they received infusions of fast acting insulin as required to keep their PG levels around 7 mM, a value slightly lower than their fasting PG concentrations (8.9 mM) upon arrival to the laboratory. In this way excessive meal-induced glucose excursions as well as hypoglycemic episodes were avoided. To allow comparisons with the healthy controls, these were infused with glucose at variable rates in order to maintain PG at similar levels.
For the present study, we developed a reproducible, stable formulation of human amylin after having characterized the amyloid formation with respect to albumin concentration, pH and salt concentration. To determine optimal conditions for prevention of amyloid formation, amylin solutions were examined at different pH values (4, 7 and 10), salt content (distilled water vs. 150 mM NaCl), human serum albumin (HSA) concentrations (0.05 and 10%) and storage temperature (25, 4 and -20°C). Each experiment was performed at least three times. The tendency for amyloid formation was tested both 5 hours after the solutions were made and after repeated freezing and thawing of the solutions. Amyloid formation was measured using Thioflavin T (ThT) fluorescence, a dye known to bind amylin amyloids (163;164). ThT fluorescence increases in a solution of freshly reconstituted amylin as amyloid fibrils grow. Measurements of amyloid formation were performed at 5 µM ThT in buffer (10 mM sodium phosphate, 100 mM NaCl), and real-time emission intensities were measured at 482 nm with excitation at 450 nm at room temperature with excitation and emission slit widths of 5 nm using a Hitachi F2000 Spectrofluorometer 000748.

SUMMARY OF RESULTS AND DISCUSSION
Our main purpose was to investigate the relationship between the incretin hormone GLP-1 and the beta cell hormone amylin on gastric emptying, appetite and food intake. For this, intravenous infusions of GLP-1 and amylin (and also the stabilized synthetic analogue, pramlintide, for comparisons with natural amylin) were carried out in both healthy subjects and C-peptide negative patients with T1DM. The latter were included in order to avoid contributions from endogenous amylin secretion. Indeed, in the T1DM patients, amylin concentrations were close to the detection limit of the assay and did not increase in response to the applied beta cell stimuli, including GLP-1.

The hormone levels obtained after the intravenous infusions corresponded well with what was intended. Thus, GLP-1 levels after the low rate infusion corresponded to what may be observed in subjects given large meals or having accelerated gastric emptying (165), while the levels after the higher rate corresponded to those observed after "therapeutic" infusions in T2DM subjects (166). The amylin levels after meal ingestion were roughly comparable to those observed in the T1DM subjects after intravenous infusion, and corresponded closely to those observed during GLP-1 infusion. In this way, it was possible to compare the effects of GLP-1 with (controls) and without amylin (T1DM), and to compare the responses to those elicited by amylin alone. In agreement with the absent contribution of endogenous amylin in the T1DM patients, plasma levels were lower both in the basal state and during infusion. However, a minor difference in amylin clearance between patients and controls (higher in the former) cannot be excluded, since the increases in plasma amylin concentrations during infusion appeared less in the patients compared to the controls.
The effects of GLP-1 in the T1DM patients corresponded to what is seen in healthy subjects (80). Both doses powerfully inhibited paracetamol absorption (gastric emptying), and inhibited subjective appetite related parameters. In addition, food intake during the subsequent ad libitum meal was significantly reduced. It can therefore be concluded that these actions of GLP-1 are exerted independently of amylin secretion. The results also document that amylin and pramlintide in physiological concentrations have very similar effects on each of these parameters (13;110;111).
GLP-1 infusions in the healthy subjects were associated with significant stimulation of endogenous amylin secretion in parallel with a strong stimulation of insulin secretion (whereas the amylin infusions had no effect on meal-induced insulin secretion). The infusions again resulted in strong inhibition of gastric emptying as well as food intake. Infusions of amylin (and pramlintide) to similar levels as observed during GLP-1 infusion in these healthy control subjects also inhibited gastric emptying, appetite parameters and food intake, but less so than GLP-1. During the GLP-1 infusions, the absolute food intake was significantly lower in the healthy subjects compared to the T1DM subjects ($P<0.03$), whereas the percentage changes from baseline (saline) between the groups were similar (P=0.13-0.15). Taken together, these results would indicate that part of the effect of GLP-1 is due to the actions of amylin, released during stimulation of the beta cells. Our studies, therefore, suggest that amylin should be counted among physiological factors regulating gastric emptying, and food intake. In addition, part of the actions of GLP-1 mimetics employed for treatment of T2DM are likely to be due to amylin to the extent that the beta cells of these patients are still capable of releasing this hormone.
Both GLP-1 and amylin have been reported to inhibit glucagon secretion, an action that is thought to contribute importantly to the antidiabetic actions of these hormones. In the present study, glucagon concentrations decreased significantly in the 30 min pre-meal period, regardless of the infusion protocol. Most likely this reflects the slightly hyperglycaemic clamp necessitated by the inclusion of the T1DM subjects. On this background, it was not possible to distinguish any effects of amylin or GLP-1 on glucagon secretion. Both patients and controls responded positively to the fixed meal ingestion during saline days, and both amylin, pramlintide and in particular GLP-1 inhibited the response significantly. However, since the same infusions also inhibited gastric emptying rates, the reduced glucagon response might merely reflect this, rather than representing a direct effect of the hormones on glucagon secretion. Indeed, the pattern of inhibition appeared to parallel the gastric emptying rates. An elucidation of the actions of amylin and GLP-1 on glucagon secretion will therefore require additional studies.
Interestingly, in the T1DM subjects, during both GLP-1 infusions, more glucose was required to maintain plasma glucose concentrations between 6 and 9 mM compared to amylin and pramlintide infusions ($P<0.05$), independent of changes in glucagon secretion or gastric emptying. Additionally, less insulin was infused during the GLP-1 infusions. This indicates that GLP-1 has effects on glucose disposal that are independent of the pancreatic hormones, an issue that has been intensely debated (80). One possible explanation could be that the glucose-lowering effect of GLP-1 is due to the metabolite GLP-1 (9-36) amide which is formed rapidly during infusion of GLP-1 (167). GLP-1 (9-36) amide appears to reduce postprandial glycaemia independently of gastric emptying and insulin secretion in both experimental animals (168) and humans (169).
As mentioned earlier, very few studies have been carried using natural amylin because of its tendency to form fibrils. Our formulation studies demonstrated that it was indeed possible to prepare human amylin in a way that prevented fibril formation, which therefore allowed infusions of amylin in physiological relevant amounts. This also allowed a determination of the short half-life of human amylin in humans (namely around 8 min) and a comparison between human amylin and pramlintide in humans, which has not been carried out before. The actions of pramlintide were rather similar to those of human amylin, confirming that the amino acid substitutions in the former have little influence on its biological activities.
In conclusion, GLP-1 mediates its effect on gastrointestinal motility, appetite, food intake and glucagon secretion directly and thereby in an amylin-independent fashion, but amylin may contribute to these effects in healthy subjects. Pramlintide has the same biological effect as human amylin.

STUDY 2

ON THE ROLE OF GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE, GIP, IN POSTPRANDIAL METABOLISM IN HUMANS

Background and aim; As mentioned, in vitro and animal studies suggest that GIP might play a role in lipid metabolism, although there is no clear evidence for this in humans. Therefore, in the present study, the effects of GIP on the plasma concentrations of triglycerides (TAG) and free fatty acids (FFA) were evaluated in healthy human subjects. Furthermore, the role of GIP in the regulation of short term energy balance was evaluated. The study was divided into two parts. In protocol 1, we studied the effect of intravenous infusions of GIP on gastric emptying, postprandial concentrations of TAG and FFA (after ingestion of a fat-rich meal), postprandial appetite sensations, energy expenditure and subsequent ad libitum food intake. In this experiment, the fixed meal elicited a robust plasma GIP response. In order to avoid the influence of endogenous GIP, protocol 2 consisted of intravenous infusions of GIP with and without infusions of Intralipid® and/or glucose to mimic postprandial lipid and glycemic levels.

Method; For the first protocol we studied 20 healthy males; mean age: 26.2 years±3.2 (mean±SD), BMI: 23.2±1.7 kg/m2 and fat mass 17.1±3.9%. We chose 20 subjects in the first protocol because more than 18 subjects are needed to detect a difference of

10 % with regard to appetite changes (170). For the second protocol, we studied 10 healthy young males; mean age: 25.1 years ± 2.5 and BMI: 22.8 ± 1.4 kg/m2. The subjects had normal levels of fasting plasma lipids.
In protocol 1, the subjects were investigated in a randomized order on two different occasions during continuous infusion of either GLP-1 (0.8 pmol/kg/min) or saline and each infusion was maintained for a period of 300 min. The protocol included ingestion of a fixed meal to allow determination of islet hormone responses and gastric emptying rates (assessed by the paracetamol absorption method), followed by a postprandial period in which we measured appetite ratings by visual analogue scales (VAS), and finally an ad libitum meal from which energy intake could be evaluated. Resting energy expenditure (REE) was measured during the study period by indirect calorimetry using an open-air-circuit, computerized, ventilated hood system (171;172).
In protocol 2, the subjects were tested on 6 different occasions in a randomized order and consisted of: an Intralipid® infusion combined with or without GIP; glucose and Intralipid® infusion combined with or without GIP and glucose infusion combined with or without GIP. Intralipid® was infused in a dose of 0.15 g/kg over 30 min followed by a continuous infusion of 0.225 g/kg/h for half an hour. Glucose (25 g) was infused over 30 min to mimic the meal-induced glucose excursions. Continuous infusion of GIP (1.5 pmol/kg/min) or saline was maintained for a period of 300 min.

Summary of results and discussion; Neither part of the present study revealed any effect of GIP alone or in combination with insulin on the levels of plasma triglyceride in humans. In agreement with a previous study (173), the kinetics of removal of TAG from the circulation was strictly first order with same rate constants on the 4 experimental days with infusion of Intralipid®. Our inability to demonstrate any effect of GIP is, therefore, unlikely to be due to saturation of the lipoprotein lipase activity due to the high plasma TAG concentration applied, because saturation would result in deviation from first order kinetics. Our finding that addition of glucose to the infusions was without effects on the TAG removal is also in accord with previous findings (174). It is not possible from the present results to identify in which tissue the TAG removal takes place, but adipose tissue, skeletal muscle and liver are the most likely sites. It has previously been shown that both adipose tissue and skeletal muscle contribute to the removal of TAG after an intravenous fat load, however, to a smaller extent than after an oral fat load (154). While there is evidence showing that the liver takes up the lipid particles from lipid emulsions without prior lipolysis, these particles are lipolysed via LPL in adipose tissue and skeletal muscle (175). The fatty acids generated due to this may either be taken up in the tissue or may escape to the circulation. The increase in circulating fatty acid concentrations found in the present experiments is probably due to the latter process. The difference between the fatty acid concentrations found under the different experimental conditions must result from differences in tissue uptake and release of fatty acids. During GIP and Intralipid infusions, insulin secretion increased significantly during the first 60 min compared to the control experiment, confirming the ability of GIP to stimulate insulin secretion at fasting glucose levels (176). We found that GIP in combination with insulin secretion decreased the plasma FFA levels. One explanation for this is that insulin inhibits adipose tissue HSL activity and thereby adipose tissue fatty acid release. In addition, insulin may enhance adipose tissue fatty acid reesterification under these conditions (154). It is difficult to distinguish between direct GIP effects on fatty acid metabolism and an indirect effect based on the insulinotropic effect of GIP. Several in vitro studies have shown that GIP exerts its effect on adipose tissue by both insulin-independent and -dependent mechanisms (144;148;149). Recent data obtained in mice with a GIP receptor knock-out showed that a high-fat diet did not lead to obesity in these animals (148). This could suggest that GIP acts as an “insulin-sensitizer” in adipose tissue. However, adding GIP to glucose and Intralipid® did not change FFA levels compared to the control experiment (Intralipid®+Glu+saline). Most likely, the insulin levels reached in response to the glucose infusion had a maximal effect on the FFA release and uptake, and therefore no further changes were seen when GIP was added. The first part of the present study showed no effect of GIP alone on the plasma concentrations of FFA compared to saline, in spite of the fact that insulin was increased. Surprisingly, adding exogenous GIP to endogenously secreted GIP did not affect the insulin secretion (in the first part of the study). One explanation could be that the GIP levels reached in response to the meal were already maximally effective so that a further increase in GIP concentrations did not result in additional effects on insulin secretion and clearance of plasma lipids. However, although the insulinotropic effects of GIP were confirmed in the second part of the study, the elevated GIP levels still did not influence triglyceride clearance. In contrast to our study, Wasada et al. found that the triglyceride levels following intravenous infusion of chylomicrons in dogs were significantly lowered by exogenous GIP (137). While some studies have indicated that Intralipid® and chylomicrons exhibit similar decay kinetics after intravenous administration in humans (177;178) other studies indicate that LPL more effectively hydrolyses chylomicrons than Intralipid® lipid droplets both in adipose tissue and in skeletal muscle. Therefore it cannot be excluded that the lack of GIP effect found in the present experiments may be due to the artificial lipid formulation applied. A similar observation was made by Jorde et al. who studied the effect of porcine GIP on clearance of plasma triglycerides after a 2-min intravenous Intralipid® infusion for a period of only 25 minutes and in only 6 subjects (139). Also in this study GIP failed to modify the levels of plasma triglycerides levels.
The infusion rate of GIP chosen in both parts of the present study raised GIP plasma levels into the high physiological range. Therefore, inappropriately low dosing of GIP does not explain the absence of GIP effects. Moreover, because of the surprising lack of effects in the first protocol, even on insulin secretion, the insulinotropic activity of the infused GIP was tested using the isolated perfused pancreas as previously described (179), demonstrating equipotence with fresh ampoules of synthetic GIP. In the first part of the study, GIP was released endogenously in response to the meal. As mentioned above, the lack of additional effect of the infused GIP could be due to endogenous GIP already reaching maximally effective concentrations. However, towards the end of the infusion the secretion of endogenous GIP appeared to have ceased, at least in the control study, leaving only exogenous GIP in the circulation, but also in this period, there was no effect on plasma triglycerides of GIP compared to saline.

The present data demonstrate no effect of GIP on gastric emptying, appetite, energy intake or energy expenditure. The lack of effect of GIP on gastric emptying is in agreement with previous studies (180;181). One previous study has shown that infusion of GIP lowered REE and increased subjective feelings of hunger in normal weight healthy subjects, an observation that we have not been able to confirm (182). However, in that study, as in ours, no effect of GIP was seen on energy intake. There was also no effect

of GIP on the secretion of GLP-2 as well as GLP-1 as previously reported (183).

STUDY 3

GIP ENHANCES FATTY ACID RE-ESTERIFICATION IN SUBCUTANEOUS, ABDOMINAL ADIPOSE TISSUE IN NORMAL-WEIGHT HUMANS.

Background and aim; In the previous study GIP did have any effect on whole body TAG clearance. In the present study we aimed to elucidate more directly the role of GIP on the regional adipose tissue and splanchnic tissue metabolism in healthy subjects.

Methods; Eight healthy males; mean age: 30 years±6 (mean±SD), BMI: 23.2±1.4 kg/m2 were studied. None of the subjects studied had a family history (first-degree relatives) of diabetes, hypertriglyceridemia or hypercholesterolemia and none were taking any medications.

Each subject participated in randomized order in four different experiments. On two of the occasions, the subjects underwent a hyperinsulinemic (150-200 pM) – hyperglycemic (6.5-7 mM) clamp technique, aiming at plasma glucose and insulin concentrations seen after ingestion of a carbohydrate rich meal, with continuous infusion of either GIP (1.5 pmol-1 kg-1 min-1), resulting in physiological concentrations, or saline infusion during 300 min. On two other occasions they received GIP (1.5 pmol-1 kg-1 min-1) or saline alone for the duration of the study.

Following arrival to the laboratory the subjects had catheters inserted into a vein draining the subcutaneous, abdominal adipose tissue on the anterior abdomin, a hepatic vein and a radial artery.

Adipose tissue blood flow measurements were performed for the duration of each experiment by recording washout of 133Xenon, which was injected in gaseous form in the adipose tissue. This technique has previously been validated in our laboratory (184). About 1 MBq gaseous 133Xenon (The Hevesy Laboratory, Risø National Laboratory, Roskilde, Denmark) was administered. Splanchic blood flow was measured by continuous infusion of indocyanine green (ICG) (185) and whole body metabolism was measured by indirect calorimetry.

Regional net substrate fluxes across the subcutaneous, abdominal adipose tissue and the splanchnic tissues were examined by direct measurements of arterio-venous concentration differences of various metabolites in combination with regional blood flow measurements (Fick's principle).

Summary of results and discussion; In this study, we investigated the effects of GIP with and without insulin and slight hyperglycemia on abdominal, subcutaneous adipose and splanchnic lipid metabolism. Since the time course of the possible metabolic effects elicited via GIP in adipose tissue in humans has not been described previously, we used a prolonged hyperinsulinemic-hyperglycemic clamp technique, raising the plasma glucose and insulin concentrations to levels seen after ingestion of a carbohydrate-rich meal.

With use of arterio-venous catheterization technique applied to the subcutaneous, abdominal adipose tissue (186), it was found that during the hyperinsulinemic, hyperglycemic clamp experiment with GIP, TAG hydrolysis increased significantly compared to the hyperinsulinemic, hyperglycemic clamp experiment without GIP. This effect was probably primarily brought about via a significant increase in the subcutaneous adipose tissue blood flow. Adipose tissue blood flow increases significantly postprandially, and this increase appears to be of particular importance in the regulation of lipid metabolism by facilitating transport and deposition of lipids in adipose tissue. A reduction in both fasting and postprandial ATBF has been observed in obesity (187-189), and this impairment seems to be associated with insulin resistance (188). A better understanding of the regulation of ATBF may, therefore, give insight into its relationship to the metabolic disturbances observed in insulin resistance. Insulin per se does not seem to affect ATBF; however, insulin may stimulate ATBF via other mechanisms. Recently, a study reported that ATBF was markedly higher with oral glucose than during the i.v. insulin-glucose infusions (161). The present findings suggest that GIP has direct or indirect vasoactive effects in adipose tissue, although the design of the study cannot rule out that other substances may play a role. Thus, C-peptide has been found to have dose-dependent vascular effects in skeletal muscle in the concentration range between 0-1 nM (190). In the present experiments the C-peptide concentration increased from about 0.4 to 0.7 nM in the hyperinsulinemic, hyperglycemic clamp experiments without a concomitant increase in adipose tissue blood flow. In the hyperinsulinemic, hyperglycemic clamp experiment with GIP, the C-peptide concentration increased more to about 1 nM. However, in the light of the missing flow increase in the hyperinsulinemic, hyperglycemic clamp experiment it seems unlikely that the flow increase found in clamp experiments with GIP primarily is elicited via C-peptide.

Simultaneously with the increase in adipose tissue blood flow, an increase in adipose tissue TAG hydrolysis took place, probably reflecting an increased substrate supply to LPL similar to the pattern seen after a mixed meal. The TAG deposited in adipose tissue after a meal arises from the pathway mediated by LPL – hydrolyzing circulating lipoprotein-TAG (189). It has been demonstrated that the effect of LPL increases after a meal, becoming maximal at ~4 h postprandially, and that this is stimulated by insulin (189). In the present study, the increase in adipose tissue blood flow began already about 30 minutes after the hyperinsulinemic, hyperglycemic clamp and GIP infusion were commenced. Samra and colleagues demonstrated that when adipose tissue blood flow was increased by infusion of adrenaline, TAG hydrolysis increased exactly in parallel with increased blood flow, implying that TAG hydrolysis is normally limited by substrate delivery, consistent with our present study (151). Recently, we found evidence for capillary recruitment taking place in adipose tissue after an oral glucose load concomitantly with the increase in adipose tissue blood flow (unpublished data, Bülow J et al., 2010). This will expand the endothelial surface for nutrient and hormone exchange from blood to tissue. The capillary recruitment may also help to expose more LPL to its substrate and thereby promote hydrolysis of circulating TAG. Whether the vascular effects found in the present experiments partly or totally are due to increased microvascular volume changes in adipose tissue remains to be elucidated.

While TAG hydrolysis was significantly higher during the hyperinsulinemic, hyperglycemic clamp with GIP compared to the clamp without GIP, the fatty acid release was lower in the clamp experiment with GIP. This suggests that fatty acids derived from LPL-mediated hydrolysis of the circulating TAG were directed into the adipose tissue, probably to be esterified and stored, similar to what has been shown previously in subjects examined in the fed state (191). Concomitantly with the fatty acid uptake, there was

an increase in glucose uptake during GIP in combination with insulin. Taken together, these results indicate that GIP directs fatty acids released by LPL-mediated TAG hydrolysis towards tissue uptake instead of escape to the circulation. Theoretically, FFAs taken up in the adipose tissue can be oxidized, however, this is hardly likely in the present study since the RER was similar during the clamp experiments with and without GIP.
The anterior, abdominal, subcutneaous adipose tissue depot has been shown to be representative of whole-body adipose tissue (192). In the present experiments, an average TAG clearance of about 3µmol min-1 (elimination rate constant multiplied with total plasma TAG content) was found during the hyperinsulinemic hyperglycemic clamp experiments with and without GIP when calculated from the decrease in circulating TAG concentration. In the experiments with GIP an average TAG hydrolysis in the examined adipose tissue depot amounted to about 100 nmol (100g min)-1. Extrapolated to the total lipid mass in the examined subjects (estimated at 20% of body weight) this corresponds to a TAG deposition rate of about 20-25 µmol min-1. Therefore, it seems that the anterior, abdominal, subcutaneous adipose tissue may be a predilection site for GIP-mediated TAG deposition under the present experimental conditions. In the experiments without GIP, the adipose tissue TAG hydrolysis rate was in the same order as found on the whole body level.
In vitro studies have shown that GIP stimulates lipolysis (193;194). However, McIntosh and colleagues found that GIP-stimulated lipolysis in 3T3 cell line was inhibited by insulin, suggesting that GIP´s lipolytic effect is weaker than the antilipolytic effect of insulin. In the present study, we could not demonstrate any lipolytic effect of GIP under any of the experimental circumstances, indicating that if GIP has a lipolytic effect in vivo in man, it is of limited biological importance. During hyperinsulinemic, hyperglycemic clamp experiment alone or in combination with GIP, we were not able to demonstrate any differences in the splanchnic TAG output, although, a tendency towards a reduced splanchnic TAG output was seen during GIP and insulin and hyperglycemia. This reduction may be explained by an increased TAG hydrolysis in visceral adipose tissue. No differences were seen in net splanchnic FFA and glycerol uptake or glucose output, indicating that GIP does not have any biologically significant effects on hepatic carbohydrate and lipid metabolism. In a recent study, we investigated the effects of GIP in combination with insulin on TAG plasma levels after a bolus infusion of Intralipid over a 5 hour study period, and found no effect of GIP on the clearance rate of TAG (195). Since the removal of Intralipid particles is more efficient in the liver, it cannot be excluded that the lack of GIP effect found in that study might have been due to the artificial lipid formulation applied.
In conclusion, GIP in combination with insulin and light hyperglycemia increased adipose tissue blood flow, increased adipose tissue glucose uptake, increased FFA re-esterification thus resulting in increased adipose tissue TAG deposition.

CONCLUSIONS

This thesis was designed to further elucidate the mechanisms of GLP-1 and amylin effects on gastric emptying, appetite and food intake, and to elucidate the role of GIP in the regulation of lipid metabolism in humans. We conducted three clinical studies in order to achieve this goal.
We showed that gastric emptying, food intake, appetite and glucagon responses were reduced equally during GLP-1, whether amylin was present or not, and conclude therefore that GLP-1 mediates its effect on gastrointestinal motility, appetite, food intake and glucagon secretion directly and thereby in an amylin-independent fashion. Amylin, however, did have a significant effect on some of these parameters in both healthy and type 1 diabetic subjects, making it likely that amylin secretion, stimulated by GLP-1 contributes to these effects. The stable human amylin preparation allowed a comparison between amylin and pramlintide. The actions of pramlintide were rather similar to those of human amylin, confirming that the amino acid substitutions in the former have little influence on its biological activities.
With regard to effects of GIP on lipid metabolism in humans we found that GIP did not have any effect on whole body TAG clearance, but diverted TAG deposition into abdominal, subcutaneous adipose tissue. GIP in combination with hyperinsulinemia and hyperglycemia increased blood flow, glucose uptake, and FFA re-esterification, resulting in increased TAG deposition in abdominal, subcutaneous adipose tissue. Furthermore, it was not possible to demonstrate any effect of GIP per se on net lipid metabolism in the splanchnic area, either during fasting conditions or in combination with hyperinsulinemia and hyperglycemia. Finally, our results demonstrated that GIP had no effect on appetite, energy intake, gastric emptying or whole body metabolism.

PERSPECTIVES AND FUTURE RESEARCH

Some of the results presented in the present thesis give rise to additional questions, which should be addressed in future studies.
The present experiments indicate that amylin inhibits glucagon postprandially. However, since the same infusion also inhibited gastric emptying rate, the reduced glucagon response might merely reflect this, rather than representing a direct effect of amylin on glucagon secretion. Indeed, the pattern of inhibition appeared to parallel the gastric emptying rates. An elucidation of the actions of amylin on glucagon secretion will therefore require additional studies.
The present experiments indicate that GIP with insulin helps to deposit fat in the subcutaneous depot. Accordingly, these new findings suggest that an absence of GIP signaling might lead to inadequate subcutaneous deposition, leading to excess fat accumulation and increased exposure of other tissues, such as the liver and skeletal muscle. This is commonly known as “ectopic fat deposition”, and it is closely associated with insulin resistance. In light of the current proposals to employ GIP antagonists in the treatment and prevention of human obesity, it appears important to clarify whether or not the absence of GIP leads to ectopic fat storage. Additional experiments are also needed to examine the effect of GIP on lipid metabolism in obese individuals with and without insulin resistance.
GIP in combination with hyperinsulinemia and hyperglycemia increased adipose tissue blood flow. The mechanisms eliciting the blood flow increase are unknown and call for further studies with respect to whether the increased blood flow is due to vasodilatation or capillary recruitment.

SUMMARY

With approximately 400 million people worldwide today being obese, we are facing a major public health problem due to the increasing prevalence of the related co-morbidities such as type 2 diabetes, hypertension and coronary heart disease. To date, pharmacological treatment of obesity has been largely unsuccessful, only achieving modest and short-lasting reductions in body weight and with adverse effects. Scientific interest in recent years has concentrated on both the secretion and function of the incretin hormones, GLP-1 and GIP, and their suitability as new target drugs.

The potential of GLP-1 to reduce gastric emptying, appetite and food intake makes it an attractive tool in the fight against obesity and several companies are developing weight lowering drugs based on GLP-1. Currently, it is not known whether the inhibiting effects of GLP-1 on gastric emptying, appetite and food intake are directly mediated by GLP-1, or if the effects are secondary to the robust insulin responses, and thereby amylin responses, elicited by GLP-1. The first study aimed to further elucidate the mechanisms of these effects in order to strengthen the development of anti-diabetic drugs with potential weight lowering capabilities. We found that GLP-1 mediates its effect on gastrointestinal motility, appetite, food intake and glucagon secretion directly and thereby in an amylin-independent fashion.
In vitro and animal studies indicate that GIP exerts direct effects on adipose tissue and lipid metabolism, promoting fat deposition. Due to its therapeutic potential in obesity treatment, a rapidly increasing number of functional studies are investigating effects of acute and chronic loss of GIP signaling in glucose and lipid homeostasis. However, the physiological significance of GIP as a regulator of lipid metabolism in humans remains unclear. In the second study, we investigated the effects of GIP on the removal rate of plasma TAG and FFA concentrations, which were increased after either a mixed meal or infusion of Intralipid® and insulin. Under these experimental conditions, we were not able to demonstrate any effects of GIP on the removal rate of either chylomicron-TAG or Intralipid®-derived TAG concentrations. However, we found evidence for enhanced FFA re-esterification under conditions with combined high GIP and insulin concentrations. Based on findings from this study, the third study was designed to evaluate the direct effects of GIP on regional adipose tissue and splanchnic metabolism. Regional net substrate fluxes across the subcutaneous, abdominal adipose tissue and the splanchnic tissues were examined by direct measurements of arterio-venous concentration differences of various metabolites in combination with regional blood flow measurements (Fick's principle). GIP in combination with hyperinsulinemia and hyperglycemia increased blood flow, glucose uptake, and FFA re-esterification, resulting in increased TAG deposition in abdominal, subcutaneous adipose tissue. Finally, it was not possible to demonstrate any effect of GIP per se on net lipid metabolism in the splanchnic area either during fasting conditions or in combination with hyperinsulinemia and hyperglycemia.

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