THE ENTEROINSULAR AXIS IN GLUCOSE DEPENDENT INSULINOTROPIC POLYPEPTIDE RECEPTOR KNOCKOUT (GIPR-/-) MICE.

by

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Abstract

The incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are gut hormones that act via the enteroinsular axis to potentiate insulin secretion from the pancreas in a glucose-dependent manner. Both GLP-1 and GIP receptor knockout mice (GLP-1R-/- and GIPR-/- respectively) have been generated to investigate the physiological importance of this axis. Studies in this thesis were carried out on GIP receptor knockout mice (GIPR-/-). Although reduced GIP action is a component of type 2 diabetes, GIP receptor-deficient mice exhibit only moderately impaired glucose tolerance. Thus, the present thesis was directed at investigating possible compensatory mechanisms that take place within the enteroinsular axis in the absence of GIP action. Fasting and 20th minute OGTT serum GIP levels as well as duodeno-jejunal GIP content were altered in GIPR-/- mice. Total serum GLP-1 levels and serum DPIV activity in GIPR knockout mice were not significantly different from those in control animals, either before or during a glucose tolerance test. However, insulin responses to GLP-1 in pancreas perfusions and static islet incubations were significantly greater in GIPR -/- than in +/+ mice ($P<0.05$), and GLP-1 induced cAMP production was also elevated in the pancreatic islets of the knockout animals ($P<0.05$). Additionally, pancreatic insulin content and insulin gene expression were reduced in GIPR-/- mice compared to wild type (+/+) mice ($P< 0.05$). There was, however, no discernible difference in GLP-1 receptor mRNA levels. Immunohistochemistry studies revealed a normal distribution and localization of endocrine cells within the pancreatic islets of GIPR-/- mice. Surprisingly, these studies showed an increase in islet area, when

compared to total pancreatic area, in the -/- mice ($P<0.05$) with less intense staining for insulin. In conclusion, the GIPR-/- mouse exhibits increased islet size and β cell sensitivity to GLP-1 despite a decrease in pancreatic insulin protein content and gene expression. These findings suggest a critical role for GIP in normal islet and β cell function and development.

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Abreviations

OGTT	Oral Glucose Tolerance Test
IVGTT	Intravenous Glucose Tolerance Test
GIP	Glucose-dependent Insulinotropic Polypeptide/Gastric Inhibitory Polypeptide. (In this manuscript GIP_{1-42} will be referred as GIP)
GLP-1	Glucagon-Like Peptide-1 (In this manuscript GLP-1_{7-36} will be referred as GLP-1)
GIPR-/-	Glucose-dependent Insulinotropic Polypeptide Receptor knockout
GLP-1R-/-	Glucagon-Like Peptide-1 Receptor knockout
WT	Wild Type, GIPR+/+
RIA	Radioimmunoassay
DPIV	Dipeptidyl Peptidase IV
RT	Room Temperature
AUC	Area Under the Curve
CCK	Cholesystokinin
PKA	Protein kinase A
PKC	Protein kinase C
DPIV	Dipeptidyl Peptidase IV
CCK-PZ	Cholesystokinin-pancreozymin
PGK	Phosphoglycerate kinase
VDF	Vancouver diabetic fatty zucker rat

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INTRODUCTION

The Enteroinsular Axis

In the beginning of the twentieth century, Bayliss and Starling (1902) reported the possible existence of a substance released from the gut after ingestion of nutrients that modulates carbohydrate disposal by generating endocrine pancreatic responses. Subsequently, in 1906 Moore et al. postulated that the "internal secretion of the pancreas might be stimulated by a chemical excitant produced by the duodenal membrane". Their attempts to treat diabetic patients with hog duodenal mucous membrane acid extracts was however unsuccessful. Two decades later this approach was pursued by Zunz and La Barre (1929), who rendered dogs hypoglycemic by i.v. injections of secretin-free intestinal extracts. In 1932, La Barre proposed that the intestinal extract contained an active substance called 'incretin' that might enhance the endocrine secretion of the pancreas by its humoral activity. Loew et al. (1939, 1940a, 1940b) after trying elaborately different methods failed to lower blood glucose levels with intestinal mucosal extracts from dogs or hogs.

For the following 20 years until the development of the radioimmunoassay technique for insulin, research on possible intestinal blood glucose lowering factors stagnated. In 1960, Yalow and Berson enabled the measurement of the circulating levels of insulin by the development of an insulin radioimmunoassay (RIA). RIA studies from two different goups, McIntyre *et al.* (1964) and Elrick *et al.* (1964) demonstrated in

humans that a glucose load that acts directly on the intestine (oral glucose or intrajejunal administration) is a more potent stimulator of insulin than glucose given intravenously. It was concluded in another study conducted on healthy subjects and patients with end to side portcaval shunts, that after intrajejunal glucose infusion, an increased insulin response and glucose clearance was due to the incretin originating from the intestinal mucosa, and not from the liver (McIntyre *et al.*, 1965). The contribution of incretins to insulin secretion was quantified by Perley and Kipnis (1967) who found that intestinal stimulation accounts for more than 50 percent of the insulin secretion following a meal in diabetic and non-diabetic subjects.

In 1969, Unger and Eisentraut proposed the term *enteroinsular axis* to describe the hormonal connection between pancreatic islet cells and the gastrointestinal tract. Ten years later Creutzfeldt (1979) enlarged the definition by including the neural and substrate influences along with the hormonal connection (Figure1). Two new terms, incretin and enteroinsular axis, ushered in a new age of research in the understanding of glucose metabolism. Two criteria were defined by Creutzfeldt to identify a substance as an incretin in the enteroinsular axis

1. The substance must be released in response to nutrients, particularly carbohydrates present in the lumen of the gut

2. When administered at physiological concentrations the insulinotropic action must be glucose concentration dependent

According to these criteria, an incretin is a gut hormone released after a meal that has insulinotropic effects on the pancreas in the presence of elevated blood glucose. Thus, a true incretin cannot by itself stimulate insulin secretion and therefore cause

hypoglycemia. Several hormones were not accepted as incretins since they did not meet the requirements (Creutzfeldt, 1979, 1992). Today two hormones of the enteroinsular axis are considered as incretins: glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP $_{1-42}$), and truncated forms of glucagon-like peptide-1 (GLP-1_{7-36} and GLP-1 $_{7-37}$) (Fehmann *et al.*, 1995; Pederson, 1994).

The work presented in this thesis concerns only the hormonal aspects of the enteroinsular axis, thus the incretins GIP and GLP-1. Therefore, the introduction will focus on the endocrine part of the enteroinsular axis.

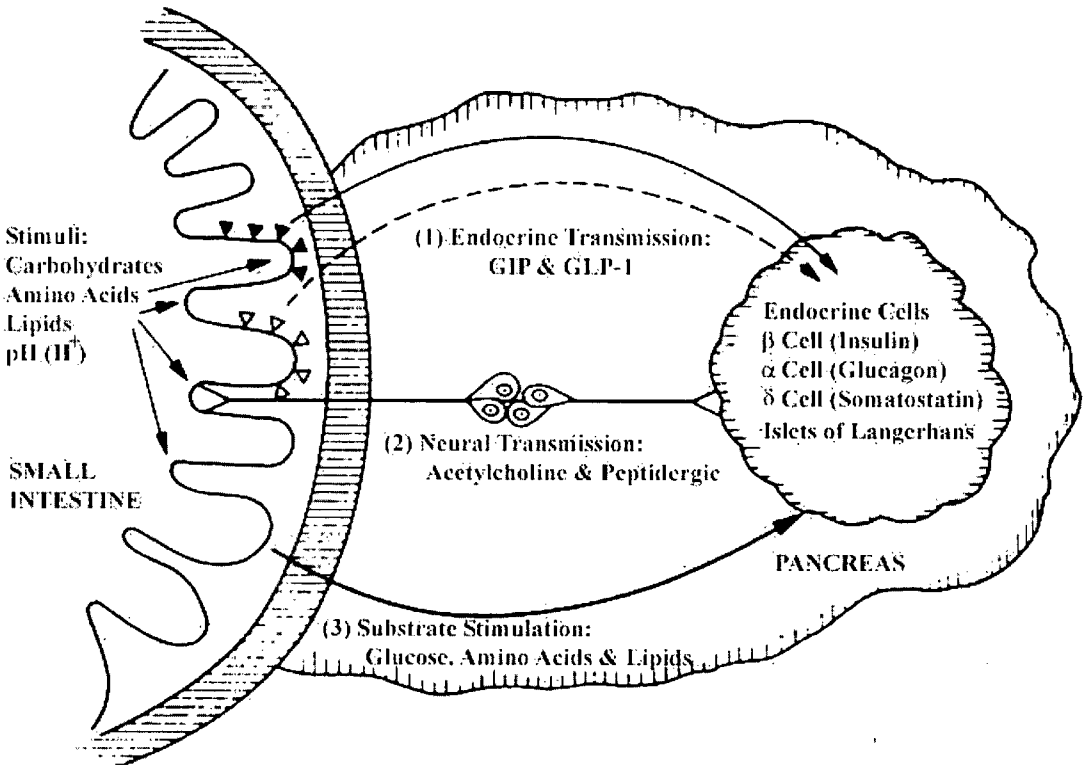


Figure 1- **The enteroinsular axis.** The hormonal, neural and substrate directed communication of the gut with the endocrine cells of the pancreas. Reproduced from Creutzfeldt, 1979.

Glucose Dependent Insulinotropic Polypeptide (GIP)

GIP was first isolated in 1970 by Brown and Pederson as an 'enterogastrone'. An enterogastrone is an endocrine substance secreted in response to fat and its digestive products present in the intestinal lumen with a primary function of inhibiting gastric acid secretion (Kosaka and Lim, 1930). GIP was isolated from impure preparations of cholesystokinin –pancreozymin that were tested for their ability to stimulate gastric acid secretion in vagally denervated canine Heidenhain pouch bioassays (Brown *et al.*, 1970, Brown and Pederson, 1970). Interestingly, the more impure preparation was found to be more effective in inhibiting gastric acid secretion. It was concluded that the CCK-PZ preparation contained an active substance that inhibits gastric acid secretion and it was given the name Gastric Inhibitory Polypeptide, GIP (Brown *et al.* 1970).

Studies demonstrating that impure preparations of CCK exhibited insulinotropic activity were first provided by Dupré and Beck (1966). Subsequently, it was shown that the insulinotropic action of these preparations could be removed by increasing the purity. This finding was very similar to what had been observed by Brown *et al.* for the gastric inhibitory action of the CCK-PZ extracts (Rabinovitch and Dupre, 1972). Shortly after, in 1973, the insulinotropic action of GIP was reported by Dupré *et al.* They infused purified preparations of porcine GIP in humans in the presence of glucose and observed that a greater amount of insulin was released when compared to glucose infusion alone. The same study also showed that GIP had no insulinotropic action during the euglycemic state suggesting that the insulin stimulatory action was glucose dependent. Insulin release was observed in the presence of at least 5.5 mM glucose in perfused rat pancreas (Pederson,

and Brown, 1976; Jia *et al.*, 1995). The glucose- dependent insulinotropic action of GIP was further verified in humans by glucose clamp studies (Elahi *et al.*, 1979).

The glucose-dependency of GIP action provides an important protection against hypoglycemia that might occur due to inappropriate insulin release in response to a low carbohydrate, high fat meal. This led Brown and Pederson (1976) to the alternate designation of GIP as glucose dependent insulinotropic polypeptide.

GIP Secretion

Polak *et al.* (1973) showed using a conventional antiserum that immunoreactive GIP was localized in the duodenum and jejenum in man and dog. Later, immunoreactive GIP cells were also located in the upper small intestine of humans, dogs, pigs, rats, and ruminants (Bufa *et al.*, 1975; Buchan *et al.*, 1982). Tseng *et al.* (1993) detected by Northern blot analysis an 800-nucleotide GIP transcript with a dense distribution through out the duodenum and jejenum and with strong attenuation in the ileum. It has been shown by Polak *et al.* (1982) in man and dog that GIP is primarly present in the cells of the midzone of the duodenal villi with a lesser intensity in the jejenum. Buchan *et al.* (1978) identified the cell of origin for GIP as the K cell of the intestinal mucosa, defined by the typical appearance of the secretory granules indicative of an endocrine cell.

The development of an RIA for GIP was a turning point in the investigation of the physiology of GIP (Kuzio *et al.*, 1974). The use of different antibodies resulted in widely varied fasting and postpandrial plasma GIP measurements. Kuzio *et al.* (1974) reported fasting GIP levels in healthy subjects of 237±14 pg/ml rising to 1200 pg/ml after a mixed meal. Although other researchers have reported different values ranging from 60-460

pg/ml for fasting and from 170-1470 pg/ml after a meal, the percentage increase relative to basal values was comparable in each case (Kuzio *et al.*, 1974; Morgan *et al.*, 1978; Jorde *et al.*, 1983; Cataland *et al.*, 1974; Sarson *et al.*, 1980; Burhol *et al.*, 1980). Despite the variability of reports, it has been shown that GIP levels rise 5 to 6 fold within an hour of ingestion of a mixed meal and the levels stay elevated for the next 2-3 hours.

To determine the effects of carbohydrate ingestion on GIP secretion Cataland et *al* (1974) administered 1g/kg glucose orally to healthy subjects and measured plasma GIP levels. The plasma glucose and insulin profile was accompanied by a similar plasma GIP profile. Shortly after, Pederson *et al.* (1975) in dogs and Falko *et al.* (1980) in humans reported a dose dependent relationship between glucose ingestion and plasma GIP levels. Studies with i.v. injection of glucose resulted in no changes in circulating GIP levels, therefore it was concluded that the luminal presence of carbohydrates is necessary to stimulate GIP secretion (Pederson *et al.*, 1975; Cataland *et al.*, 1974; Andersen *et al.*, 1978). Furthermore, small intestinal perfusions with glucose, sucrose, and galactose resulted in GIP secretion, whereas, fructose, mannose, and lactose perfusions had no effect (Sykes *et al.*, 1980). Evidence was reported by Kieffer *et al.* (1994, 1995) in studies on canine endocrine cells and on a tumor derived cell line that glucose acts directly at the level of K cells to stimulate GIP release. The intracellular mechanisms of this stimulation are not clear as yet, however it has been demonstrated that sodium-dependent transport of the carbohydrates is necessary, and when glucose transport is inhibited so is glucose stimulated GIP secretion (Hopfer, 1987)

Intraluminal triglycerides have been also shown to stimulate GIP release in man, dogs, and rats (Cleator, 1975; Krarup *et al.*, 1985; Pederson, 1975). The delays caused by

lipids on the absorption and on gastric emptying results in a different GIP secretory profile than with carbohydrates. Hence, fat ingestion results in delayed but more potent and prolonged secretion of GIP (Falko *et al.*, 1975; Brown and Otte, 1978). It has been shown that only long chain fatty acid were able to stimulate GIP secretion whereas, medium and short chain fatty acids have little effect (O'Dorisio *et al.*, 1976; Ross and Shaffer, 1981).

Although the first aproaches in the early1970s by Brown *et al.*, and Cleator and Gourlay failed to detect the stimulation of GIP secretion by proteins, later on, it was shown that, in addition to glucose and fat, the intraluminal administration of protein also stimulates GIP secretion (Brown, 1974; Cleator and Gourlay, 1975). Thomas *et al.* (1976, 1978) observed GIP secretion when they perfused the duodenum with an amino acid mixture containing arginine, histidine, isoleucine, lysine, and threonine. The rise in serum GIP levels occurred within 5 min of administration of the perfusate and reached peak values within 30 min. In contrast perfusate containing methionine, phenylalanine, tryptophan, and valine, a mixture known to stimulate CCK release, failed to stimulate GIP secretion (Thomas *et al.*, 1978).

Structure of GIP

The complete amino acid sequence of porcine GIP_{1-42} was first described by Brown and Dryburgh (1971). Later, it was shown that this initial sequencing did not correspond exactly to the structure of GIP due to some impurities in the extract. The sequence was corrected by Jörnwall *et al.* in 1981. The human GIP cDNA was sequenced by Takeda *et al.* (1987) and the complete GIP peptide sequence was reported by Moody

et al. (1984) from human intestinal tissue. Shortly after, the rat GIP gene was also isolated and sequenced (Inagaki *et al.*, 1989). The mouse GIP sequence was reported recently by Schieldrop *et al.* in 1996. Comparison of GIP sequences among species revealed more than 90% conservation at the amino acid level. The human sequence differs by only two amino acids from the porcine and rat sequences and by three amino acids from mouse and bovine sequences (Figure2). It is also important to note that size differences exist in the GIP gene among species, the human GIP gene spans about 10 kbp wheras rat GIP gene is only 8.2 kbp (Inagaki *et al.*, 1989; Higashimoto and Liddle, 1993). It has been also shown that these size differences are mainly due to the arrangements and variations in the lengths of the introns (Higashimoto *et al.*, 1992; Inagaki et al., 1989).

The understanding of the structure of the preproGIP gene and the computer analysis of the amino acid sequence of GIP have shown that structural homology exists between GIP and other members of the secretin family including secretin, glucagon and glucagon-like peptides (GLP-1 and GLP-2). (Bell, 1986; Campbell and Scanes, 1992; Tseng *et al.*, 1993).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Human	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met
Porcine														
Rat														
Mouse														

	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Human	Asp	Lys	Ile	His	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala
Porcine				Arg										
Rat				Arg										
Mouse				Arg										

	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Human	Gln	Lys	Gly	Lys	Lys	Asn	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln
Porcine						Ser								
Rat												Leu		
Mouse		Arg				Ser								

Figure 2- **Homology for GIP amino acid sequence between species.** This comparaison is based on human sequence, only amino acids that differ from the human sequence are shown. Sequences are from Moody *et al.* (1984), Jörnvall *et al.* (1981) Higashimoto *et al.* (1992), and Schieldrop *et al.* (1996) for human, porcine, rat and mouse respectively.

The GIP Receptor

The GIP receptor is a member of the secretin-VIP family of G protein-coupled receptors with seven transmembrane domains, an intracellular carboxy (C) terminus and an extracellular amino (N) terminus that is involved in ligand binding (Usdin *et al.*, 1993; Gelling *et al.*, 1997; Ulrich *et al.*, 1998; Fehmann *et al.*, 1995). In 1993 Usdin *et al.* analyzed a partial cDNA from rat cerebral cortex and a full length cDNA from a rat tumor cell line (RINm5F) encoding a putative seven transmembrane G-coupled receptor

of 455 amino acids and demonstrated a 44% homology with the glucagon receptor and 40% homology with the GLP-1 receptor.

The GIP receptor was first shown to be present in hamster insulinoma cells, and hamster β cell line and later in human insulinomas and β-TC3 cell lines (Maletti *et al.*, 1984; Amiranoff *et al.*, 1984, 1985). These studies suggested that GIP acts directly on the β cell of the pancreas to stimulate insulin release.

Multiple studies have shown that stimulation of the GIP receptor results in activation of the adenylyl cyclase- cAMP pathway resulting in elevated intracellular cAMP levels in islet tumor cell lines and pancreatic islets leading to insulin secretion (Amiranoff *et al.*, 1984; Siegel and Creutzfeldt, 1985; Wheeler *et al.*, 1995). In addition, it has been also shown by several groups that GIP binding to its receptor increases intracellular Ca^{2+} levels in HIT-T 15, RINm5F, and COS cell lines (Wheeler *et al.*, 1995; Wahl *et al.*, 1992; Usdin *et al.*, 1993; Lu *et al.*, 1993 a). It is believed that this increase is partly due to the increased Ca^{2+} uptake and or direct stimulation of Ca^{2+} channels. Although there is no evidence of activation of PI3 kinase directly by GIP, Kubota *et al.* (1997) recently reported that GIP activates mitogen activated protein kinase via a wortmannin (a phosphatidylinositol 3-kinase inhibitor)-sensitive pathway. Ehses *et al.* (2001) suggested a new signalling pathway for GIP in the β-TC3 cell line in which GIP stimulates phospholipase A2 and arachidonic acid production leading to the secretion of insulin via a Ca^{2+} independent pathway.

Studies with Northern blot analysis, RT-PCR, and *in situ* hybridization have reported a ubiquitous distribution of GIP receptor mRNA in rat tissues, specifically in pancreas, stomach, intestine, adipose tissue, adrenal cortex, heart, lung and endothelium

of major vessels (Usdin *et al.*, 1993; Yasuda *et al.*, 1994). Interestingly, although GIP receptor mRNA transcripts are present in several regions of the brain, multiple studies were unable to detect GIP mRNA or GIP itself in the brain (Tseng *et al.*, 1993; Higashimoto *et al.*, 1992; Buchan *et al.*, 1982).

GIP Actions on the pancreas

The actions of GIP on pancreatic β cells has been discussed previously. Additionally, GIP also has effects on other endocrine cells of the pancreas.

GIP has been reported to stimulate glucagon release from islet α cells (Pederson and Brown, 1978). This effect was observable only when glucose levels were below 5.5 mM in perfused rat pancreas. Studies in humans by Elahi et *al.* (1979) have shown that although GIP was able to stimulate glucagon release in the presence of higher glucose concentrations, glucose-inhibited glucagon release was not restored by GIP. However, GIP was able to stimulate glucagon release in perifused mouse islets (Opara and Go, 1991). Verchere (1991) reported that stimulation of isolated rat islets with GIP following 2 days of incubation increases glucagon, insulin and somatostatin release. It seems that the action of GIP on α cells is variable between species.

GIP has also been shown in the perfused rat pancreas to be a weak stimulator of somatostatin release from islet δ cells (Schmid et al., 1990). No conclusion has been reached on whether this stimulation is physiologically significant or not.

Extrapancreatic actions of GIP

As discussed earlier, GIP receptors are widely distributed in the body. Although, the physiological importance is yet unclear this suggests that GIP might have other roles in addition to enterogastrone and incretin actions. Recently, attention has focused on the actions of GIP on adipose tissue. The actions of GIP on lipid metabolism have been reviewed by Morgan *et al.* (1996) and Wolfe *et al.* (1999). Wasada *et al.* (1981) have shown in dogs that GIP increases the clearance of injected chylomicron triglycerides. Similar results have been reported from porcine GIP infusion into rats receiving intraduodenal lipid (Ebert *et al.*, 1991). However, in humans exogenous porcine GIP and endogenous GIP released after a mixed meal had no effect on the clearance rate of soy oil given by *i.v.* injection (Jorde and Burhol, 1984). Furthermore, treatment of rat adipocytes with GIP improved insulin stimulated glucose uptake and increased insulin receptor affinity (Starich *et al.*, 1985; Hauner *et al.*, 1988). It has been also shown that GIP increases lipoprotein lipase activity in a concentration dependent manner in cultured 3T3-L1 cell line (Eckel *et al.*, 1981). Using the same cell line, McIntosh *et al.* (1999) suggested that GIP stimulates lipolysis and this could be inhibited by insulin via a wortmannin-sensitive pathway. Taken together these results strongly implicate GIP in the regulation of fat metabolism.

Although there is no evidence of GIP receptor mRNA in the liver, GIP has been reported to inhibit glucagon stimulated hepatic glucose production in human and rat liver (Hartmann *et al.*, 1986; Elahi *et al.*, 1986). The mechanism of this action is still unclear but it may be an indirect effect or signaling via glucagon receptors (Wolfe *et al.*, 1999).

Glucagon-Like peptide-1 (GLP-1)

Following the discovery of GIP, it was observed that, despite the removal of GIP action by immunoneutralization, the incretin effect was still conserved (Ebert *et al.*, 1983). The possibility of a second incretin was resolved in the early 1980's with the development of recombinant DNA technology that enabled the isolation of cDNA encoding the preproglucagon from the pancreata of the anglerfish (Lund *et al.*, 1983). The strong resemblance between the glucagon related peptide section of preproglucagon and GIP led this group to conclude that glucagon related peptide might be the second incretin in the enteroinsular axis (Lund *et al.*, 1982). It was concluded that the anglerfish glucagon related peptide-1 is a homolog of mammalian GLP-1. It has been shown by Bell *et al.* (1983) in hamsters that GLP s are encoded by the glucagon gene and are a product of post translational processing of proglucagon (160 aminoacid length peptide; PG1-160). Upon analysis of the glucagon gene in different species, two carboxy-terminal glucagon like sequences were identified: GLP-1 and GLP-2 (Bell *et al.*, 1983; Lopez *et al.*, 1983; Heinrich *et al.*, 1984).

GLP-1 is widely distributed throughout the body, the majority being located in L cells of the small and large intestine, pancreatic α cells, and in the brain (hypothalamus, thalamus, and septal regions)(Mojsov *et al.*, 1990; Orskov *et al*, 1992, 1994; Shimizu *et al.*, 1987; Jin *et al.*, 1988). The L cells are mainly localized in the distal jejenum and ileum with an increasing abundance in the colon and rectum (Eissele *et al.*, 1992). Despite this wide distribution, the majority of circulating GLP-1 is expressed from the proglucagon gene in the α cells and in the L cells by a tissue-specific process (Figure3) (reviewed by Kieffer and Habener, 1999, Fehmann *et al.*, 1995; Orskov, 1992).

The intestinal processing of PG-1 160 leads to glicentin (PG1-69), oxyntomodulin (PG33-69), intervening peptide 2, GLP-1 (1-37), and GLP-2. (Figure3). It has been shown that GLP-1 undergoes further proteolytic cleavage between the sixth and seventh amino acid leading to the predominant end product of intestinal PG1 processing: GLP-1 (7-36) (Schmidt *et al.*, 1985; Mojsov *et al.*, 1986; Orskov *et al.*, 1989). This truncated form has greater homology with GIP and enhanced insulinotropic activity (Schmidt, 1985; Holst, 1987). It has been shown by several groups that GLP-1 (7-36) is the principal GLP-1 related peptide secreted after a meal from the gut and it corresponds to 80% of circulating GLP-1 levels in humans (Fehmann *et al.*, 1995) .

The pancreatic α cell processing of PG-1 leads to different post-translational end products with pancreatic glucagon (PG33-61) as the main end product (Figure3). Other products are secreted in equimolar amounts: glicentin related pancreatic polypeptide (PG1-30), intervening peptide-1 (PG64-69), and the major proglucagon fragment (PG72-158) corresponding to GLP-1 and GLP-2 (Figure3)(Orskov *et al.*, 1994; Holst *et al.*, 1994; Mojsov *et al.*, 1986).

Proglucagon processing in the brain is similar to that in the intestine, leading to oxyntomodulin, glincentin, GLP-1 and GLP-2 (Figure3) (Larsen *et al.*, 1997; Yoshimoto *et al.*, 1989). The *in situ* hybridization studies revealed proglucagon mRNA in the cell bodies of paraventricular nucleus, medulla oblongata, dorsal and ventral part of medullary reticular nucleus. It is believed that the processing of the proglucagon occurs within these cells (rewieved by Kieffer and Habener, 1999).

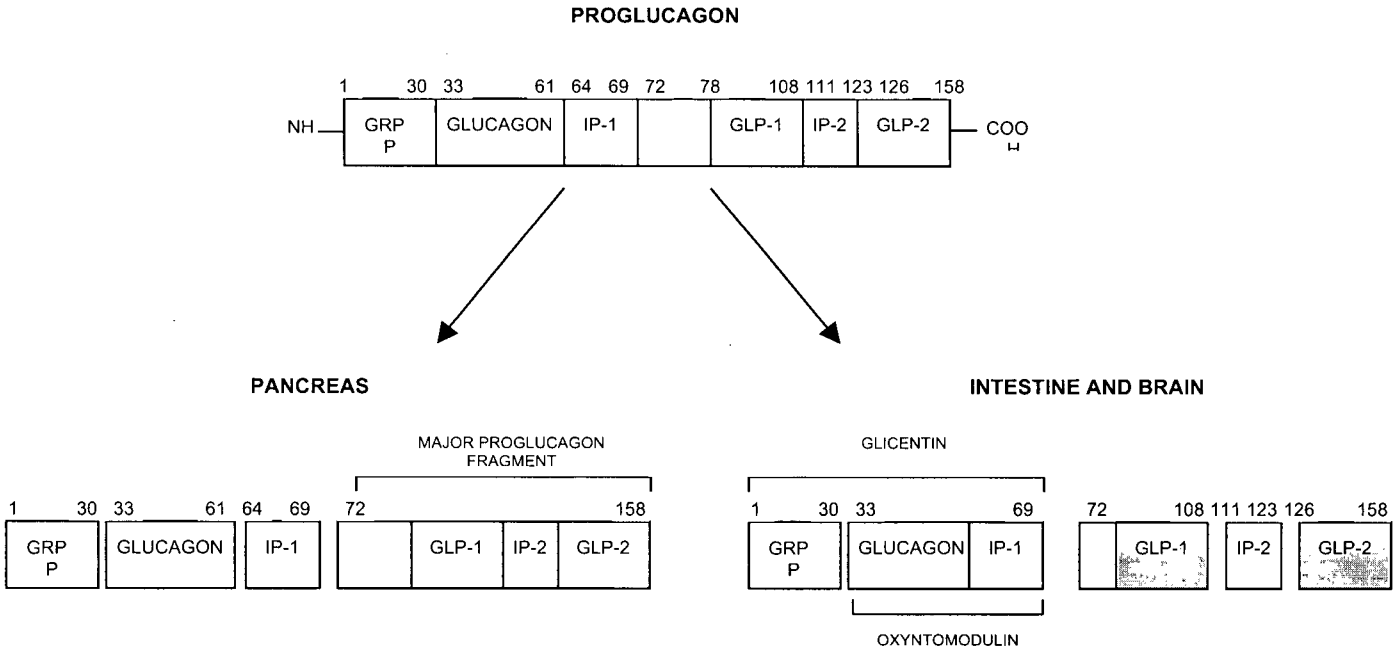


Figure 3- **Postranslational processing of proglucagon in pancreas, intestine, and brain.** Glucagon is the main product resulting from processing of proglucagon in α cells. In intestine and brain GLP-1 and GLP-2 are formed following similar enzymatic cleavages. More than 80% of the circulating GLP-1 levels in humans correspond to GLP-1 (7-37 amide) form produced mainly in the intestine. Adapted from Fehmann *et al.* (1995) and Mojsov *et al.* (1986)

GLP-1 Secretion

As discussed previously, small quantities of GLP-1 are produced in α cells and cosecreted with glucagon, whereas the main portion of circulating GLP-1 originates in intestinal L cells. It has been shown that ingestion of nutrients stimulates intestinal GLP-1 secretion, although, the L cells of the distal intestine are not directly exposed to nutrients

(Elliott *et al.*, 1993; Morgan *et al.*, 1993; Orskov *et al.*, 1994). As will be discussed below, this led to the suggestion that GLP-1 secretion is a combined result of nutrient, hormonal and neural stimulation.

Circulating GLP-1 levels have been shown to rise within 15-30 min. and reach peak levels 90 min. after ingestion of a mixed meal in normal man (Orskov *et al.*, 1994). As is the case with GIP, reported GLP-1 levels differ from study to study according to the antisera used, with reported peak levels of about 6 times the basal levels reaching up to 50 pM (Orskov *et al.*, 1994). To investigate the mechanism of GLP-1 secretion, *in vitro* techniques such as development of intestinal cell lines, and tumor derived cell lines expressing the glucagon gene products (i.e. GLUTag, and STC-1) have been used (Brubaker *et al.*, 1992; Drucker *et al.*, 1992). *In vitro* studies demonstrated that PKA and PKC are important intracellular regulators of GLP-1 secretion. PKA has been shown to stimulate GLP-1 gene expression and secretion in intestinal cell cultures of canine, rat and mouse ileum, and in GLUTag tumor cell line (Damholt *et al.*, 1998; Saifia *et al.*, 1998; Drucker *et al.*, 1994, Brubaker *et al.*, 1998). PKC has also been shown to stimulate GLP-1 release in various experiments with intestinal cell cultures and GLUT-ag and STC-1 cell lines, but it was unable to stimulate peptide synthesis (Drucker *et al.*, 1994; Brubaker *et al.*, 1998; Abello *et al.*, 1994). Additionally, increased intracellular Ca^{2+} was shown to inhibit GLP-1 release suggesting a role for calcium in the basal regulation of GLP-1 secretion (Brubaker, 1988).

Oral ingestion or intestinal injection of glucose stimulates GLP-1 release in humans, pigs, dogs, rats, and, mice (reviewed by Kieffer and Habener, 1999; Fehmann *et al.*, 1995). It has been shown that intestinal administration of glucose must be

accompanied by sodium in order to stimulate GLP-1 release (Hermann *et al.*, 1995; Sasaki *et al.*, 1993). This implies that glucose absorption is necessary to stimulate GLP-1 secretion and that this occurs through sodium/glucose transporters on intestinal villi. This is consistent with the information that intestinal infusion of the non-transportable carbohydrates (e.g. fructose, and lactose) does not stimulate GLP-1 release (Kong *et al.*, 1999; Horowitz *et al.*, 1996). In contradiction, recently, Rayner *et al.* (2000) compared the effects of glucose and fructose on GIP and GLP-1 secretion in healthy humans. Intraduodenal infusions of glucose and fructose stimulated similar levels of GLP-1 secretion. Furthermore, a recent study by Damholt *et al.* using canine L cells has shown that GLP-1 secretion is not affected by different concentrations of glucose (Damholt et al., 1998b). This implies that factors other than direct stimulation of L cells by glucose are in place since carbohydrates are digested before reaching the distal intestine. Additionally, the rapid rise in GLP-1 levels, after an oral glucose load suggests that glucose alone cannot account for direct GLP-1 stimulation.

In addition to glucose, the ingestion of tryglycerides or fatty acids stimulates GLP-1 secretion from the gut. Studies in humans, dogs and rats have shown that as for glucose ingestion, intraluminal administration of fats and or triglycerides stimulates GLP-1 release (Hermann *et al.*, 1995; Elliott *et al.*, 1993, Layer *et al.*, 1995). Additionally, it has been shown that fatty acids are able to act directly on L cells (Rocca and Brubaker, 1995; Brubaker, 1991). Interestingly, despite the increasing abundance of L cells in the distal intestine, studies have shown that administration of fat to the duodenum or to the ileum does not modify the magnitude of GLP-1 secretion (Roberge and Brubaker, 1993; Brubaker, 1991). This observation led Roberge and Brubaker (1993) to suggest that there

is a proximal-distal loop regulating nutrient stimulated GLP-1 secretion from the L cells. Neural, and endocrine factors are thought to contribute to this loop. Hence, the rapid rise in circulating GLP-1 levels after administration of a mixed meal may be partly explained by the operation of the proximal–distal loop. In support of this, it has recently been shown that disruption of the subdiaphragmatic vagus nerve in rats abolishes fat induced GLP-1 release (Rocca and Brubaker, 1999).

A wide range of studies in different species have reported that proteins when administered alone do not stimulate GLP-1 secretion (Hermann *et al.*, 1995; Layer *et al.*, 1995). However, recently, Cordier-Bussat *et al.* (1998) have shown that peptones are able to stimulate GLP-1 secretion from the perfused rat intestine, and murine STC-1 cells. The same group has also been able to show an increase in proglucagon gene expression in STC-1 and GLUT-ag cell lines.

In addition to nutrients, various hormones have been reported to feedback on GLP-1 secretion. Both *in vitro* and *in vivo* insulin has been reported to inhibit GLP-1 secretion (Matsuyama *et al.*, 1975; Brubaker and Vranic, 1987). Intestinal somatostatin, a general endocrine inhibitor, has been reported to inhibit GLP-1 secretion (Brubaker, 1991, Damholt et al., 1998b). Interestingly, recent studies with immunocytochemistry and intestinal cell cultures in dogs have shown that GIP stimulates GLP-1 secretion (Damholt *et al.*, 1998a, 1999). These studies have suggested that glucose stimulates GIP secretion from the proximal intestine, which in turn acts on L cells to stimulate GLP-1 release: the concept of proximal-distal loop. However, the existence of a similar loop was not shown in humans. In addition Rocca and Brubaker have recently shown that disruption of several hepatic vagal branches abolishes GIP induced GLP-1 release and

stimulation of subdiaphragmatic vagus nerve also stimulates GLP-1 secretion (Rocca and Brubaker, 1999). Taken together, these data suggest GIP stimulated GLP-1 secretion. The pathway of this stimulation is yet still unclear. It might be a paracrine interaction between K and L cell or might involve vagus mediated signaling. (reviewed in Kieffer and Habener, 1999).

The GLP-1 receptor

GLP-1 receptors are present on tumor derived β and α cell lines, pancreatic islets, lung membranes, stomach, intestine, heart, kidney and brain (Shimizu *et al.*, 1987; Richter *et al.*, 1990; Wei and Mojsov, 1995; Bullock *et al.*, 1996; Yamato *et al.*, 1997; Gros *et al.*, 1992). Despite existence of reports on the actions of GLP-1 on liver, adipose, and skeletal tissue, investigations involving RT-PCR and in situ hybridization have failed to find GLP-1 receptors on these tissues (Wei and Mojsov, 1995; Bullock *et al.*, 1996). Intra-islet distribution of GLP-1 receptors has also been studied. Bullock *et al.* (1996) reported the colocalization of insulin and GLP-1 receptor mRNA. They were unable to determine whether the mRNA was present in the α cells or not. Recently, Heller *et al.* (1997b), using GLP-1 receptor and islet hormone specific antibodies on dispersed islet cells, reported that the GLP-1 receptor was present on 90% of insulin positive cells, 76% of somatostatin positive cells and 20% of glucagon positive cells.

As has been previously discussed for the GIP receptor, the GLP-1 receptor is also a member of the G protein coupled family of receptors including glucagon, VIP, secretin, GIP, PACAP, GHF, calcitonin and PTH (reviewed in Kieffer and Habener). The 463 amino acid receptor has eight hydrophobic segments with an extracellullar N terminus

responsible for ligand binding and an intracellular C terminus responsible for intracellular signaling (reviewed in Kieffer and Habener). Ligand-binding studies have shown that GLP-1 receptors have a specificity for GLP-1 in the nanomolar range whereas other peptides from the glucagon family do not bind to the receptor (Fehmann *et al.*, 1994; Kieffer *et al.*, 1996). Recently Moens *et al.* (1998) reported that glucagon binds to GLP-1 receptors on islet β-cell cultures with very low affinity (millimolar range).

As for all other members of VIP-secretin receptor family, the primary signaling pathway is believed to be via the adenylate cyclase-cAMP pathway. For the GLP-1 receptor this has been demonstrated in β cell lines, isolated β cells and isolated islets (Drucker *et al.*, 1987; Ahren *et al.*, 1996; Moens *et al.*, 1996). The receptors present on the surface of the cells are coupled to stimulatory G proteins leading to the stimulation of adenylyl cyclase and a rise in intracellular cAMP levels. cAMP in turn stimulates the protein kinase A and cAMP dependent Ca^{2+} pathways that both conribute to the rise of intracellular Ca^{2+} leading to insulin secretion (reviewed in Kieffer and Habener, 1999 and in Gromada *et al.* 1998). Recently, it has been shown that activation of the cAMP/PKA pathway by GLP-1 improves the effectiveness of the K-ATP channel-independent action of glucose (Yajima *et al.*, 1999). Studies have shown that activation of cAMP/PKA pathway in β cell results in increased intracellular Ca^{2+} levels only in the presence of glucose (Yajima *et al.*, 1999; Gromada *et al.*, 1998). It has been proposed that GLP-1 contributes to β cell glucose responsiveness (glucose competence of the β cell) mainly by this process.

GLP-1 Actions on the pancreas

The insulinotropic action of GLP-1 was first demonstrated in the isolated perfused pancreas of rat, pig and dog (Holst *et al.*, 1987; Mojsov *et al.*, 1987; Kawai *et al.*, 1989). Later, in humans it was shown that the presence of glucose and GLP-1 in the circulation stimulates insulin release (Kreymann *et al.*, 1987). In vivo studies using the GLP-1 receptor antagonist exendin (9-39) have shown that GLP-1 accounts for an important portion of the incretin effect in rats, baboons and recently in man (reviewed in Kieffer and Habener, 1999). Several studies have shown that GLP-1 also exhibits glucose dependency, the threshold glucose dose varying between species and experiments (using different doses of GLP-1) between 2.8 and 5.0 mM (Fridolf and Ahren, 1991, Hargrove *et al.*, 1995; Ahren *et al.*, 1995) The glucose dependence of GLP-1 is an important measure to protect from the hypoglycemia caused by elevated circulating insulin levels. The interdependence between glucose and GLP-1 action implicates a synergism between glucose metabolism and activation of GLP-1 receptor signaling cascades leading to the glucose competence concept where glucose is required for GLP-1 action and GLP-1 is required to improve β cell responsiviness to glucose (Holz *et al.*, 1993).

GLP-1 has also been shown to stimulate proinsulin gene transcription and biosynthesis in islets and insulinoma cell line (Fehmann and Habener, 1992; Drucker *et al.*, 1987). Recently, it has been also demonstrated that GLP-1 contributes to the proliferation and neogenesis of β cells in rodents by activation of cell proliferation and differentiation transcription factors such c-fos, c-jun, jun B (Xu *et al.*, 1999; Edvell and Lindstrom, 1999; Susini *et al.*, 1998).

GLP-1 also has effects on the other endocrine cells of the pancreas. There is evidence that GLP-1 inhibits glucagon secretion in vivo. Perfused pancreas studies in pigs, rats and dogs resulted in up to an 80% decrease in pancreatic glucagon output (Orskov *et al.*, 1988; Kawai *et al.*, 1989). Later, these reports were supported with similar findings in human islet studies (Fehmann *et al.*, 1995b). The glucagonostatic action of GLP-1 is believed to contribute to its glucose lowering effects.

As has been previously introduced, GLP-1 receptors are also present on the surface of δ cells. Perfused pancreas and islet studies have reported that GLP-1 stimulates somatostatin secretion (Kawai et al., 1989; Schmid, 1990). Additionaly, this effect has been shown in human islet studies (Fehmann et al., 1995b). It is widely discussed whether the glucagonostatic effects of GLP-1 are mediated directly on the α cell or via the stimulation of islet somatostatin. In either case GLP-1 has a role in intra islet hormone regulation.

Extrapancreatic actions

In addition to its insulinotropic action, GLP-1 has well documented extrapancreatic actions. As discussed previously, tryglicerides and fats are potent stimulators of GLP-1 secretion. The presence of partially digested fat in the ileum contributes to the *ileal brake* by slowing gastric emptying and jejunal motility (Layer and Holst, 1993; Lin *et al.*, 1997; Layer *et al.*, 1995). It has been shown in humans that GLP-1 infusion in an amount equal to postpandrial plasma GLP-1 concentrations, results in inhibition of gastric acid secretion and gastric emptying (Willms *et al.*, 1996; Schirra *et al.*, 1996; Nauck *et al.*, 1997b). The mechanisms underlying these effects remain unclear

and confusing since possible mechanisms differ from species to species. In rats, there is evidence suggesting that the gastric action of GLP-1 is mediated through inhibition of gastrin secretion and stimulation of gastric somatostatin (Eissele *et al.*, 1990; Jia, 1994). Whereas in humans and pigs the involvement of central nervous system seems more probable (reviewed in Kieffer and Habener, 1999; Wettergren *et al.*, 1998).

Interestingly, despite the absence of evidence showing the existence of GLP-1 receptors on liver, fat and muscle tissue, GLP-1 has been shown to have glycogenic and lipogenic actions in these tissues (reviewed in Kieffer and Habener, 1999). The reported effects of GLP-1 on hepatic glucose metabolism are controversial. GLP-1 has been reported to improve or to not change insulin-independent hepatic glucose absorption (Gutniak *et al.*, 1992; D'Alessio *et al.*, 1994). This information led researchers to focus on the possible insulin like effects of GLP-1. Initial reports on diabetic models suggested that GLP-1 was able to increase peripheral glucose uptake (D'Alessio *et al.*, 1994, 1995). However, subsequent studies in healthy man were unable to reproduce the insulin-like effects of GLP-1 (Ryan *et al.*, 1998; Toftnielsen *et al.*, 1996). Since all these studies were carried out with healthy subjects, the effects of GLP-1 on peripheral glucose disposal in diabetic state remains unclear. In this perspective a new study from, Sandhu *et al.* (1999) reported that in depancreatized dogs, GLP-1 was able to potentiate insulin action during a hyperinsulinemic clamp.

A new area of interest is GLP-1 action in the brain. Multiple studies have reported that the intracerebral ventricular administration of GLP-1 decreases the food intake (reviewed in Kieffer and Habener, 1999; Turton *et al.*, 1996). The intraperitoneal administration of GLP-1 to test the same intent was however unsuccesful suggesting that

GLP-1 action on the brain might be effected by locally produced GLP-1 (Turton et al., 1996). Furthermore, subcutaneous infusions of GLP-1 in humans did result in the reduction of food intake suggesting that intestinal GLP-1 might be crossing the blood-brain barrier (Toft-Nielsen *et al.*, 1999). It is also still unclear whether the reduction of food intake is a result of food aversion or a satiety effect.

Dipeptidyl Peptidase IV (DPIV)

Both incretins after their secretion are metabolized through several processes; renal clearance, hepatic clearance, and proteolytic processing in the circulation. A plasma enzyme, dipeptidyl peptidase IV (DPIV), has been demonstrated to be responsible for rendering the incretins biologically inactive by removing the N terminal dipeptide. Mentlein *et al.* (1993) reported that incubation of GIP or GLP-1 with either serum or purified DPIV results in production of the inactive forms of both hormones; GIP_{3-42} and $GLP\text{-}1_{9-36}$. Later it was shown by Kieffer *et al.* (1995) that this degradation is very rapid with more than 50% of the circulating intact peptide hydrolysed to the inactive form in less than 2 minutes. Degradation by DPIV in the circulation is believed to be the primary mechanism for the physiological inactivation of incretins.

DPIV is a serine protease most abundantly present in intestinal enterocytes, proximal tubule of kidney and placental tissue (Yaron and Naider, 1993). It has been also found in liver, stomach, spleen, lung, bone, testes, gall bladder, large intestine, vascular endothelium and pancreatic islets (Vanhoof *et al.*, 1992; Poulsen *et al.*, 1993; Mentzel *et al.*, 1996).

The inhibition of DPIV activity plays an important role in the diabetes research over the past decade. It has been shown that in pigs the inhibition of DPIV activity potentiates the insulin response to GLP-1 (Deacon *et al.*, 1998). More recently, Deacon *et al.* (2001) have shown in pigs with the use of valine pyrolidide (DPIV inhibitor) that DPIV inhbition reduces the degradation and clearance of GIP and potentiates its insulinotropic and antihyperglycemic effects. Mice with genetic disruption of the DPIV gene exhibit increased levels of bioactive GIP and GLP-1 along with enhanced glucose clearance after an OGTT (Marguet *et al.*, 2000). Furthermore the oral administration of a DPIV inhibitor isoleucine thiazolidide to Zucker fatty rats has been reported to improve glucose tolerance mainly by increasing the plasma half-life of both incretins GIP and GLP-1 (Pederson *et al.*, 1998). Very recently, Pospisilik *et al.*, (2002) reported imporoved glucose tolerance and insulin sensitivity in long term P 32/98 (DPIV inhibitor) treated VDF (Vancouver Diabetic Fatty) Zucker rats. Ahren *et al.* (2000) reported improved glucose tolerance and insulin secretion by inhbition of DPIV with valine-pyrolidide in mice.

Incretins and type 2 Diabetes Mellitus

The incretin effect after an oral glucose load may account for up to 50% of insulin secretion. Alterations in GIP and GLP-1 secretion and/or modifications in incretin action are important issues to address in the investigation of the pathophysiology of type 2 (non-insulin dependent) diabetes mellitus.

Fasting GIP levels have been reported to be normal or elevated in type 2 diabetic patients when compared with healthy individuals (Ebert *et al.*, 1976; Mazzaferri *et al.*,

1985; Osei *et al.*, 1986). Postpandrial GIP secretion has also been reported to be normal, increased or decreased in type 2 diabetes patients (Ahren *et al.*, 1997; El-Salhy, 1998; Nauck, 1986). Nauck *et al.* (1986) reported that although GIP secretion after an OGTT was not different between type 2 diabetic and normal patients the insulinotropic action of GIP was abolished in diabetic patients. This suggests an impaired responsiveness of the β cell to GIP stimulation. Creutzfeldt *et al.* (1983) identified two distinctive hyper- and hypo-secreters of GIP among 141 indiviuduals with type 2 diabetes that both exhibit loss of incretin effect. It has been argued that the β cells in the hypersecretors group are overstimulated perhaps leading to the impairment of the GIP receptors on the cell surface.

Recent studies have demonstrated that GIP receptors exhibit similar characteristics as other G protein coupled receptors in terms of ligand binding, desensitization and internalization (Gelling *et al.*, 1997; Wheeler *et al.*, 1999; Tseng and Zhang, 1998). Tseng *et al.* (1996) reported that preincubation of L cells with high concentrations of GIP decreased cAMP production in response to subsequent GIP stimulation. The same group reported that supraphysiological infusions of GIP in rats resulted in the ablation of the incretin effect of GIP. A recent study on isolated rat islets and an insulinoma cell line reported desensitization of the GIP receptor after high dose GIP preincubation (Hinke *et al.*, 2000) Collectively, these findings suggest that elevated serum GIP levels in diabetic patients might induce chronic desensitization of the receptor leading to the loss of incretin effect. Furthermore, recently, Lynn *et al.* (2001) reported that GIP action was abolished in VDF rats due to decreased GIP receptor gene expression in the islets. This suggests down regulation of the pancreatic GIP receptor may contribute to the pathology of type 2 diabetes.

Studies in type 2 diabetic patients have shown that, while GIP loses its insulinotropic action, GLP-1 preserves its incretin effect. It is still controversial whether GLP-1 levels are normal or elevated in type 2 diabetes. Therfore it is not possible to conclude a role for over or under production of GLP-1 in type 2 diabetes. (reviewed in Kieffer and Habener, 1999)

Recent studies in diabetic rodents with exendin 4 (a GLP-1 agonist) have shown reduced blood glucose, increased insulin secretion and insulin mRNA, and pronounced weight loss, and reduced adipose tissue content (Greig *et al.*, 1999; Stoffers *et al.*, 2000; Szayna *et al.*, 2000). Intravenous, subcutaneous, and oral administration of GLP-1 has been shown to improve both fasting and postpandrial glycemia in type 2 diabetics (Gutniak *et al.*, 1994, 1996, 1997; Nauck *et al.*, 1997a; Rachman *et al.*, 1996; Willms *et al.*, 1998). These findings strengthen the potential role of GLP-1 as a therapeutic agent in type 2 diabetes. The glucose dependence of incretin action differentiates GLP-1 from sulfonylureas and makes this peptide a good candidate for the treatment of type 2 diabetes (rewieved in Kieffer and Habener, 1999 and Wolfe *et al.* 1999).

GLP-1 receptor knockout mice

In 1996, to investigate the physiological importance of GLP-1 Scrocchi *et al.* generated GLP-1 receptor knockout mice. The mouse GLP-1 receptor gene was isolated and homologous recombination in mouse embryonic stem cells was used to generate mice with null mutations in both GLP-1 receptor alleles (Scrocchi *et al.*, 1996). The targeting vector was designed to inactivate the GLP-1 receptor gene by replacing exons encoding for intra-membrane and intra and extracellular domains of the receptor. Since

1996, numerous studies have been pursued by different groups with the GLP-1R-/- mice to identify the physiological importance of the lack of GLP-1 action.

GLP-1R-/- mice exhibit modest impaired glucose tolerance and diminished plasma insulin levels, after oral and intravenous administration of glucose (Scrocchi *et al.*, 1996). Interestingly the heterozygous GLP-1 receptor +/- mice also exhibited an abnormal glycemic response and reduced circulating insulin levels in response to a glucose challenge. These facts strongly suggest that besides its effects as an incretin on the β cell, GLP-1 also exerts actions peripherally, possibly through regulation of glucose disposal. However, no change in feeding behaviour nor in body weight was detected between GLP-1R-/- and wild type mice for 24 weeks (Scrocchi *et al.*, 1996). This study was supported by the findings of Scrocchi and Drucker (1998), who examined the effects of aging and high fat diet on body weight in GLP-1R-/- mice. They were unable to relate GLP-1 to obesity.

The glucose competence concept was also investigated in GLP-1R-/- mice. Flamez *et al.* (1998) perifused GLP-1R-/- mouse islets to assess insulin secretion in response to different concentrations of glucose. No difference in terms of insulin secretion has been observed between wild type and GLP-1R -/- mice. Accordingly, islet and pancreatic insulin content were found to be very similar. Flamez *et al.* concluded that pancreatic β cells exhibit preserved glucose competence in mice with a null mutation in the GLP-1 receptor. Pederson *et al.* (1998), however, reported a 30% decrease in pancreatic insulin content with an equivalent decrease in pancreatic insulin gene expression in GLP-1R-/- mice.

Pederson *et al.* (1998) also reported that the absence of GLP-1 action was compensated for in part by upregulation of the GIP-insulin axis. The enhanced insulin response to pancreas perfusion and islet perifusion with GIP in the GLP-1R-/- mice suggests that some compensations occurs within the β cell. Flamez *et al.* (1999) reported that disruption of GLP-1 receptor signaling leads to reduced basal but enhanced GIP stimulated cAMP production and to altered basal and GIP stimulated cytosolic Ca^{2+} accumulation. Thus, it appears evident that in the absence of GLP-1 action, GIP compensates with adaptive changes mainly occurring within the β cell.

Very recently, Ling *et al.* (2001) have reported that the distribution of β cells is shifted from large to small and medium-sized islets in the absence of GLP-1R signaling Furthermore, GLP-1R-/- islets exhibit abnormalities in cell topography, with two to threefold more centrally located α cells detected in GLP-1R-/- islets. These alterations in α- and β -cell location indicate that basal levels of GLP-1 signaling are important to maintain normal cellular organization of the endocrine pancreas (Ling *et al.*, 2001).

GIP receptor knockout mice

In 1999, Miyawaki *et al.* generated GIP receptor knockout mice. The exons 4 and 5 of the GIP receptor gene that encode the N-terminal extracellular region were replaced by phophoglycerate kinase cassettes. The embryonic stem cells were transfected with the construct and transmitted to germ lines.

GIPR-/- mice have been shown to have impaired glucose tolerance after an OGTT accompanied by decreased insulin levels at the 15^{th} and 30^{th} OGTT time point. The intravenous administration of glucose did not alter the glycemic profile in the GIPR-/-

mice. A high fat diet for three weeks in GIPR-/- mice resulted in increased plasma glucose levels during an OGTT but suprisingly the insulin secretory profile did not change. Weight gain for the high fat diet conditions was not different in wild type versus knockout animals (Miyawaki *et al.*, 1999).

Objectives and Hypothesis of this thesis

GIPR-/- mice represent an important animal model to investigate the physiological importance of GIP and its contribution to the enteroinsular axis. The studies reported in this thesis had two principal aims: first, assessment of general characteristics that contribute to the enteroinsular axis of GIPR-/- mouse and second, investigation of compensatory mechanisms that might have taken place in the continuous absence of functional GIP receptors.

The first aim was to investigate the physiological characteristics that are critical for the enteroinsular axis. It is important to outline the normal and abnormal profiles that might be present in the absence of a GIP-insulin axis. Plasma glucose, insulin and incretin levels were assessed under fasting and postpandrial conditions. Finally, duodeno-jejunal GIP and GLP-1 content were also measured.

It is the main hypothesis of this thesis that GIPR-/- mice exhibit compensatory changes in the enteroinsular axis. However, it was difficult to predict where these changes might occur. Therefore, the experimental design of the second aim was developed based on the preliminary findings of the general physiological characteristics. β cell responsiveness to different secretogogues (different doses of glucose, and arginine) was assessed. *In vitro* insulin and cAMP responses to GLP-1 were measured. Pancreatic

insulin protein content and gene expression were investigated. RT-PCR was used to determine insulin and GLP-1 gene expression in pancreatic islets. Immunohistochemistry was used to assess any alterations in the islet structure of the GIPR-/- mouse pancreas.

The Enteroinsular axis in the GIPR-/- mice.

Background

GIPR-/- mice were generated by Miyawaki *et al.* (1999) at the University of Kyoto in Japan. This group has published the details of the generation of mutant animals and the description of the targeting construct. This protocol leads to a null mutation where mutant animals were identified by the length of their GIP receptor. As has been pointed out in the Introduction, the exons coding the N terminal extracellular region of the GIP receptor gene were replaced by a PGK neo-cassette. The transmission of the mutant allele was confirmed by DNA blotting analysis using a 3' probe set in the external region of the targeting construct. The heterozygote mice islets did not respond to GIP indicating that the mutated GIP receptor does not have a dominant –negative effect but has lost its function.

Three heterozygote (GIPR+/-) pairs were sent to our laboratory at UBC from the University of Kyoto in March 2000. To genotype the litters derived from GIPR+/- intercross, the genomic DNA was probed and identified by Southern blot analysis. Eco RI digested genomic DNA using a 3' external probe confirmed that homologous recombination results in a 3kb length GIP receptor instead of a 7 kb wild type. In our laboratory the Southern blot protocol was adapted and optimized by Francis Lynn.

The GIPR-/- mouse colony developed in Japan exhibited impaired glucose tolerance after an OGTT along with a normal glucose excursion after an IVGTT (Miyawaki *et al.*, 1999). The first step was to assess the physiological characteristics that

are thought to be important in the regulation of glucose homeostasis. Various protocols have been used to investigate glucose tolerance, plasma circulating insulin and incretin levels during an OGTT, and intestinal GIP and GLP-1 content. The possibility of a compensatory mechanism similar to what has been shown in GLP-1R-/- mice was also investigated (Pederson *et al.* 1998). The experimental approach was to characterize the beta cell insulin reponse and cAMP production in response to different secretagogues by pancreas perfusion, and static islet stimulation. The islet insulin and GLP-1 receptor gene transcription as well as insulin protein content were also measured. Immunohistochemistry was also performed to assess the morphological status of the islets in GIPR-/- mice.

METHODS

Preparation of Genomic DNA from mouse tissue

Four week old mice were rendered unconscious under CO_2. A tail segment (5-8 mm) was cut and the ears were pierced according to a numbering system. Tissues were digested overnight at 55 ^{0}C in 700 µl of tail buffer, 50 mM Tris pH=8, 100mM ethylenediamine tetraacetic acid (EDTA), 0.5% SDS and in 35 µl of 10 mg/ml proteinase K (Gibco). The next day digestion was stopped with a 5 M NaCl wash at 12,000 g for 5 min. The supernatant was precipitated in isopropanol with 30 min centrifugation at 12,000 g. The pellet was washed with 70% ethanol and air dryed. For DNA quantification the pellet was resuspended in 10 mM TrisHCl at 4 ^{0}C overnight. The

concentration was determined with sample absorbance at A_{260} and A_{260}/A_{280} ratios. The DNA used for Southern blot had a A_{260}/A_{280} ratio lower than 1.6.

Southern Blotting and Hybridization of Genomic DNA

Ten μg of genomic DNA was restriction digested overnight at 37 ^{0}C with ECO RI (Gibco Life Technologies). The reaction was stopped with 3M NaOAc and the digests were ethanol (100%) precipitated on dry ice for 10 min and centrifuged for 20 min at –4 ^{0}C. After ethanol wash the pellet was resuspended in 20 μl Tris/EDTA buffer, 10 mM TrisCl, 1 mM EDTA pH=8. The fragments were separated overnight at 15 V in a 0.7% agarose gel with TAE buffer, 40 mM Tris-acetate, 1 mM EDTA, 40 mM glacial acetic acid. The next day the gel was depurinated in 0.25 M HCl for 15 min and neutralized with the transfer buffer 0.4 M NaOH. An alkaline transfer with a of positively charged membrane, where the transferred DNA becomes covalently linked to the membrane was chosen. The gel was transferred to a positively charged nylon membrane (Roche Diagnostic GmbH, Germany) in a pyramid type structure (Figure 4) for two hours. The membrane was then washed with 2X sodium chloride/sodium citrate (SCC), and baked in an oven at 120 ^{0}C for 30 min to immobilize the DNA on the membrane.

The DNA was then hybridized with the 3' probe to allow detection of the segment on the membrane. After 15 min of prehybridization at 42 ^{0}C, the membrane was hybridized overnight with labeled 3' directed probe at 60 ^{0}C in APH, 5X SCC, 5X Denhardts, (for 100X, 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (Pentax fraction V: Miles lab), H_20 to 500 ml), 1% SDS, 150μg/ml herring sperm DNA. The next day the membrane was washed first in 2XSCC with 0.1%SDS than

with 0.2XSCC with 0.1%SDS at 42 ^{0}C and at room temperature. The final radioactivity was kept under 0.05 mrad/hr and the membrane was exposed for three days at -70 ^{0}C with the use of Kodak Biomax film.

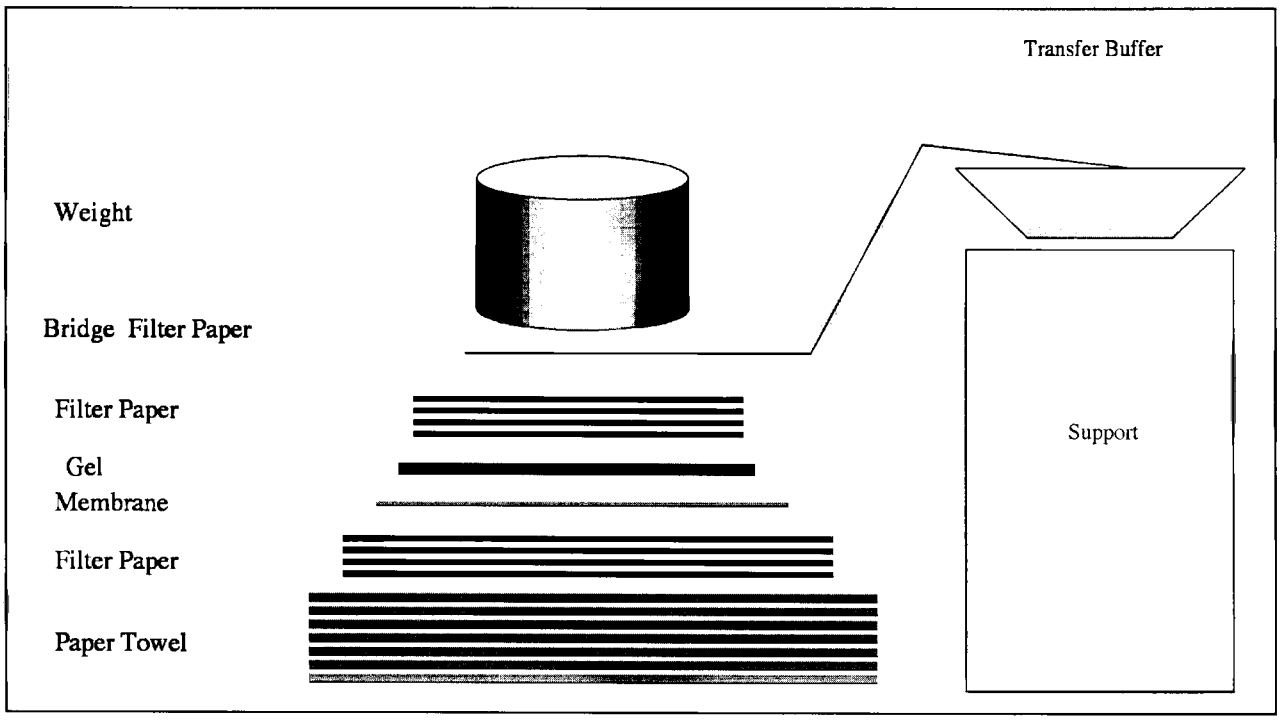


Figure 4- **Passive transfer by denaturing agarose blotting for Southern blots.** This low-tech method for agarose transfer is by a passive, alkaline, downward elution. The pyramid like setting prevents the shortcircuit between the membrane and the layers, and allows a safe and complete transfer

Labeling of DNA by Random Oligonucleotide-Primed synthesis

Random oligonucleotide-primed synthesis is an alternative to nick translation for producing uniformly radioactive DNA of high specific activity (Feinberg and Vogelstein, 1983). To allow double strand labeling, 100 ng gel purified wild type genomic DNA

fragment was combined with 4 μg random hexanucleotides (Life Technologies, Burlington, ON, Canada) followed by 10 min boiling and cooling. Subsequently the mixture was incubated at 37 ^{0}C for 2 hours with 0.5 M 3dNTP mix (minus dATP) same volume of 10X Klenow fragment buffer (Gibco), 50 μCi [α-^{32}P] dATP (Amersham Pharmacia), and 6 U DNA polymerase large fragment (Klenow). The probe was purified with QIAQUICK PCR Spin kit, following the instruction manual.

Oral Glucose Tolerance Test

Age matched 9-14 weeks old male WT and GIPR-/- mice were used for this experiment. Animals were fasted overnight (16-18 hours). The next morning the mice were weighed and allowed 20 minutes of acclimatization to the laboratory. Blood samples were collected from the tail veins. Blood glucose was measured individually for each mouse using a handheld glucose meter (Surestep®, Lifescan INC., Burnaby, BC, Canada). Baseline, fasting glucose was measured before the administration of glucose. Just before the administration by gavage of 1 g/kg glucose as a 10% solution, each animal was rendered unconscious under CO_2 for a very short period of time (less than 1 minute) to facilitate the feeding. Blood glucose was measured at the 10, 20, 30, 45, 60, and 120 minute- time points.

Measurement of Fasting and 20th minute OGTT Blood Glucose, Plasma Insulin, GIP, and GLP-1.

This experiment was designed to investigate the concurrent levels of insulin and incretins that are important in the control of glucose homeostasis. The 20th minute was

chosen as the peak period of glucose and hormone release. The OGTT protocol described above was used to assess plasma GIP, GLP-1, and insulin levels. To obtain a sufficiently large blood sample, four groups of 5-6 animals each were used. Blood was pooled at t=0 and t=20 min from each group. Plasma was than separated by centrifugation at 12000 xg for 15 min at 4 °C and stored at –20°C for RIA. Insulin was measured with a rat sensitive Linco kit (Linco St Charles, MO, USA) requiring 50 µl of plasma. Total GLP-1 (GLP-1_{1-36} and GLP-1_{7-36}) was measured with a carboxy terminal directed Linco kit requiring 150 µl of plasma. To indirectly assess plasma active GLP-1 levels, plasma DPIV activity was measured. Plasma GIP levels were also measured as described below

Measurement of Immunoreactive GIP

Samples were diluted in assay buffer, 5% charcoal extracted plasma (CEP), 2% Trasylol, 0.4 M PO_4 buffer (pH=6.5). GIP standards were serially diluted from 7.8 pg to 2000 pg to constitute the standard curve. On day 1 samples were incubated with GIP antisera (1:30,000) RK343F (Linda Morgan, University of Surrey, Guilford, Surrey). On the second day, radiolabelled GIP (5000 cpm/ tube) was added to the incubation tubes. On day 3, to separate the antisera bound GIP was separated from the unbound with 25% Polyethylene Glycol 8000 leading to a total volume of 1 ml in the assay tube. Radioactivity was assessed with a gamma counter,(LKB, Wallac 1277 , Gammamaster)

DPIV Activity Assay

A colourimetric assay was used to assess mouse plasma DPIV activity. H-Gly-Pro-pNA (Sigma G-0153-100 mg), a substrate for DPIV, is hydrolyzed to Gly-Pro and

–pNA whose yellow appearance can be monitored by spectrophotometer. A final concentration of 0.40 mM of substrate was prepared in 2.4% HEPES, 1.9% KCl, at pH=7.6. The standart, porcine kidney DPIV, (Probiodrug) was used at a serial dilution starting at 80 mU/ml. The formation of yellow product was measured at 405 nm with a kinetic reading for 30 min using an SP8 100 uv spectrophotometer (Pye Unicam). The sample activity values were calculated using linear regression based on the standards.

Measurement of Duodeno-jejunal GIP and GLP-1 content

This experiment was designed to determine if there were any alterations in intestinal incretin content in the absence of GIP signaling.

Seven male age-matched 12-14 week mice were anesthesized with 15% sodium pentobarbital (65 mg/kg) administered intraperitoneally. Segments of intestine (10 cm) starting from the pyloric antrum were isolated. Each segment was cut open and flushed with ice cold PBS to remove any intestinal contents. The clean segments were blotted to remove any liquid and weighed. The segments were boiled for 10 min in 5 times of their volume. The tissue was than homogenized in a polytron homogenizer. The homogenate was centrifuged at 10 000 g for 30 min. The supernatant was collected and stored in 4 ^{0}C. The pellet was boiled in 1M acetic acid for 10 min. To allow the homogenate to cool and equilibrate it was kept at 4^{0}C for an hour prior to 30 min centrifugation at 10,000g. The supernatant was combined with the first supernatant and stored at –20 ^{0}C until assayed for GIP and GLP-1 as described previously. To normalize the hormonal content, tissue total protein content was also measured with the use of a Bicinchoninic Acid (BCA) kit (Pierce, Rockford, IL, USA) according to the instructions.

In vitro Mouse Pancreas Perfusions

The mouse pancreas perfusion was performed with some modifications similar to rat pancreas perfusion as previously described (Pederson *et al.*, 1982). Briefly, overnight fasted male mice were anethesized by intraperitonial injection of 80 mg/kg sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada) prior to surgery. The abdominal aorta and the portal vein were cannulated with PE: 50 tubing (Cole-Parmer, Chicago). The perfusate consisted of a Krebs-ringer bicarbonate buffer supplemented with 3% dextran and 0.2% bovine serum albumin (BSA, Fraction V, RIA grade, Sigma) gassed with 95% CO_2 to achieve and maintain pH=7.4. The flow rate was maintained at 1 mL/min and the outflow was collected at 1 min intervals. GIP (the final perfusate concentration being 1nM) and GLP-1 (the final perfusate concentration being 1 nM) were delivered by side arm infusion in the presence of 16.6 mM glucose, whereas arginine was mixed directly into the perfusate (final concentration 10 mM) containing 8.8 mM glucose.

Isolation and Culture of Pancreatic Islets

Age matched male mice were anesthetized with Somnotol. The pancreas was exposed by midline laparotomy. The distal end of the pancreobiliary duct was clamped at the entrance to the duodenum. The common bile duct was punctured under a dissecting microscope with a $30^{1/2}$ gauge needle as close to the liver as possible. The pancreas was then inflated with ice cold collagenase (type V, 2mg/ml, Sigma) in Hank's Balanced Salt Solution (HBSS, GIBCO/Life Technologies, Burlington, ON, Canada) supplemented

with 10 mM HEPES, 2 mM L-glutamine, and 0.2% BSA, was injected into the common bile duct in order to inflate the pancreas. The pancreas was then removed and digested with collagenase in a 37°C water bath for 10 min. The digest was than dispersed mechanically with a Pasteur pipette, washed three times with 1 min centrifugation (1200 g) at each wash, and filtered through a 1 mm nylon screen, and washed again. Islets were separated by centrifugation at 1800 rpm for 20 min in a discontinuous dextran gradient (1.10 g/ml, 1.085 g/ml, 1.075 g/ml, 1.045 g/ml, and EC-Benz alone) diluted in EC Benz composed of 15 mM KCl, 15 mM KH_2PO_4, 40 mM K_2HPO_4, 10 mM $NaHCO_3$, 0.08% Benzamidine, and 0.2 M D- glucose. Using a pasteur pipette the islets were picked from gradient interfaces and washed two times with ice cold HBSS. Hand picked islets were cultured overnight in RPMI 1640 with 8.8 mM glucose, 10% fetal calf serum (Cansera, Rexdale Ontario, Canada), 5% vol/vol antibiotics (50 U/ml of each penicillin G and streptomycin), 0.07% human serum albumin, 0.0025% human apotransferrin, 25 pM sodium selenite and 10 μM ethanolamine.

Static Stimulation of Pancreatic Islets for Insulin

Approximately 100 islets were collected after overnight culture, washed twice with Krebs-Ringer buffer, and incubated for 45 minutes in 4.4 mmol glucose containing Krebs-Ringer, 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_20$, 24.9 mM $NaHCO_3$ supplemented with 0.1% BSA. After a brief centrifugation the medium was replaced with Krebs-ringer containing either 16.6 mM glucose alone or 16 mM glucose plus 10 nM GIP or 10 nM GLP-1. After 45 minutes of stimulation, islets were lysed by boiling for 5 min in 1 M acetic acid, and insulin RIA

(Linco, St. Charles, MO, USA), was conducted on all the supernatants according to the instruction manual.

Static Stimulation of Pancreatic Islets for cAMP

Each experimental condition was tested at the minimum in duplicate for each animal. After overnight culture, 15-18 islets were selected, washed twice with 3 min centrifugation at 1,200 g with 0.5 mL of Krebs-ringer supplemented with 0.2% BSA. To allow the islets to equilibrate in the experimental media, they were incubated at 37°C for 30 min. Thereafter, islets were stimulated for 30 min at 37 °C with either 0.5 ml of 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 10 μM forskolin, or IBMX with either 10 nM GIP, or GLP-1 in the presence of 16.7 mM glucose. After a 2 minute centrifugation the medium was replaced with 0.05N HCl, and islets were lysed by boiling for 3 min. Samples were subsequently dried overnight by vacuum centrifugation (Speed-Vac, Sorvall, Farmingdale, NY, USA) and stored at –20°C until assayed for cAMP (Biomedical Technologies, Stoughton, MA, USA) following the instructions in the manual.

Pancreatic Insulin Measurements

Age matched male animals were rendered unconscious with CO_2 and exsanguinated. Upon removal the pancreas was blotted dry and weighed. The tissue was homogenized with a polytron homogenizer in 2 M acetic acid and boiled for 5 min. Homogenates were centrifuged at 15,000 rpm for 15 minutes and the supernatant stored at –70 °C. Total protein levels were measured with a Bicinchoninic Acid (BCA) kit

(Pierce, Rockford, IL, USA), and insulin was measured with a kit (Linco, St. Charles, MO, USA). Values were normalized to total protein content.

Isolation and Measurement of Islet Insulin and GLP-1 Receptor mRNA by Reverse Transcriptase, Real-Time Polymerase Chain Reaction (PCR)

Mouse islet RNA was isolated using Trizol (GIBCO). Following RNA isolation, 50 ng of islet RNA was subjected to reverse-transcription (RT). Total RNA was reverse transcribed in a volume of 10 ml containing, 0.5 mM deoxynucleotide triphosphate, 15 pmol gene specific primer targeted at the carboxy termini of the mouse GLP-1 receptor (ACC AAC AGG GAG GAC CGG) or the mouse Ins II (GTA GTT CTC CAG CTG GTA GAG GG) 100 U Superscript II RNAse H^- Reverse Transcriptase (GIBCO), 10 U RNAse inhibitor (RNA Guard; Amersham-Pharmacia), 1 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$. Following RT, 10 ng (2 mL) of mouse cDNA was used in the real-time PCR reaction to measure insulin and GLP-1 receptor expression. The PCR reaction mix consisted of 1x TaqMan Buffer A (PE Applied Biosystems, Foster City, CA, USA), 10 mM $MgCl_2$, 200 mM dATP, dCTP, dGTP and 400 mM dUTP, 200 nM mouse GLP-1 receptor 5' forward primer (5'-CAG GGC TTG ATG GTG GCT ATC-3') or 200 nM mouse Ins II 5' forward primer (5' TGG AGG CCC GGG AGC), 200 nM mouse GLP-1 receptor 3' reverse primer (5'- CGC TCC CAG CAT TTC CG -3') or 200 nM mouse Ins II 3' reverse primer (5'- ATC TAC AAT GCC ACG CTT CTG C - 3'), and 100 nM GLP-1 receptor probe co-labelled with the fluorescent dyes VIC and TAMRA (5'- ACT GCT TTG TCA ACA ATG AGG TCC AGA TGG -3') or 100 nM Ins II probe co-labelled with fluorescent dyes TET and

TAMRA (5'-ACC TTC AGA CCT TGG CAC TGG AGG TG-3'), 0.01 U/ml AmpErase" uracil N-glycosylase (UNG, PE-Applied Biosystems), and 0.025 U/ml of AmpliTaq Gold" (PE Applied Biosystems). PCR reactions were carried out in triplicate in the PE Applied Biosystems 7700 sequence detection system. The reaction profile included a 10 minute preincubation at 50 °C to allow the UNG to degrade any uracil containing nucleic acids and a further 10 minute incubation at 94 °C to activate the AmpliTaq Gold. Following these preincubations, a two-step PCR protocol was carried out, which included a denaturation step at 94 °C for 15s followed by a 1 minute annealing/extension step at 60 °C. Fluorescence was measured during the annealing/extension steps over 40 cycles and used to calculate a cycle threshold (Ct), *i.e.* the point at which the reaction is in the exponential phase and is detectable by the hardware. All reactions followed the typical sigmoidal reaction profile, and Ct was used as a measure of amplicon abundance (Freeman and Varana, 1999).

Immunohistochemistry

Age matched male mice were fasted overnight, anesthesized under CO_2 before being exsanguinated. The pancreases from wild type and knockout mice (n = 5) were isolated and cut into head and tail segments. Each segment was fixed separately in Bouin's solution for 1 hour at room temperature and washed three times with 70% ethanol. The tissue was embedded in paraffin wax, and kept at –20 ^{0}C prior to cutting. Three consecutive 5 μm sections were taken 300 μm apart and mounted on glass slides. The study was carried out blind; thus, the slides were coded to prevent identification of +/+ and -/- tissues prior to quantification. The sections were dewaxed in xylene (2X5

min) and cleared in petroleum ether for 5 min. (Fluka). The sections were then rehydrated in phosphate-buffered saline PBS (80 mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.86mM KCl, 137 mM NaCl). To control for intra-immunostain variability, all the sections were incubated in the same batch of solutions and stained at the same time. The insulin cells were detected by overnight incubation with a polyclonal rabbit antibody (1:100, Santa Cruz). The next day bound antibodies were localized by incubating sections with a biotinylated goat anti-rabbit secondary antibody (1:300 , Jackson Labs, PI) for 90 min at room temperature. Sections were then washed to remove unbound antibody and incubated in the Avidin Biotin Peroxidase complex (ABC, Vector Labs, Ontario) at a dilution of 1:1000 in PBS supplemented with 5% horse serum. The peroxidase reaction was developed with 2% diaminobenzidine tetrahydrochloride in 0.05 M Tris pH 7.5 with 0.2% H_2O_2. After counterstaining with hematoxylin for 5 min the sections were dehydrated through graded alchohol and coverslips applied with Permount (Fisher Scientific). The sections were analyzed using the NIH image software (shareware) and data analyzed as islet area over total pancreatic area. Once the quantification had been completed the source of the sections (head or body, +/+ or -/-) was identified and significance assessed.

Data Analysis.

All the data are expressed as mean ± standard error of the mean. Unpaired Student's 't' test and Mann-Whitney U test (exclusively for immunocytochemistry) were used to compare the control values to GIPR-/- mice values, where $P<0.05$ was considered

statistically significant. The data analysis and area under the curve calculations were carried out using graph analysis software (Graphpad, Prism, San Diego, CA, USA).

RESULTS

The mice were classified as homozygote dominant (WT), heterozygote (GIPR+/), and homozygote recessive (GIPR-/-) according to the length of their GIP receptor DNA: 7 kb alone, 7 and 3kb combined and 3 kb alone respectively (Figure 5). After one year of breeding a colony was established with sufficient animal numbers to allow the experiments to start .

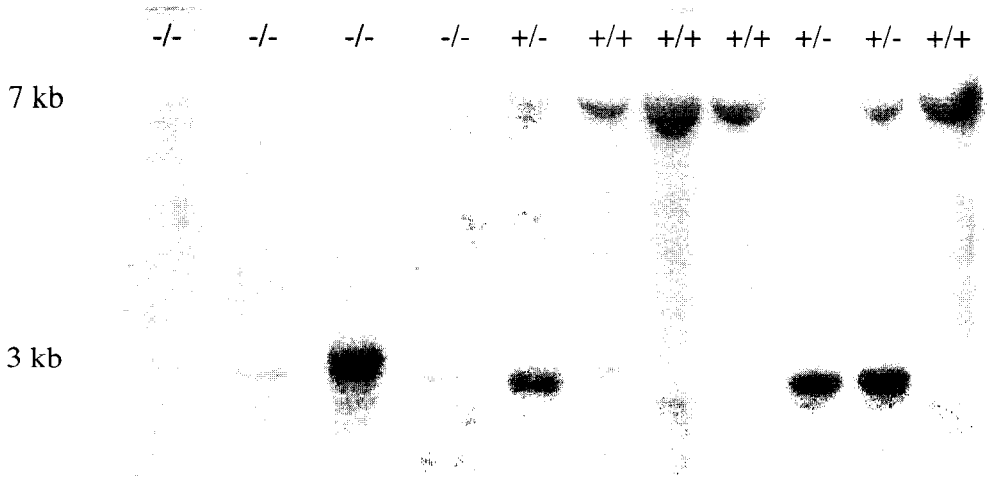


Figure 5- **Southern blot with Eco RI digested genomic DNA.** The sizes of 7 kb (Wild Type and 3 kb (GIPR-/-) fragments are indicated. +/+ refers to GIPR+/+ (WT), +/- to heterozygote GIPR+/- and -/- to GIPR-/- knockout mice.

Oral Glucose Tolerance Test

Oral administration of 1 g/kg glucose resulted in very similar blood glucose profile in both GIPR-/- and WT mice (Figure 6). The fasting values were normal (3.8 mM) and not different in WT and GIPR-/- mice. Both groups reached peak values of blood glucose at 15 min. The blood glucose values were 17.9±0.6 mM for WT and 22.7±0.7 mM for GIPR-/- ($P<0.05$). Although, statistically significant the peak glucose values for knockout mice were only 12% greater than wild type. For the 30th minute, blood glucose values were 14.3±1.0 mM for WT and 17.8±1.5 mM for the GIPR-/- mice ($P<0.05$). The two curves merged after 40 minutes and both groups returned to their basal levels after 1 hour.

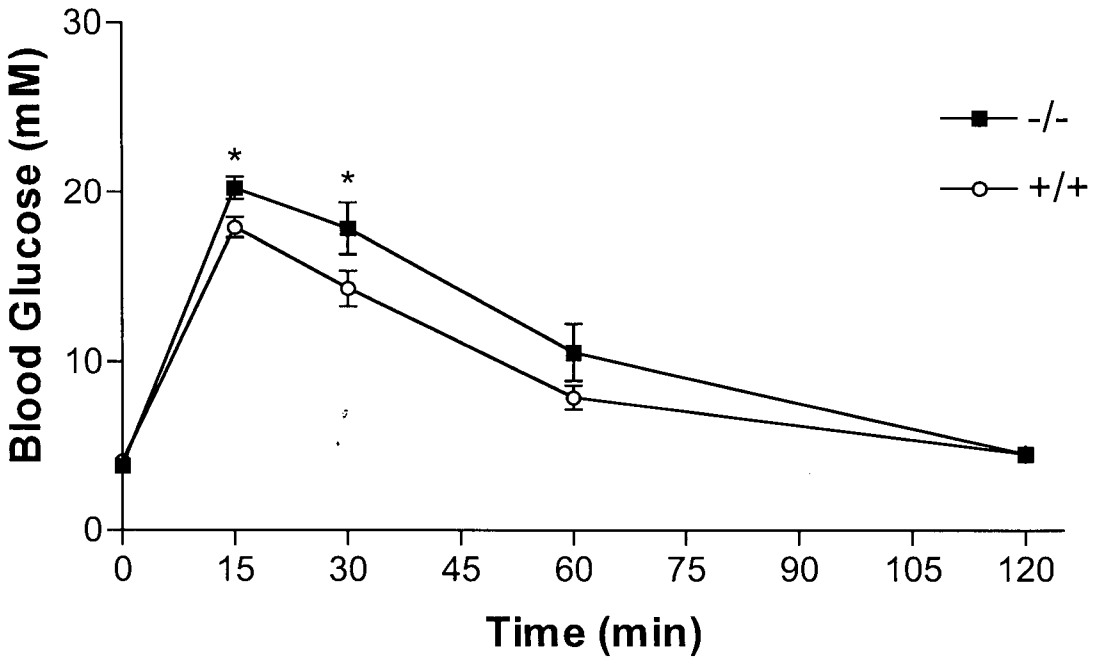


Figure 6- **Oral Glucose Tolerance Test.** Blood glucose was measured in response to oral glucose load (1g/kg) in WT (O, n=7) and GIPR-/- (■, n=11) mice. Values are mean ±SEM. * denotes $P<0.05$

Plasma Insulin, GIP, GLP-1 and DPIV levels for fasting and 20th minute OGTT

Fasting blood glucose was not different between groups (Figure 7A). This is consistent with the full profile OGTT data (Figure 6). Following an oral glucose challenge, 20th minute blood glucose levels were 18% higher in GIPR-/- mice than in

wild type mice (P<0.05) (Figure 7A). Fasting insulin levels did not differ between groups (Figure 7B). However, 20th minute insulin levels were 45% lower in GIPR-/- mice than in wild type mice; values were 1.6±0.3 ng/ml for WT and 0.9±0.1 ng/ml for GIPR-/- (P<0.05) (Figure 7B).

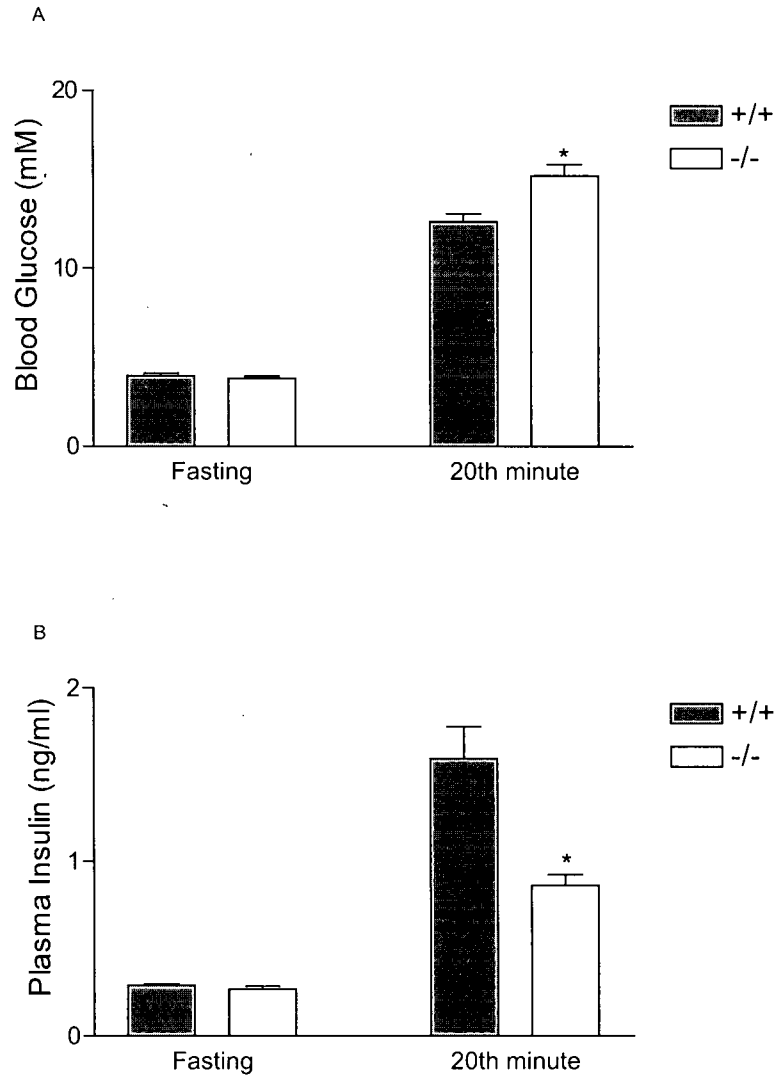


Figure 7- **Fasting and 20th minute OGTT blood glucose and plasma insulin levels. A:** Blood glucose levels in WT (■, n=4) and in GIPR-/- (□, n=4) mice. **B:** Plasma insulin levels in WT and GIPR-/- mice. Values are mean ± SEM. * denotes P<0.05

To further assess the hormonal components of the enteroinsular axis in GIPR -/- mice, fasting and 20th minute plasma GLP-1, DPIV, and GIP levels were investigated. It was hypothesized that DPIV would be downregulated to compensate for the lack of GIP action. This would lead to greater circulating active GLP-1. Neither total GLP-1 nor DPIV levels in GIPR-/- mice differed from those in wild type animals either for fasting or for the 20th minute OGTT time point (Figure 8A, 8B). However, fasting plasma GIP levels were elevated (17%) in GIPR-/- mice (Figure 8C). Values were 2217±50 pg/ml in GIPR-/- and 1862±99 pg/ml in WT mice ($P<0.05$). For the 20th minute, plasma GIP levels were lower by 18% in GIPR-/- when compared to WT mice (Figure 8C). Values were 3266±258 pg/ml for GIPR-/- and 3973±260 ng/ml for WT ($P<0.05$).

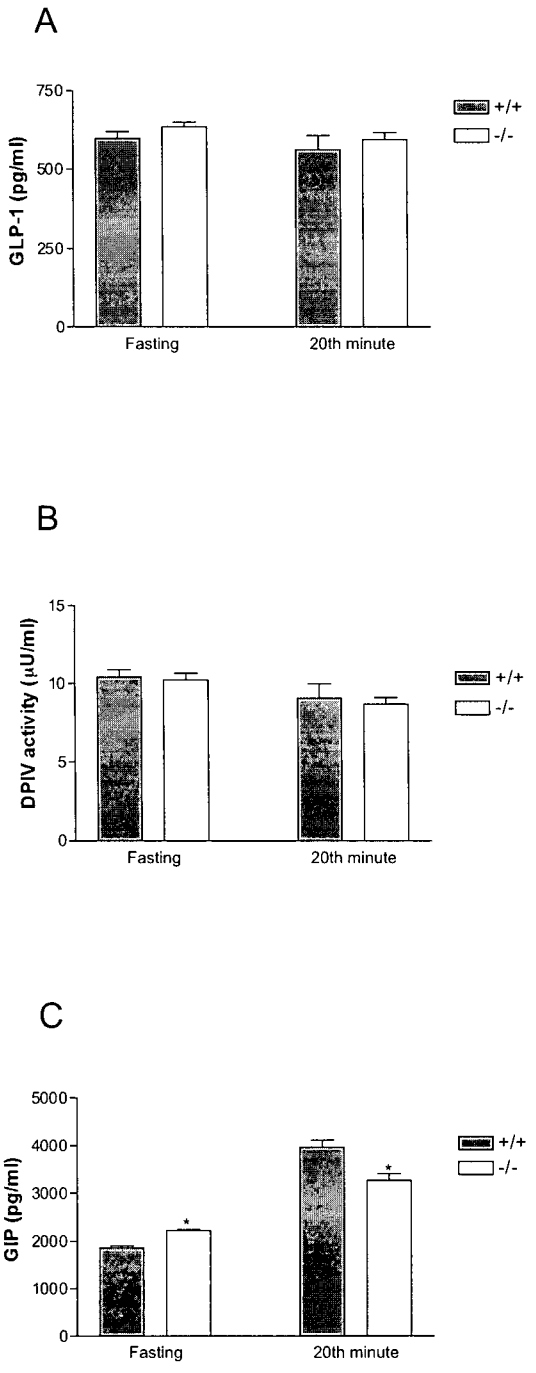


Figure 8- **Fasting and 20th minute OGTT plasma GLP-1, DPIV and GIP levels. A:** Plasma total GLP-1 levels in WT (O, n=4) and GIPR-/- (■, n=4) mice. **B:** Plasma DPIV activity in WT (O, n=4) and GIPR-/- (■, n=4) mice. **C:** Plasma GIP levels in WT (O, n=4) and GIPR-/- (■, n=4). Values are mean±SEM. * denotes $P<0.05$

Duodeno-jejunal GLP-1 and GIP content

It has been shown in dogs and rats that GIP release from the upper intestine in response to luminal glucose in turn stimulates GLP-1 release (Pederson *et al.* 1975; Roberge and Brubaker, 1993; Brubaker, 1991). To assess any alterations in intestinal cell distribution that might result from the absence of GIP signaling, incretin content was measured in duodenum and jejunum. Hormone levels were expressed over tissue total protein. Duodeno-jejunal GLP-1 levels were 50% reduced in knockout mice. GLP-1 levels were 10.5±1.5 pg/μg protein for WT and 5.05±0.4 pg/μg protein ($P<0.05$) (Figure 9A). Interestingly GIP levels were also slightly reduced in GIPR-/- mice. Values were 67.5±8.2 pg/μg protein for WT and 59.7±4.8 pg/μg protein, less than a 10% reduction($P<0.05$) (Figure 9B).

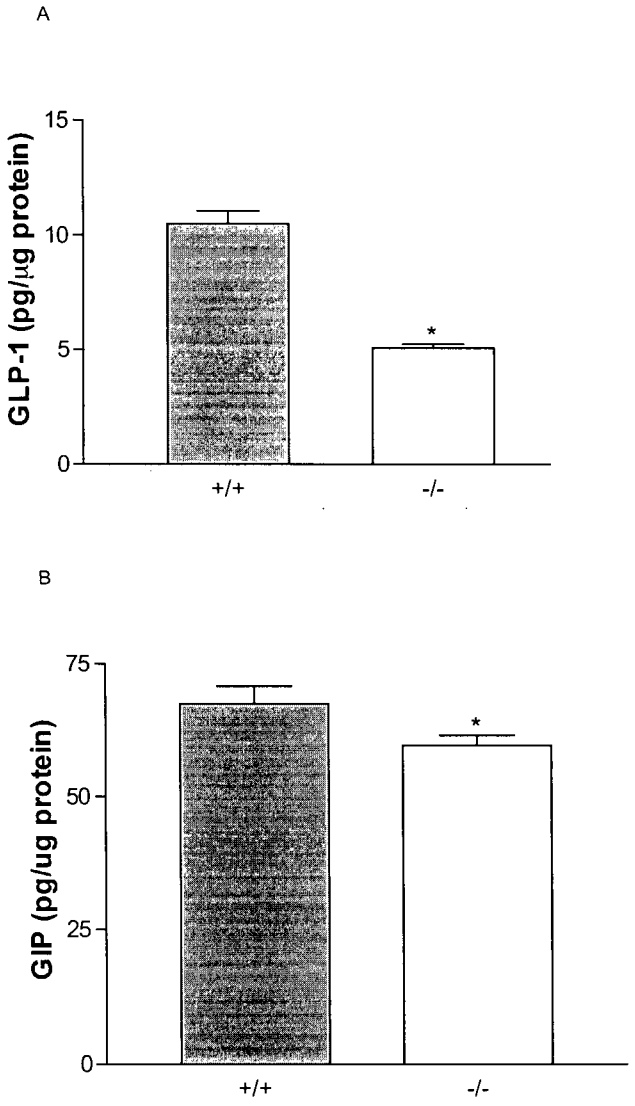
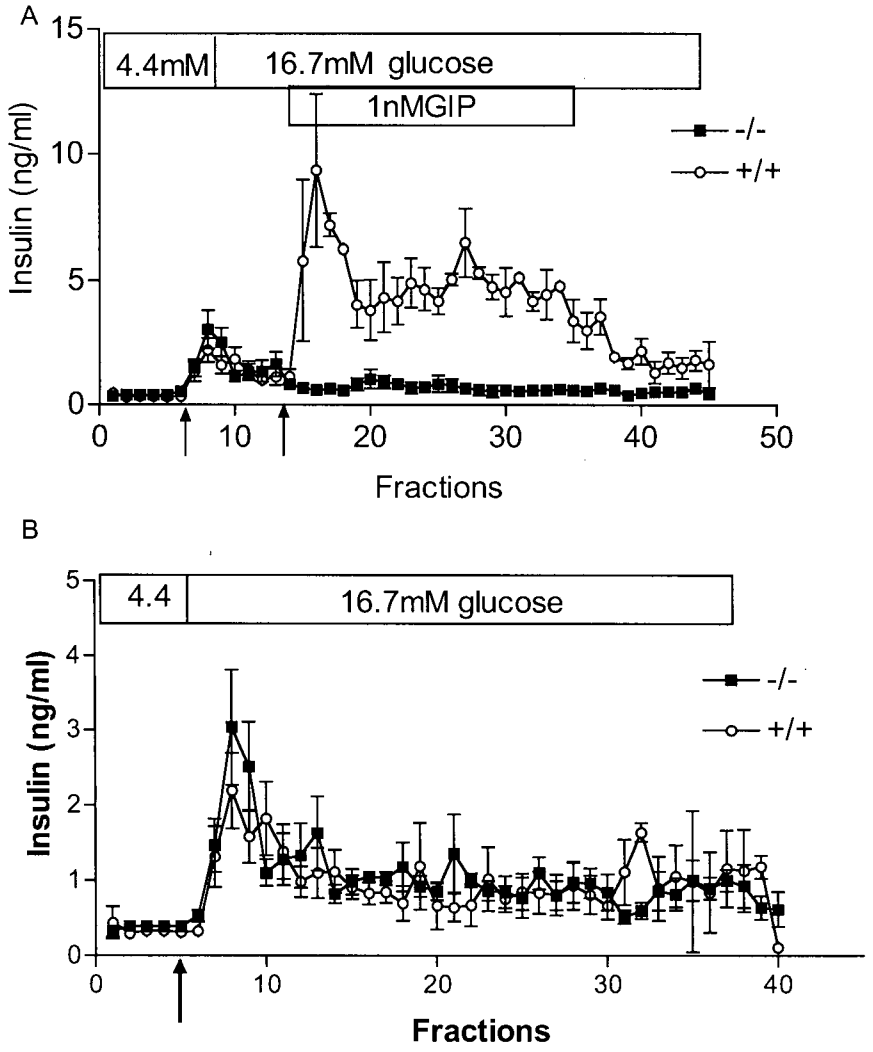


Figure 9- **Duodeno-jejunal GIP and GLP-1 content. A:** Duodeno-jejunal GLP-1 content in WT (■, n=7) and in GIPR-/- (□, n=7) mice. Hormonal content is normalized over tissue total protein content. **B:** Duodeno-jejunal GIP content in WT (■, n=6) and in GIPR-/- (□, n=7) mice. Values are mean± SEM, * denotes $P<0.05$.

Insulin Responses from pancreas perfusions of GIP, Glucose, and Arginine

To ensure that GIP receptors were non-functional and GIP was ineffective at stimulating insulin secretion from the pancreases of -/- animals, pancreas perfusions were carried out with GIP in the perfusate (Figure 10A). As expected, 1 nM GIP generated no insulin response from the perfused knockout pancreases whereas WT mice responded with a first phase insulin peak followed by a sustained second phase insulin secretion (Figure 10A). To determine if absence of GIP receptors influenced the β cell responsiveness to glucose, low (4.4 mM) and high (16.7 mM) glucose perfusions were carried out (Figure 10B). Glucose- stimulated insulin secretion in WT and GIPR-/- mice were comparable with an area under the curve of 29.1±6.1 ng/ml and 33.7±4.5 ng/ml respectively (Figure 10C). The peak values however of the first phase insulin secretion was significantly greater in GIPR-/- than in WT mice ($P<0.05$). The integrated insulin response as well as the peak value to high dose arginine (10 mM) perfusion in the presence of 8.8 mM glucose were not different between groups (Figure 10D and 10E). In conclusion, no significantly different insulin response profile was observed in response to insulin secretagogues, glucose and arginine in GIPR-/- versus WT mice.


A
4.4mM
16.7mM glucose
1nMGIP
-/-
+/+
Insulin (ng/ml)
Fractions
B
4.4
16.7mM glucose
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Insulin (ng/ml)
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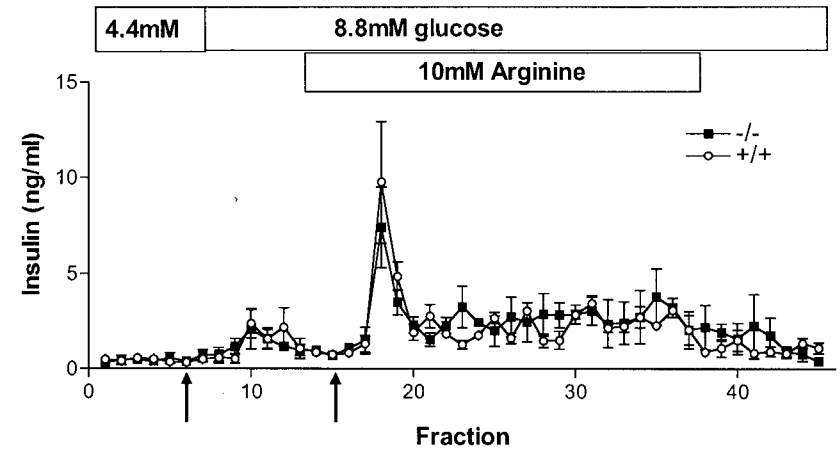
C
AUC Insulin (ng/ml)
+/+
-/-

D



E

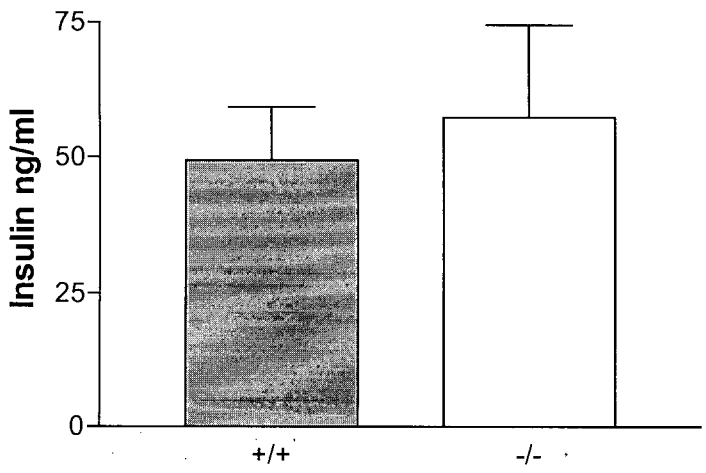


Figure 10- **Insulin response to pancreas perfusions with different secretogogues**. A: Insulin response to pancreas perfusion with GIP. WT (O, n=4) and GIPR-/- (●, n=4) B: Insulin response to pancreas perfusions with low (4.4 mM) and high (16.7 mM) glucose. WT (O, n=4) and GIPR-/- (●, n=5) C: Integrated insulin values for glucose alone pancreas perfusion. D: Insulin response to pancreas perfusion with 10 mM arginine in the presence of 8.8 mM glucose. WT (O, n=4) and GIPR-/- (●, n=4) E: Integrated insulin response for arginine perfusion. Arrows indicate the switch between conditions.

Insulin Responses to GLP-1

Pancreas perfusions were performed to assess any changes in the islet insulin response to GLP-1.The data are presented as percentage of the mean basal (in response to 4.4 mM glucose) insulin secretion. GIPR -/- mice exhibited a greater first phase and second phase insulin release in response to 1nM GLP-1 perfusion (Figure 11A). The first phase peak values were, 8.4±2 for WT and 26.7±10.9 fold basal for GIPR-/- ($P<0.05$). Furthermore, as is shown by the integrated insulin response, total insulin secretion during 1 nM GLP-1 perfusion was 60% greater in GIPR -/- mice compared to wild-type mice ($P<0.05$) (Figure 11B). The mean basal insulin secretion was not different between groups, 0.40±0.09 ng/ml in WT versus 0.42±0.03 ng/ml in GIPR-/- (Figure 11C).

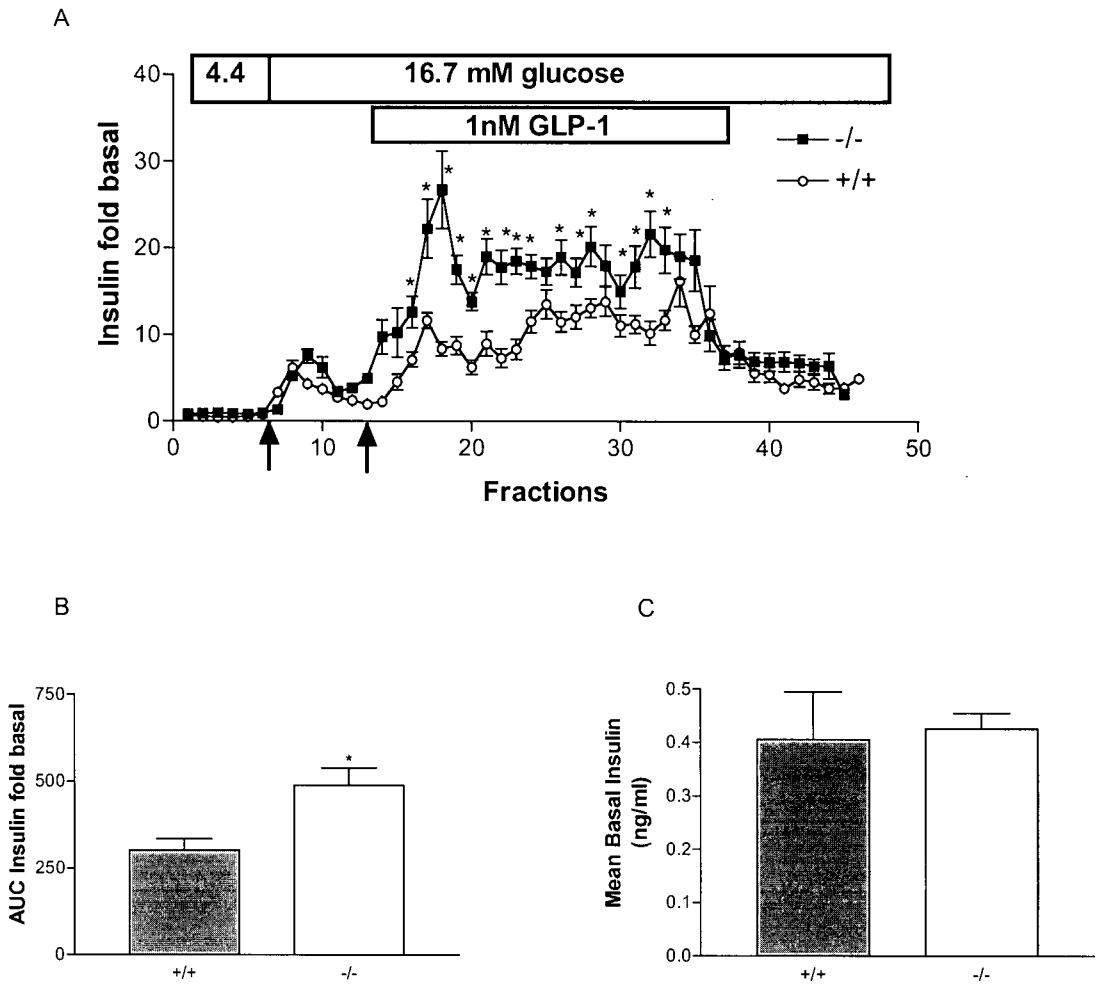


Figure 11- **Insulin response from GLP-1 perfused WT and GIPR-/- mice pancreas.** A: The perfusion profile for 50 minutes with 4.4 mM glucose, 16.7 mM glucose, and 1nM GLP-1 in the presence of high glucose. WT (O, n=6) and GIPR-/- (●, n=6) Arrows indicate the scwitch between concentrations. B: The integrated insulin response. C: Mean basal insulin values for WT and GIPR-/- mice during the perfusions.

To determine if these results were due to change in islet physiology, isolated islets were stimulated with low (4.4 mM) and high (16.7 mM) glucose alone or in the presence of either 10 nM GIP or GLP-1 (Figure 12). Insulin secreted over 45 minutes was 40% greater in response to GLP-1 stimulation for -/- (0.25±0.05 ng/ml) vs. +/+ (0.182±0.02 ng/ml) islets ($P<0.05$). As expected, GIP did not stimulate insulin release from -/- islets whereas it elicited a response comparable to GLP-1 stimulation from +/+ islets (0.10±0.01 vs. 0.18±0.05 respectively). The insulin response to 16.7 mM glucose was comparable in both groups.

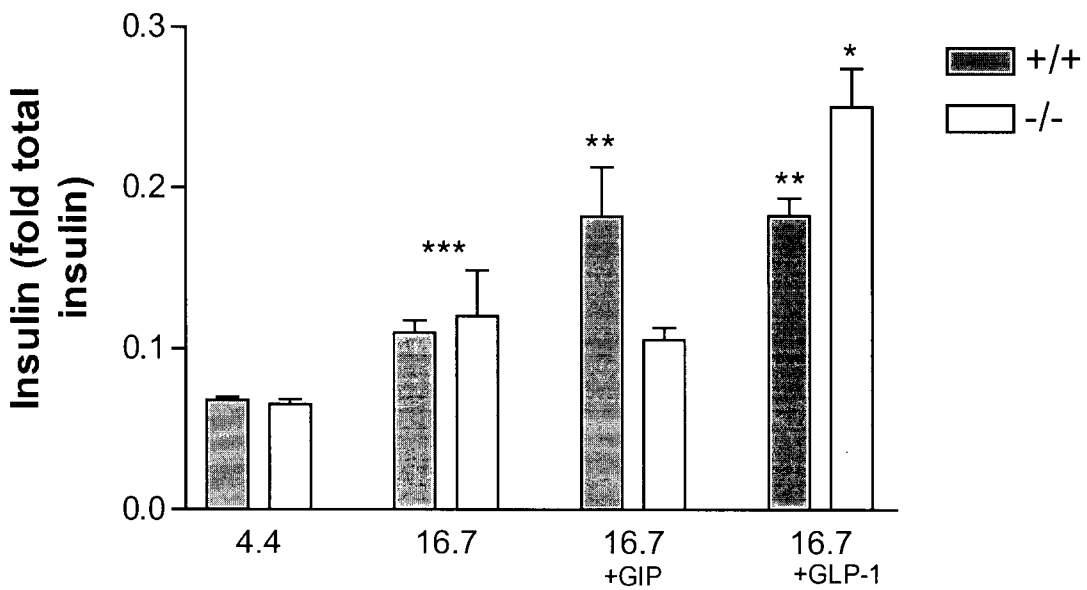


Figure 12- **Insulin responses to different stimulation of islets of WT and GIPR-/- mice.** Islets were stimulated with 4.4 mM, and 16.7 mM glucose alone, and with high glucose in the presence of either 10 nM GIP or GLP-1. WT (■, n=6) and GIPR-/- (□, n=6). * denotes $P<0.05$ for GLP-1 stimulated insulin release between WT and GIPR-/-. *** denotes $P<0.05$ for 16.7 mM glucose stimulated insulin release between WT and GIPR-/-. ** denotes $P<0.05$ for 16.7 mM glucose, GIP and GLP-1 stimulated insulin release for GIPR+/+ mice .

Intracellular cAMP Production

Both GIP and GLP-1 receptors are coupled to cAMP dependent signaling pathways in the beta cell (see Introduction for references). To correlate GLP-1-stimulated insulin release with receptor activation, cAMP production was measured in response to GLP-1 in isolated islets (Figure 13). A high concentration of forskolin (adenylate cyclase stimulator) was used as a positive control to stimulate near maximal levels of cAMP production. The cAMP values obtained from GIP and GLP-1 stimulation were normalized to forskolin-stimulated cAMP to account for differences in islet size and number. Interestingly, control (16.7 mM) cAMP levels were slightly increased in GIPR-/- mice compared to wild type ($P<0.05$). GLP-1 stimulated cAMP production was 100% increased in knockout animals (0.16 ± 0.01 fold forskolin/islet) versus wild type (0.08 ± 0.006 fold forskolin/islet) mice ($P<0.05$).

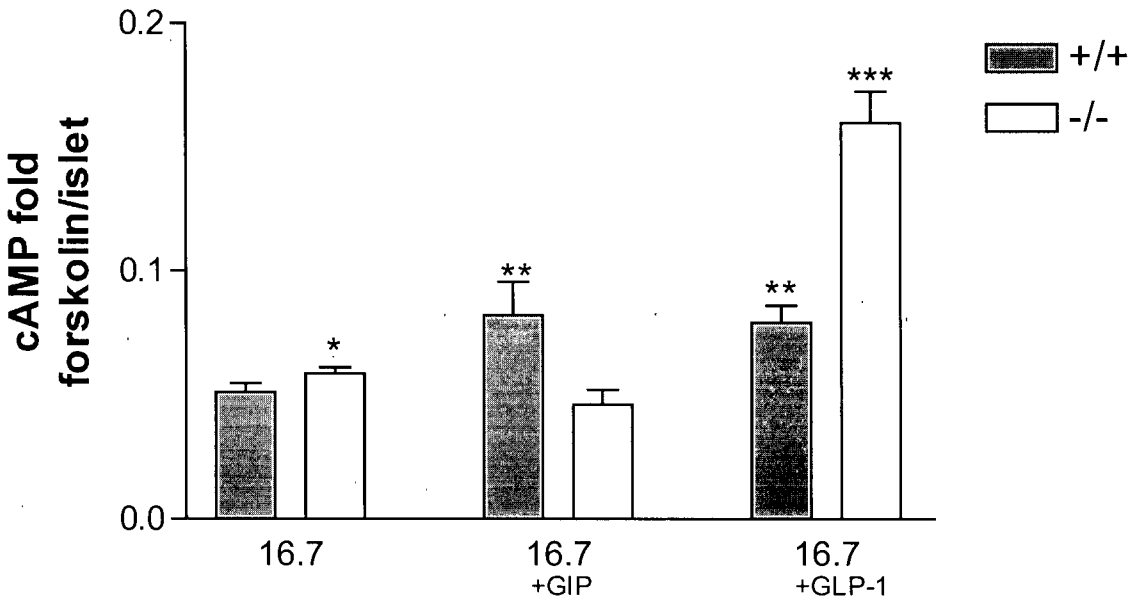


Figure 13- **cAMP production in the islets of WT and GIPR-/- mice** in response to 16.7 mM glucose alone , 10 nM GIP with 16.7 mM glucose, and 10 nM GLP-1 with 16.7 mM glucose. WT (■, n=5) and GIPR-/- (□, n=4) Basal levels were 0.051±0.003 (n=8) in GIPR+/+ vs. 0.059±0.002 (n=8) in GIPR-/-. (*P<0.05). * denotes P<0.05 for basal values between WT and GIPR-/- mice. GIP and GLP-1 stimulated cAMP production was significantly different from basal values in GIPR+/+ mice (**P<0.05).*** denotes P<0.05 for GLP-1 stimulated cAMP production in GIPR+/+ versus GIPR-/- mice. Values are mean ±SEM.

Pancreatic insulin content and islet insulin and GLP-1 mRNA content

Both GIP and GLP-1 stimulate insulin gene transcription and protein synthesis in the beta cell (Drucker *et al.*, 1987; Fehmann *et al.*, 1995a). Thus, we hypothesized that absence of GIP action may lead to alterations in insulin gene transcription and therefore in pancreatic insulin content. The total insulin content from fed mice pancreata was significantly lower (40%) in GIPR-/- mice than in +/+ mice (1.1±0.1 ng/μg protein vs. 0.75±0.07 ng/μg protein) ($P<0.05$, Figure 14A). These data are supported by findings that insulin mRNA levels were significantly reduced (40%) in isolated islets of GIPR -/- mice (1.06±0.09 relative to WT) when compared to control (0.74±0.11 relative to WT) (Figure 14B, $P<0.05$).

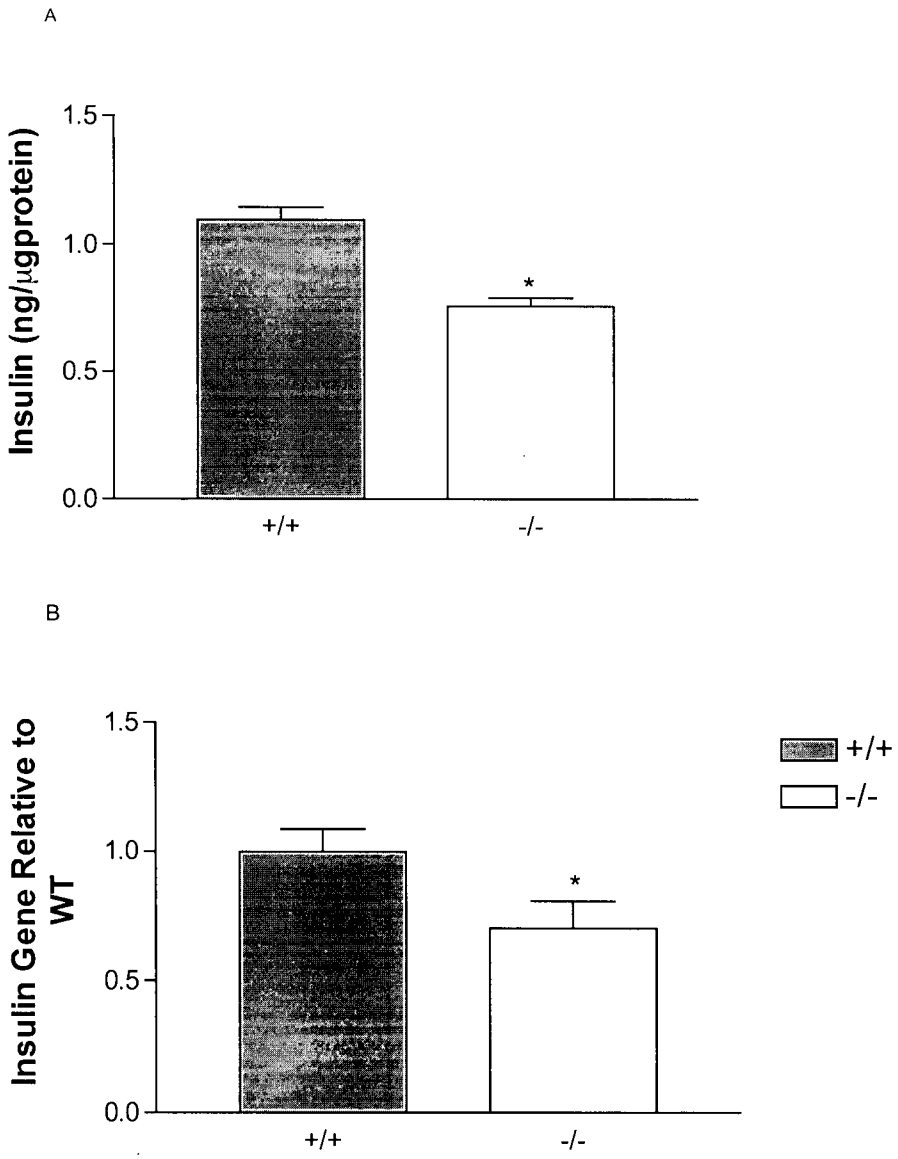


Figure 14- Pancreatic insulin content and islet insulin gene expression in WT and GIPR-/- mice. A. Pancreatic insulin content is expressed as a percentage of pancreatic total protein content. WT (■, n=5) and GIPR-/- (□, n=5). B Islet insulin gene expression in WT (■, n=10) and GIPR-/- (□, n=9). * denotes $P<0.05$

Interestingly, assessment of GLP-1 receptor mRNA levels revealed that, despite an increase in GLP-1 sensitivity, no increase in GLP-1 receptor mRNA was detected in the islets of GIPR-/- mice (Figure 15).

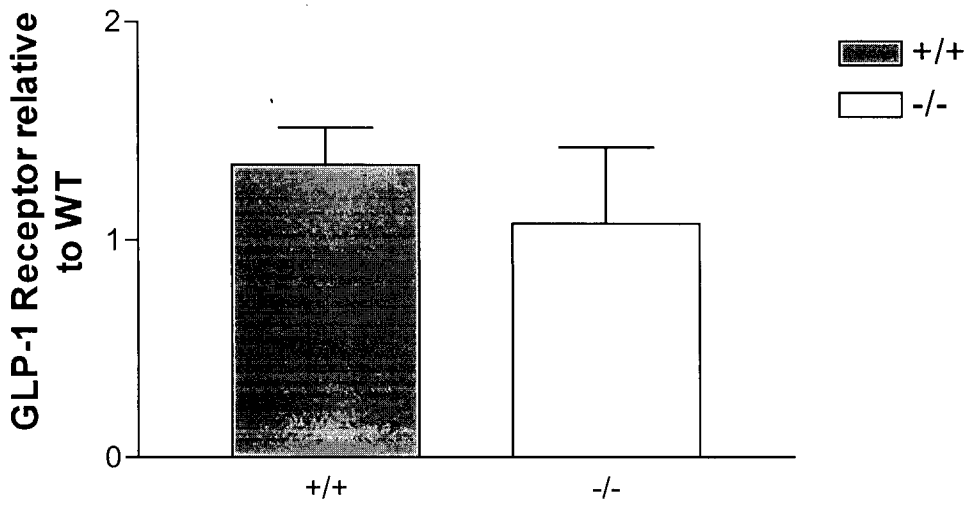


Figure 15- **GLP-1 receptor gene expression** in the islets of WT (■, n=11) and GIPR-/- (□, n=12) mice.

Immunohistochemistry

Immunhistochemical studies were carried out to assess the effect of GIP receptor deficiency on islet and pancreas morphology. Islet area over the total pancreatic area was

significantly increased (45%) in knockout versus wild-type mice ($P<0.05$), (Figure 16A). The head and tail analysis have shown that this increase was mainly due to the alterations occurring in the head of the pancreas where the islet area over total pancreatic area was dramatically altered whereas no significant difference has been observed for the tail segment (Figure 16B). Additionally, under identical experimental conditions, the staining intensity for insulin was reduced for -/- islets (Figure 17). This finding suggests that the insulin content is reduced in GIPR-/- islets. Glucagon and somatostatin staining have shown that the distribution of these cells within the islets is consistent with the literature, non beta cells occupy the periphery of the islet. (Figure18 and 19).

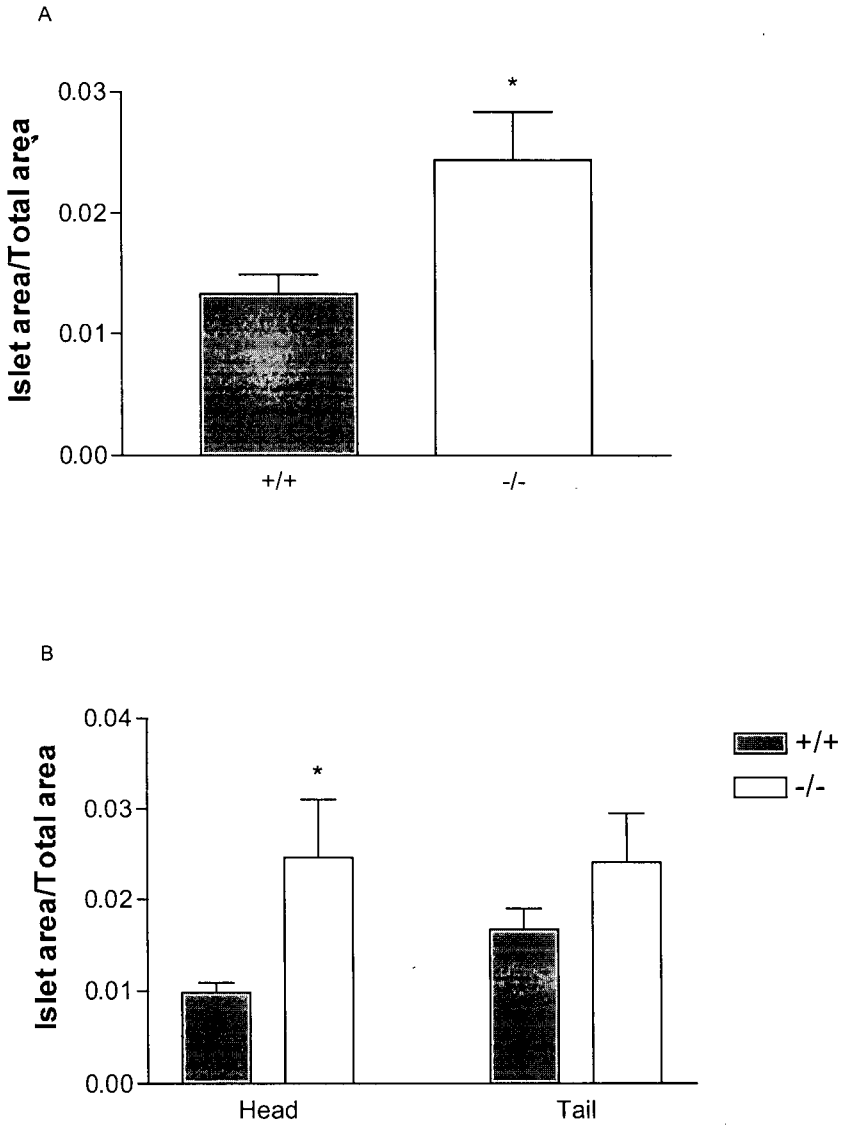


Figure 16- **Quantitative expression of islet area over total pancreatic area** in WT (■, n=5) and GIPR-/- mice (□, n=5). A: Islet area in the total pancreas in WT and GIPR-/- mice. B: The distribution of the Islet area in the head and tail of the pancreas in WT and GIPR-/- mice.

Figure 17- Pancreatic islets immunostained for insulin (peroxidase) in WT (top) and GIPR-/- (bottom) mice .

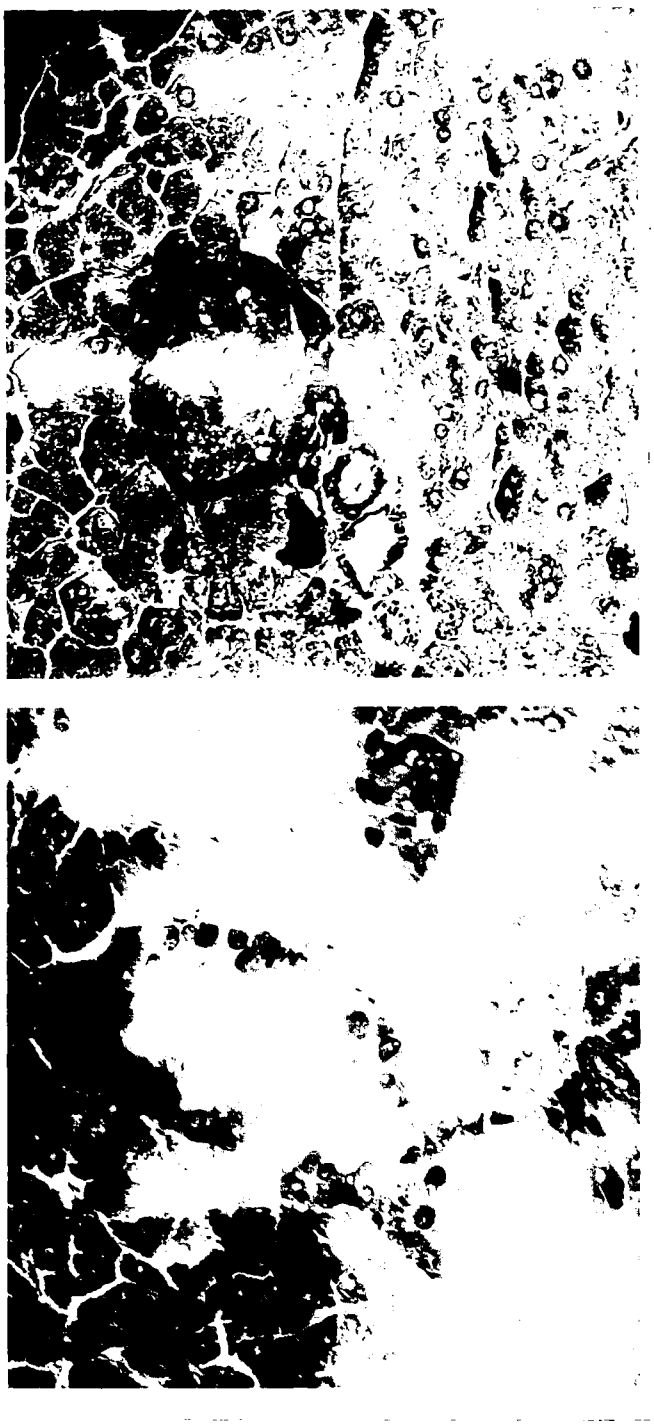


Figure 18- Glucagon immunostaining of WT (top) and GIPR-/- (bottom) mice islets.

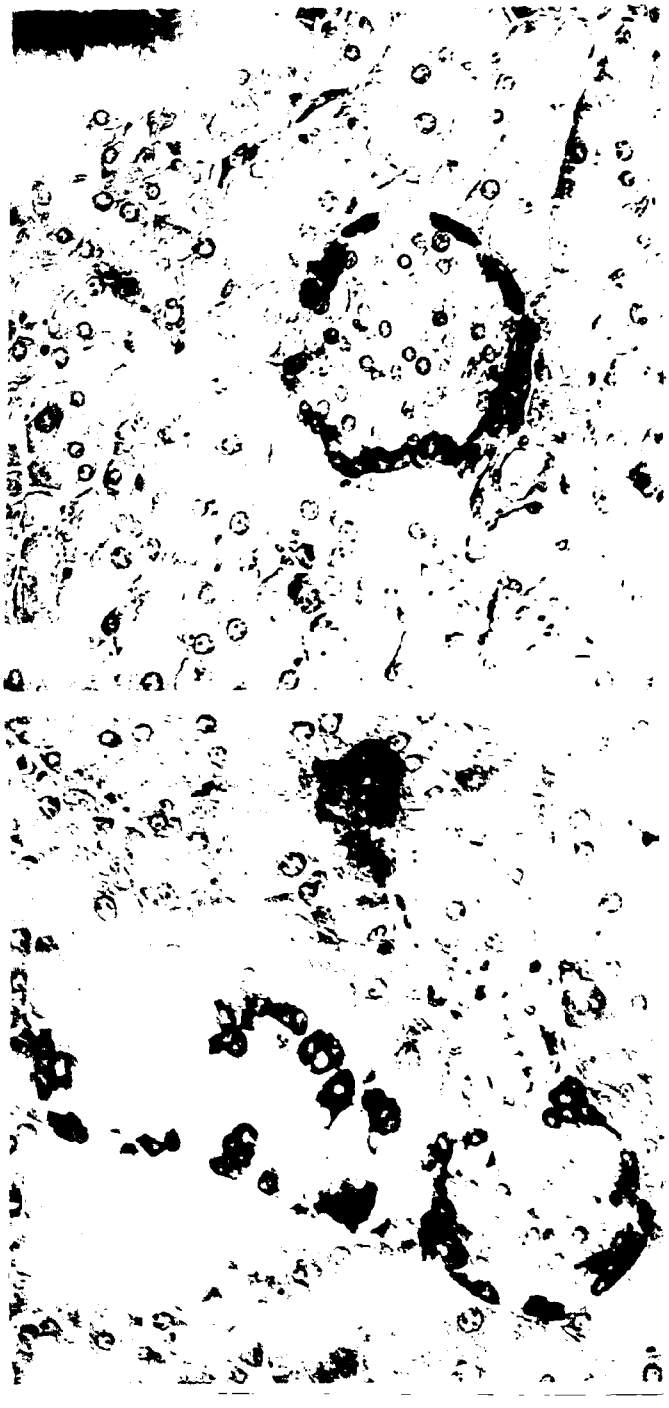


Figure 19- Somatostatin immunostaining in WT (top) and GIPR-/- (bottom) mice islets

DISCUSSION

Type 2 diabetes is a polygenic disease characterized by elevated blood glucose levels due to insulin resistance and impaired insulin secretion. In human type 2 diabetes, the insulinotropic action of GIP, but not GLP-1, is reduced (Jones *et al.*, 1989, Nauck et al., 1993). In addition, postprandial circulating GIP levels in type 2 patients have been reported as being elevated, normal or reduced (Mazzaferri *et al.*, 1985; Service *et al.*, 1984; Nauck *et al.*, 1986). These measurements were all made with C-terminal directed antibodies and therefore do not reflect the circulating levels of intact peptide. Hence, it is difficult to assess the contribution of serum GIP levels to the etiology of the disease. No consensus has been reached regarding the possibility that GIP receptor desensitization may contribute to the etiology of type 2 diabetes. However, this possibility was recently addressed in a model of type 2 diabetes, the VDF rat, in which it was demonstrated that GIP receptor expression is reduced (Lynn *et al.*, 2001). Mice with a targeted disruption of the GIPR gene (GIPR-/-) provide a useful model for studying the potential implications of a lack of GIP signaling on glucose homeostasis and the development of type 2 diabetes.

As has been discussed in the introduction, GIPR-/- mice are mildly glucose intolerant with normal fasting glucose and insulin levels (Miyawaki *et al.* 1999). However the oral glucose-stimulated insulin response is reduced while the insulin response to an intravenous glucose tolerance test (IVGTT) is intact, demonstrating a defective enteroinsular axis. Despite reduced insulin levels, glucose levels remain close

to the normal range. The mild degree of glucose intolerance suggests that, as with GLP-1R-/- mice, these animals compensate for the absence of incretin action.

A role for GIP in the maintainance of glucose homeostasis in the fasting state is under investigation. A recent study by Baggio *et al.* (2000) reported that chronic exposure of GLP-1R-/- and +/+ mice to a GIP receptor antibody (GIPR Ab), showed no differences in fasting glucose levels between groups. A similar study with GIPR Ab was also conducted in rats, and Lewis *et al.* (2000) have reported that inhibition of GIP action had no effect on fasting plasma glucose levels (Lewis *et al.*, 2000). Accordingly, the Japanese study and the current thesis (Figure 7) report normal fasting plasma glucose and insulin levels in GIPR-/- mice demonstrating that absence of functional GIP receptors has no effect on the fasting state. However, it is also possible that this might be a consequence of compensatory changes. One such change might be an increased sensitivity to GLP-1. GLP-1 has been shown to regulate fasting glycemia by Baggio *et al.* (2000). It is unclear why fasting GIP levels are increased in GIPR-/- mice (Figure 8C). This increase might be partly explained by the absence of somatostatin-related regulation of the K cells. Somatostatin secreting D cells are distributed throughout the small intestine (Larsson *et al.*, 1979). There is evidence that GIP secretion is inhibited by somatostatin (paracrine interaction), which in turn is stimulated by GIP (Jia *et al.*, 1994; Rossowski *et al.*, 1998).

OGTT plasma GIP levels at the 20th minute, were decreased in GIPR-/- mice. These results might be partly explained by the reduced duodenal GIP content observed in GIPR-/- animals (Figure 8C), where even a potent stimulus would result in relatively reduced peptide secretion. On the other hand, if the peripheral sensitivity to GLP-1 is

increased, delayed gastric emptying may blunt the peak values of plasma GIP. Hence, this observation might only reflect a right shift on the time axis. Therefore, it is very important to assess circulating GIP and GLP-1 levels during an OGTT with shorter time intervals for blood sampling. This would allow a more complete profile for both incretins. In this thesis the restriction on animal numbers precluded an experimental design in which sufficient numbers of animals could be sacrificed at each time point in order to collect enough blood for glucose and hormone measurements.

Although our GIPR-/- mice colony was developed from heterozygote pairs from the original Japanese colony, the OGTT profiles for the two colonies vary in terms of peak values and curve trends. The two hour OGTT profiles for GIPR-/- and +/+ mice are remarkably similar in the current study however, Miyawaki *et al.* reported more dramatic differences in GIPR-/- versus +/+ mice than was observed in the Vancouver colony. In both studies blood glucose reached peak values for both groups of animals at the 15 minute time point. In our study the curves merged after 40 minutes whereas in the Japanese study this did not occur until the 90 minute time period. Additionally, they reported a more pronounced increase in plasma glucose level for the 15^{th} and 30^{th} minutes of OGTT than in our study. Interestingly, despite the variations in glucose profiles, the insulin profiles were very similar in the two groups of animals. Alterations in diet and environment, along with genetic shift from inbreeding might result in different adaptations occurring in the development of these mice as compared to other colonies.

Despite the abscence of GIP receptors, the modest increase (18%) in the 20^{th} minute OGTT blood glucose levels does not correlate with the more profound reduction (55%) in plasma insulin levels observed in the GIPR -/- animals (Figure 7). However, in

the current study, it was found that the sensitivity of the pancreas to GLP-1 is increased in GIPR-/- mice (Figures 11-13), although no changes in total GLP-1 levels or in DPIV activity were observed (Figure 8A and B). As previously discussed in the introduction, controversy exists as to whether GLP-1 is capable of exerting insulin-like effects on peripheral tissue in addition to its well-studied insulinotropic effects. Studies have shown that GLP-1 improves glucose disposal in type 2 diabetes, by enhancing insulin-stimulated glucose utilization (Sandhu et al., 1999) and these effects have been shown to be independent of the amount of insulin secreted (D'Alessio *et al.*, 1995; Gutniak et al., 2001). Recently, Ahren *et al.* (1999) reported that in mice, the effects of GLP-1 on glucose homeostasis were mainly insulin mediated and the use of a GLP-1 receptor antagonist, exendin-3 (9-39), reversed these actions (Ahren and Pacini, 1999). Additionally, Baggio *et al.* (2000) demonstrated in mice that basal levels of circulating GLP-1 are essential for glycemic control in the fasting and postpandrial state (Baggio et al., 2000). It is still to be determined whether these actions of GLP-1 could be explained by mechanisms other than insulinotropic action such as improvement of glucose dependent glucose uptake. Therefore, it is possible, that the relatively small change in glucose disposal observed in GIPR-/- mice results from increased extrapancreatic sensitivity to GLP-1 in peripheral tissues, thereby augmenting glucose clearance despite reduced insulin secretion, or as a result of increased insulin sensitivity of extrapancreatic tissues.

Similar to GIPR-/- mice, GLP-1R-/-mice exhibited modest glucose intolerance, with upregulation, in this case, of the GIP component of the enteroinsular axis (Pederson *et al.*, 1998). In keeping with this finding, the glucose excursion after an I.V. glucose

challenge, bypassing the enteroinsular axis, was normal in GIPR-/- mice (Miyawaki *et al.*, 1999) suggesting that the incretin effect is responsible for both reduced circulating insulin levels (absence of GIP action) along with only modest changes in plasma glucose levels (due to enhanced GLP-1 action). Pancreas perfusions (Figure 11), static islet stimulation (Figure 12), and cAMP production (Figure 13) data presented here support our original hypothesis that the GLP-1 component of the enteroinsular axis in GIPR-/- mice is upregulated. This increased sensitivity to GLP-1 is more pronounced if we take into consideration the decrease in islet insulin gene transcription and total pancreatic insulin content in GIPR-/- mice (Figure 14). The fact that *in vitro* insulin responses to high glucose and arginine are similar, as well as islet GLP-1 receptor mRNA levels in GIPR-/- and in GIPR+/+ mice suggests that compensation occurs distal to the GLP-1 receptor in the beta cell. Both incretins act through G-protein coupled receptors and signal via the adenylyl cyclase-cAMP system (Wheeler *et al.*, 1995; Drucker *et al.*, 1987; Moens *et al.*, 1996). This being the case, it could be hypothesized that permanent absence of the GIP receptor leads to increased availability of the cAMP signaling system to GLP-1 receptors.

Reduced insulin gene expression and protein content, might be interpreted as a reduction in either islet size or beta cell number in the islet. We have shown that decreased islet size is not a valid explanation since GIP receptor null mice displayed increased islet area when compared to total pancreatic area (Figure. 17). This raises the possibility that other adaptive changes within the beta cell of GIPR-/- mice have taken place, since no differences in insulin responses to high glucose and arginine pancreas perfusions were observed between the two groups.

Both GIP and GLP-1 stimulate insulin gene expression (Fehmann et al. 1995a; Drucker *et al.*, 1987). It might be predicted that, an increase in beta cell GLP-1 sensitivity in the absence of GIP action could protect against a decrease in islet insulin mRNA and protein levels. However, this study shows a 40% decrease in both insulin mRNA expression and pancreatic protein content. This is comparable to the 35% decrease that was shown by Pederson *et al.* (1998) in GLP-1R-/- mice. Hence, absence of either of the incretins results in abnormalities within the β cell, leading to impaired insulin content. The compensation by GLP-1 or by GIP at the beta cell level, in GIPR-/- and in GLP-1R-/- mice respectively, is insufficient to regulate downstream signaling from the receptor, and subsequent activation of the transcription factors necessary for insulin biosynthesis.

It was proposed that GLP-1 could regulate β cell function by maintaining it in a glucose competent state (Holz et al., 1993). However studies with GLP-1R-/- mice have reported that these mice exhibit preserved glucose competence with preserved insulin storage capacity and glucose dependent insulin secretion. It has been shown by Pederson *et al.* (1998) that GLP-1R-/- mice exhibit compensatory changes in the form of enhanced GIP action. Since GIP and GLP-1 activate the same type G coupled receptors and its intracellular effectors, it is possible that in GLP-1R-/- mice the enhanced GIP action could contribute to the well-preserved glucose responsiveness of the β cell. In this regard, we have shown that β cell glucose responsiveness to different doses of glucose was intact in GIPR-/- mice. An enhanced GLP-1-pancreatic axis might be responsible for preserving the β cell in a glucose competent state despite decreased insulin gene transcription and biosynthesis.

Recently, GLP-1 has been shown to be involved in the morphological developement of the islets of Langherhans. In immunohistochemical studies, Ling *et al.* (2001) demonstrated alterations in beta cell distribution and in islet size in GLP-1R-/- mouse pancreata which may in part explain the reduced insulin gene expression. Our studies on GIPR-/- mice, with the same objective, also showed alterations in islet morphology mainly occurring in the head (ventral bud) of the pancreas (Figure 16). Reduced insulin gene expression and insulin content correlated with less intensely stained islets in GIPR-/- mice (Figure 17). The glucagon and somatostatin staining of pancreatic islets (Figure 18 and 19) showed that α and δ cells were localized on the periphery of the islet in both GIPR-/- and wild type animals; only the core of the islet staining for insulin. This suggests that an increase in either α or δ cells cannot account for larger islets with less insulin content in GIPR-/- mice. It would seem paradoxical to have increased islet size along with reduced insulin content. This suggests that these β cells have been depleted of insulin.

The current concept of the β cell mass is that it is dynamic, increasing and decreasing to maintain glucose homeostasis within a narrow physiological range (reviewed in Bonner-Weir, 2000). Hyperplasia and hypertrophy of the β cell mass is part of the continued turnover taking place during the lifespan. The turnover is dependent on changes in replication rate, cell death rate and in rates of differentiation from precursor cells. Studies with mice with disrupted insulin receptor substrate-1 (IRS-1) showed that the increased insulin demand to overcome the insulin resistance was answered by up to a 40 fold increase in β cell mass (Bruning *et al.* 1997). The increased β cell area over total pancreatic area observed in GIPR-/- mice (Figure 16) might be part of the compensatory

changes that occur in these mice. However to discuss β cell mass in detail one must take into consideration pancreatic volume as well as weight and three-dimensional localization.

Very recently it has been shown that GLP-1 has growth hormone-like effects on pancreatic islets (Holz and Leech, 2001). Enhanced GLP-1 action on the GIPR-/- mouse pancreas could thus also result in increased islet size. The immunohistochemistry studies presented in this thesis support this hypothesis. Islet size was determined by insulin immunostaining and was expressed as a percentage of the total pancreatic area, therefore in this study only the core of the islet accounts for islet size. The increase in islet area over total pancreatic area does not take into consideration changes in α and δ cell mass. It is important to note that GLP-1 receptors are abundantly present on β cells and their activation results also in the activation of growth, proliferation, and differentiation transcription factors such as c-jun, c-fos and jun B (Xu *et al.*, 1999; Edvell and Lindstrom, 1999; Susini *et al.*, 1998). Therefore, an upregulation of GLP-1 receptor signaling should mainly affect β cells. Hence, it is possible to partly explain the increase in islets size by the increased sensitivity of the β cell to GLP-1. However it is also important to determine whether α and δ cells are affected by the upregulation of GLP-1 action.

The development of the pancreas begins with the dorsal (tail) and ventral (head) protrusion of a region of the primitive gut epithelium (Edlund, 2001). The dorsal and ventral bud development of the pancreas as well as cell differentiation and specification processes remain unclear. The initiation and the development of the ventral and dorsal pancreatic buds are believed to be different. In mice the first endocrine cells appear in the

dorsal bud before the differentiation of the cells in the ventral bud (Lewis, 1996). Additionally, adult mice have fewer α cells in the ventral bud. In the current study it was observed that in GIPR-/- mice, islet area was increased mainly in the head (ventral) part of the pancreas. The developmental biology of the pancreas might account for the islet size difference observed between head and tail pancreatic segment of the GIPR-/- mice.

As has been discussed in the introduction, two recent studies by Damholt *et al.* (1998 and 1999) proposed a role for GIP in the direct secretion of GLP-1. K cells are more abundantly distributed throughout the duodenum with a decreasing density in jejenum and ileum (Polak *et al.*, 1982). L cells are mainly localized in the ileum with decreasing concentration in duodenum and jejenum (Eissele *et al.*, 1992; Damholt *et al.*, 1999). It is proposed that GIP secreted after a meal stimulates GLP-1 secretion. Immunohistochemical evidence suggests that in the canine upper intestine, K cells and L cells are adjacent to each other indicating a paracrine interaction (Damholt *et al.* 1999). In the current study, the absence of functional GIP receptors resulted in a 40% decrease in the duodeno-jejunal GIP and GLP-1 content. This implies either a relocalization of K and L cells and/ or a decrease in GIP and GLP-1 biosynthesis and storage. The data presented in this thesis are preliminary and further investigation is required to elucidate the effects of the absence of functional GIP receptors on the regulation and intestinal distribution of K and L cells

Which incretin makes a more important contribution to the enteroinsular axis? This question has been discussed for more than 15 years. Studies with GLP-1R-/- and GIPR-/- mice should shed light in this debate. In GIPR-/- and GLP-1R-/- mice GIP and GLP-1 compensate for each other by enhancing the β cell sensitivity partly by altering

the cAMP-adenylyl cyclase pathway (Figure 13, Pederson *et al.*1998, Flamez *et al.* 1999). The physiological significance of this compensation is still unclear. The ability of one component of the enteroinsular axis to compensate for a missing partner requires further investigation.

GLP-1R-/- mice exhibit plateau like peak glucose levels following an OGTT and the glucose levels do not return to normal for 90 minutes. The GIPR-/- mice exhibit a single peak at 15 minute and the levels return to normal within 40 minutes. GIPR-/- mice also exhibit normal fasting glucose and insulin levels, while the absence of GIP action is reflected in a 50% decrease in 30 minute OGTT plasma insulin levels. These profiles suggest that impairment of glucose tolerance is more pronounced in GLP-1R-/- mice. It is important to note that this statement does not take into consideration different backgrounds of these mice (CD1 vs C57BL/6) but only a comparison between control and test animals. Baggio *et al.* (2000) suggests a role for GLP-1 but not GIP in the regulation of fasting glycemia but other reports indicate that neither fasting glucose levels nor insulin levels are altered in either GIPR-/-, or GLP-1R-/- mice (Pederson *et al.* 1998, Scrocchi and Drucker, 1998). GLP-1R-/- mice compensate for the absence of GLP-1 action partly by upregulating the GIP-insulin axis. As demonstrated in this thesis, GIPR-/- mice compensate for the absence of GIP action by increased β cell sensitivity to GLP-1.

GIPR-/- and GLP-1R-/- mice exhibit impaired glucose tolerance with different degrees of severity. It is arguable that the absence of one of the incretins is sufficient to suppress the enteroinsular axis by reducing glucose stimulated insulin release up to 50% in GLP-1R-/- and GIPR-/- mice. However it is interesting that in both animal models the

β cell responsiveness to high and low glucose is preserved (Figure 10 and Pederson *et al.* 1998). Insulin gene expression and biosynthesis were reduced in comparable amounts in both animal models (current thesis and Pederson *et al.* 1998) suggesting that both incretins have similar effects on the β cell function. Pederson *et al.* (1998) have reported that first phase of the glucagon stimulated insulin secretion is altered in GLP-1R-/- mice. However they reported no effects of GLP-1 on pancreatic glucagon content. These parameters should be compared in GIPR-/- mice.

In summary, this thesis demonstrates that disruption of the GIP component of the enteroinsular axis in mice results in alterations in the beta cell that lead to decreased insulin gene transcription and protein biosynthesis, increased islet sensitivity to GLP-1, and changes in islet structure. It was demonstrated that compensation for the absence of a functional GIP receptor occurs in part by up-regulation of the GLP-1 component of the enteroinsular axis. The physiological changes that take place in both the GIPR-/- and GLP-1R-/- strains of knockout mice suggest that the incretins act in concert to maintain glucose homeostasis. The balance between GIP and GLP-1, where chronic disruption of one results in upregulation of the other one, appears to be part of the homeostatic system in the enteroinsular axis.

FUTURE DIRECTIONS

The research presented in this thesis was aimed at determinining general characteristics of GIPR-/- mice in order to have a better understanding of GIP physiology and its contribution to the enteroinsular axis. The few reported studies on GIPR-/- mice dictate a wide range of important topic still to be investigated. Several research directions are outlined below, which are based on the work presented in this thesis.

Glucagon and Somatostatin secretion and biosynthesis

Although GIP receptors are present on α and δ cells, the effects of GIP on glucagon secretion are ambiguous (see introduction). GIPR-/- mice present an important opportunity to investigate the role of GIP on glucagon and somatostatin secretion and biosynthesis. In vitro pancreas perfusion and static islet stimulations could bring more clarity on this subject.

The enteroinsular axis of GIPR +/- mice

Although Miyawaki *et al.* (1999) reported that truncated GIP receptor did not have dominant-negative effect it is clear that GIPR+/- animals would have less functional receptors. This coincides with the findings of reduced GIP receptor gene expression in the fatty Zucker rats (Lynn *et al.* 2001). The enteroinsular axis of the GIPR+/- could be assessed with similar studies presented in this thesis.

Islet β cell signaling

The study of intracellular signaling in the isolated β cells could provide more information about the mechanisms of the compensation by the enhancement of GLP-1-insulin axis in these animals. Transcription factors and the mechanisms that led to the gigantic islets are also in interest since the role of GIP in cell growth and proliferation is under investigation.

Duodenal and intestinal immunohistochemistry

Data presented here suggest that the morphology of the intestine has been modified. Immunocytochemistry studies with GIP and GLP-1 staining could provide more information about the distribution and localization of K and L cells in GIPR-/- mice.

SUMMARY

The studies presented in this thesis were targeted at developing a better understanding of GIP physiology and its contribution to enteroinsular axis. Using *in vivo* and *in vitro* techniques in GIPR-/- mice, it was demonstrated that the absence of GIP action affects glucose homeostasis by altering glucose disposal and insulin gene transcription and secretion without changing the β cell responsiveness to different secretagogues. It has been also reported that these effects were alleviated in part due to the compensation by the enhancement of the GLP-1-insulin axis in this animal model. The insulin, glucagon and somatostatin staining of the pancreatic islets have shown that islet structure and topography was also altered suggesting a role for GIP in islet cell organization. These studies indicate that the absence of GIP action modifies the structure of the enteroinsular axis in the GIPR-/- mice.

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