REGULATION OF GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) RECEPTOR EXPRESSION IN THE PANCREATIC β-CELL

by

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Abstract

Glucose-dependent insulinotropic polypeptide (GIP) is a peptide hormone that is released postprandially from the small intestine and acts to potentiate glucose induced insulin secretion from the pancreatic β–cell. In type 2 diabetes (T2D) there is a decreased responsiveness of the pancreas to GIP. The literature suggests that the ineffectiveness of GIP in T2D may be a result of chronic homologous desensitization of the GIP receptor (GIPR); however, there has been no conclusive evidence suggesting that GIP levels are elevated in diabetes. The first hypothesis of this thesis is that one cause of decreased responsiveness to GIP in T2D is an inappropriate expression of the GIPR on the pancreatic islet. This hypothesis was tested using the Vancouver Diabetic Fatty (VDF) strain of Zucker rats. The VDF rats were unresponsive to a GIP infusion during an intraperitoneal glucose tolerance test (IPGTT). GIP did not alter insulin[*] secretion or cAMP production in isolated VDF islets, nor did it stimulate insulin secretion from perfused VDF pancreata. The expression of GIPR mRNA and protein in islets from VDF rats was significantly reduced. The second hypothesis is that hyperglycemia and hyperlipidemia are able to regulate β-cell expression of the GIP receptor. High glucose was able to significantly reduce GIPR mRNA levels in INS(832/13) cells after only 6 hours. Palmitic acid and the PPARα activator, WY 14643, produced an approximate doubling of GIPR expression in INS(832/13) cells under 5.5 mM but not 25 mM glucose conditions, suggesting that free fatty acids can regulate GIPR expression via PPARα in a glucose-dependent manner. A dominant negative form of PPARα transfected into

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[*] Circulating hormone levels determined by radioimmunoassay are most accurately described as *immunoreactive* (IR) peptides (eg IR-GIP or IR-insulin). For the sake of brevity, the prefix IR- has been omitted from "insulin" in this text; although, in most cases insulin levels are measured by radioimmunoassay.

INS(832/13) cells caused a significant reduction in GIPR expression in 5.5 but not 25 mM glucose. In hyperglycemic clamped rats, there were reductions in GIPR expression in the islets and in GIP-stimulated insulin secretion. Thus, evidence is presented that the GIPR is controlled at normoglycemia by the fatty acid load on the islet; however, when exposed to hyperglycemic conditions the GIPR is down-regulated. The final hypothesis of this thesis is that glycosylation of the GIPR is able to control receptor expression on the cell surface. Here we demonstrate that cell surface expression of the GIPR and GIP-stimulated insulin secretion are dependent on glycosylation of the GIPR. Furthermore, the asparagine-linked glycosylation sites on the GIPR include Asn-59, Asn-69, and Asn-200 and alteration of any of these sites decreased total cell surface GIPR expression. Overall, this thesis presents evidence that the GIPR is regulated negatively by glucose and positively by free fatty acids. Additionally, it is demonstrated that hyperglycemia leads to downregulation of the GIPR in models of T2D. This glucose-induced downregulation is a result of a decrease in transcription of the receptor as well as a glucose-induced defect in glycosylation of the receptor.

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Abbreviations

AA	arachidonic acid
1-Oct	octamer-1
ACC	acetyl CoA carboxylase
ACS	acyl-CoA synthase
AOX	acyl-CoA oxidase
aP	adipocyte fatty acid binding protein
ATP	adenosine triphosphate
AUC	area under the curve
BCA	bicinchoninic Acid
Bis	bisindolylmaleimide
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
cPCR	competitive RT-PCR
cpm	counts per minute
CPT-1	carnitine palmitoyl transferase-1
CRE	cAMP response element
Ct	cycle threshold
C-terminal	carboxy-terminal
DMEM	Delbucco's modified eagle media
dNTP	deoxyribonucleotide triphosphates
DP IV	dipeptidyl peptidase 4
EC_{50}	effective concentration where a 50 % maximal response occurs
ECL	enhanced chemi-luminescence
EMSA	electromobility shift assay
ERK	extracellular regulated protein kinase
FAM	1-Dimethoxytrityloxy-3-[O-(N-carboxy-(di-O-pivaloyl-fluorescein)-3-aminopropyl)]-propyl-2-O-succinoyl-long chain alkylamino
FAS	fatty acid synthase
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
FFA	free fatty acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GHRH	growth hormone releasing hormone
GIP	gastric inhibitory polypeptide/Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLP	glucagon-like peptide

GPCR	G-protein coupled receptor
GRE	glucose response element
HBSS+	Hank's Balanced Salt Solution supplemented with 10 mM HEPES, 2 mM L-glutamine and 0.2 % BSA
HEK	human endothelial kidney
HPLC	high performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	inhibitory concentration where 50 % maximal binding occurs
IJ	intrajejunal
IP	intraperitoneal
IP_3	inositol trisphosphate
IPGTT	intraperitoneal glucose tolerance test
IR	immunoreactive
IV	intravenous
K_{ATP}	inwardly rectifying potassium channel (Kir) 6.2
Kb	kilobase
Kd	dissociation constant
kDa	kilodaltons
KRBH	Krebs-Ringer bicarbonate HEPES buffer
LACS	long-chain acyl-CoA synthetase
LC-CoA	long chain acyl-CoA esters
L-FABP	liver fatty acid binding protein
LPL	lipoprotein lipase
MAP	mitogen activate protein
mRNA	messenger ribonucleic acid
NPY	neuropeptide Y
N-terminal	amino-terminal
OGTT	oral glucose tolerance test
ORF	open reading frame
PCR	polymerase chain reaction
PEPCK	phophoenolpyruvate carboxy-kinase
PK	pyruvate kinase
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PNGase	peptide:N-glycosidase F
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element
PUFA	polyunsaturated fatty acid
RIA	radioimmunoassay

rPCR	real-time RT-PCR
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
sh	synthetic human
sp	synthetic porcine
SRE	sterol regulatory element
SREBP/ADD1	sterol regulatory element binding protein/adipocyte determination differentiation-dependent factor 1
T2D	type 2 diabetes
TAMRA	1-Dimethoxytrityloxy-3-[O-(N-carboxy-(Tetramethyl-rhodamine)-3-aminopropyl)]-propyl-2-O-succinoyl-long chain alkylamino
Taq	*T.* aquaticus DNA polymerase
TBST	tris-buffered saline with 0.5 % Tween 20
TF	transcription factor
UCP1	uncoupling protein 1
UNG	uracil N-glycosylase
USF/MLTF	upstream stimulatory factor/major late transcription factor
UTR	untranslated region
VDCC	voltage-dependent calcium channels
VDF	Vancouver diabetic fatty
VIP	vasoactive intestinal polypeptide
WT	wild-type
ZDF	Zucker diabetic fatty

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Chapter 1 – Introduction

1.1 Overview

Gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) is a 42 amino acid polypeptide that is synthesized and released by the K-cells of the duodenal and jejunal mucosa (Brown *et al.*, 1981; Buchan *et al.*, 1978; Jornvall *et al.*, 1981). GIP is released following ingestion of a meal by direct stimulation of the K-cell with the digestive products of glucose, fat, and protein (Dupre *et al.*, 1973; Falko *et al.*, 1975; Pederson *et al.*, 1975; Thomas *et al.*, 1978; Wolfe & McGuigan, 1982; Wolfe *et al.*, 2000). One of the main physiological actions of GIP following its release is potentiation of glucose-induced insulin secretion from the endocrine pancreas (Beck, 1989; Pederson, 1994; Pederson & Brown, 1976; Pederson *et al.*, 1975). For this reason it is termed an incretin and, along with its partner incretin, glucagon-like peptide-1, comprises the endocrine axis of the physiological connection between the gut and the endocrine pancreas, known as the enteroinsular axis (Kieffer & Habener, 1999).

Type 2 diabetes (T2D) is characterized by impaired glucose tolerance and thereby, an inability to properly secrete insulin following a glucose load. Because both GIP and GLP-1 are insulin secretagogues, there is considerable interest in using these polypeptides in the treatment of diabetes. One characteristic of T2D is the apparent loss of a GIP stimulated insulin response; however GLP-1, which signals through similar transduction pathways, seems to retain full potency (Elahi *et al.*, 1994; Krarup *et al.*, 1987; Meneilly *et al.*, 1993; Nauck *et al.*, 1993b).

Early studies attempted to link the lack of potency of GIP in T2D to a defective GIP receptor. Two studies, in Japanese and Danish populations, demonstrated several

mutations in the GIP receptor (A207 V, E354Q and C198G) that did not have prevalence in T2D populations (Almind *et al.*, 1998; Kubota *et al.*, 1996). The Japanese group, but not the Danish group, showed that there was a decreased ability of GIP (~ 70-fold right-shift of the dose-response relationship) to stimulate cAMP accumulation in CHO K1 cells transfected with the E354Q form of the receptor when compared to wild-type conditions. However, since none of these mutations seem to be linked directly to the development of overt diabetes, it appears as if a mutation in the GIP receptor is probably not responsible for the decreased insulinotropic potency of GIP in T2D.

Another possible cause for a loss of potency of GIP in T2D is a decrease in cell surface expression of the receptor in this disease which could occur via desensitization and internalization followed by downregulation (Hinke *et al.*, 2000a; Tseng *et al.*, 1996a; Tseng & Zhang, 1998a, b). It has been hypothesized that receptor downregulation is a major pathway by which GIP actions become attenuated (Holst *et al.*, 1997; Livak & Egan, 2002).

1.2 The Incretin Concept

The field of endocrinology began in 1902 when Bayliss and Starling reported that a substance from the gut could influence secretion of pancreatic juice. These initial studies demonstrated that hydrochloric acid, when introduced into the duodenum of dogs with denervated small intestines produced an increase in the volume of secretion from the exocrine pancreas into the small intestine. Furthermore, when these same investigators infused a duodenal extract into these dogs, there was a similar increase in pancreatic secretion. They called this substance secretin (Bayliss & Starling, 1903).

At around the same time, investigators were postulating that the "internal secretion" from the pancreas could control blood glucose. In 1906 Moore *et al.* hypothesized a role for gut secretions in the stimulation of the "internal secretion" from the pancreas. They were unable to show any effect of porcine gut extracts on the hyperglycemia of diabetic individuals, probably because of a total absence of β-cells. It was not until 1921 that Banting and Best isolated insulin and proved that it was the elusive "internal secretion." This discovery led to a revival of interest in the effects of duodenal extracts on hyperglycemia. To this end, La Barre and colleagues showed that a crude extract of secretin, when injected intravenously, could lower blood glucose levels in some dogs (LaBarre & Still, 1930; Zunz & LaBarre, 1929). They concluded from these studies that the secretin extract contained another substance which they termed incretin for its ability to stimulate release of the "**internal secretion**" *i.e.* insulin from the endocrine pancreas (LaBarre, 1932).

In the ensuing years, studies by Loew, Gray and Ivy demonstrated that an incretin secreted from the gut did not have a blood glucose lowering effect (Loew *et al.*, 1939, 1940a, b). It was not until development of the radioimmunoassay (RIA) in (1960) that the insulinotropic effects of duodenal extracts were studied. McIntyre *et al.* (1964) reported that intrajejunal (IJ) administration of glucose in two healthy subjects resulted in a more profound insulin response and more rapid return to basal glycemia than an equal intravenous (IV) dose. They hypothesized that this was a result of a substance that was released from the small intestine in response to glucose that stimulated insulin secretion from the endocrine pancreas (McIntyre *et al.*, 1964). The following year the same group ruled out liver as a potential site for the release of an insulintropic substance by carrying

out similar IV vs IJ experiments and obtaining similar results in healthy control patients and those with end-to-side portacaval shunts (McIntyre *et al.*, 1965). These studies supported the hypothesis of LaBarre (1932) that an incretin substance was released from the intestinal mucosa, not the liver. Perley and Kipnis (1967) quantified insulin responses in diabetic, non-diabetic, obese and normal individuals and demonstrated that the response to oral glucose was 60-70 % greater than the response to IV glucose. In a seminal review, Unger and Eisentraut (1969) brought together the ample physiological evidence to coin the term enteroinsular axis to describe the endocrine connection between the gut and the endocrine pancreas. The definition was later broadened by Creutzfeldt (1979) to include both neural and substrate stimulants of insulin secretion.

1.3 Glucose-Dependent Insulinotropic Polypeptide

1.3.1 The Discovery of GIP

GIP was initially isolated for its ability to inhibit gastric acid secretion. This followed a long search for enterogastrone: an inhibitory messenger that was secreted from the small intestine in response to intraluminal fat and acted via the blood to decrease gastric secretion (Gray *et al.*, 1937; Greengard *et al.*, 1946; Kosaka & Lim, 1930). Studies by Brown and Pederson (1970) suggested that different preparations of CCK, when given in doses that stimulated equal gallbladder contractile activity, had differing inhibitory effects on pentagastrin-induced acid secretion from canine stomach pouches. In these studies, the 40 % pure CCK preparation was not able to inhibit pentagastrin-induced gastric acid secretion to the same degree as an equimolar dose of the 10 % preparation. The authors proposed that this was due to the presence of an inhibitor of gastric acid secretion that was in greater concentration within the less pure preparation.

Concomitant to these studies in dogs, GIP was chemically isolated from porcine

duodeno-jejunal mucosa by standard biochemical methods. GIP and CCK were then

separated using Sephadex G50, and amino acid composition was determined (Brown *et*

al., 1969).

1.3.2 GIP Sequence and Homology

The amino acid sequence of porcine GIP was initially described by Brown and

Dryburgh (1971) who reported that GIP was a 43 amino acid polypeptide. An error in

the intial sequence of porcine GIP was later corrected by removal of a glutamine residue

at amino acid 30 (Jornvall *et al.*, 1981), leaving a 42 amino acid polypeptide with an

apparent molecular weight of approximately 5 kDa. Sequence identity analysis indicated

that GIP was highly conserved between species; human GIP having 95 % sequence

identity with the porcine and rat forms of GIP and 91 % sequence identity with the

bovine and mouse sequences. This high conservation of sequence identity may indicate

that GIP is an important regulatory hormone. Additionally Jornvall *et al.* (1981)

demonstrated that there was a minor component of the porcine GIP preparation with a 2

amino acid deletion at the amino-terminus producing GIP_{3-42}.

The structure of the GIP gene puts it in the growth hormone releasing hormone

(GHRH) superfamily of genes, which is thought to have evolved during a gene

duplication event in invertebrates between 500 million and 1 billion years ago. This gene

duplication event resulted in the formation of the GHRH/VIP gene family and the

glucagon gene families (Campbell & Scanes, 1992; Inagaki *et al.*, 1989). Sequence

similarity indicates that the GIP gene was then a result of a further series of gene

duplication events early within the existence of the glucagon family of genes (Irwin, 2002).

1.3.3 GIP Gene Structure and Posttranslational Processing

The human GIP gene spans approximately 10 kb and contains 6 exons encoding a 153 amino acid prepro-form of GIP. Similar to other members of the GHRH and glucagon families of genes, each exon in the GIP gene codes for a specific region of the peptide: exon 1 encoding the majority of the 5' untranslated region of the mRNA (UTR), exon 2 encoding the remainder of the 5' UTR and the signal peptide; exon 3 encoding the majority of GIP, exon 4 and 5 encoding the remainder of GIP and exon 6 encoding the 3' UTR (Inagaki *et al.*, 1989). The rat gene structure is similar to that of the human gene; although, the rat gene product is a 144 amino acid peptide that is primarily an amino-terminal deletion of the human ortholog (the result of a splice site shift) (Higashimoto & Liddle, 1993; Tseng *et al.*, 1993). Exon 1 of the GIP gene contains putative TATA and CCAAT boxes, sites that are often necessary for the initiation of transcription, although it has been reported that the TATA box in the rat GIP gene is not active in the adult animal (Higashimoto & Liddle, 1993; Inagaki *et al.*, 1989).

Human preproGIP is posttranslationally processed by removal of the 21 amino acid signal peptide (at glycine-21), and the intervening 30 amino acid N-terminal peptide, as well as the removal of the 60 amino acid C-terminal peptide by proteolytic cleavage at single arginine residues (Arg51, Arg94) flanking the mature 42 amino acid peptide (Inagaki *et al.*, 1989; Takeda *et al.*, 1987). The posttranslational processing of rat preproGIP is similar to that of the human peptide (Higashimoto & Liddle, 1993).

1.3.4 Tissue Distribution, Release and Gene Expression

GIP mRNA has been localized primarily to cells in the gastrointestinal tract such as the duodenum (Inagaki *et al.*, 1989) and the stomach (Cheung *et al.*, 2000; Yeung *et al.*, 1999). A recent study has demonstrated GIP mRNA to be present in the duct cells of the submandibular glands, although the physiological role of GIP in these cells is unknown (Tseng *et al.*, 1995). GIP-like immunoreactivity has been localized to the K-cells of the duodenum and jejunum in humans but immunoreactive GIP (IR-GIP) has been observed in the ileum in rats and dogs but not in the colon (Buchan *et al.*, 1982; 1978; Polak *et al.*, 1973).

GIP is released from cells that have been "defined by the characteristic appearance of the intracellular secretory granules having a small electron dense core surrounded by a concentric electron-lucent halo (Pederson, 1994)." These putative K-cells of the duodenum and ileum are located within the intestinal mucosa, and respond to stimulation by luminal nutrients (Buchan *et al.*, 1982; 1978; Dupre *et al.*, 1973; Falko *et al.*, 1975; Pederson *et al.*, 1975; Thomas *et al.*, 1978; Wolfe & McGuigan, 1982; Wolfe *et al.*, 2000). GIP levels have been reported to increase from 12-92 pM basally, to 35-235 pM postprandially: with the great degree of variability coming about as a result of the affinities of different antibodies for human GIP (Alam & Buchanan, 1993). In any case, most of the literature agrees that GIP levels increase 5-6 fold basal following a mixed meal (Pederson, 1994). A more recent study by Hoffmann *et al.* (2002), reported that fasting bioactive GIP levels in normal humans were in the low pM range and that the peak bioactive GIP level of 45 pM occurred 30 min following initiation of an OGTT.

Total GIP levels (inactive +active) rose to approximately 150 pM at the 30 min timepoint.

The literature reports that many of the constituents of a mixed meal stimulate GIP release to varying degrees. One potent stimulus of GIP release, and appropriate for its role as an incretin, is glucose. IR-GIP has been reported to increase in response to an oral glucose load in humans, dogs, rats, and mice (Cataland *et al.*, 1974; Pamir *et al.*, 2002; Pederson *et al.*, 1982; Pederson *et al.*, 1975) as well as isolated canine K-cells and from a mouse intestinal cell line (Kieffer *et al.*, 1994; 1995a). IR-GIP is not released in response to IV glucose, indicating that luminal stimulation of the K-cell is necessary for release. The exact mechanism for this release involves uptake of glucose into the enterocytes, as phloridzin an inhibitor of sodium-dependent glucose transport abolished the GIP secretory response to glucose in the perfused rat intestine (Sykes *et al.*, 1980).

Probably the most potent stimulant of GIP release is ingestion of triglycerides. The GIP response to oral triglycerides is more prolonged and often greater in magnitude, which may be a result of the decreased rate of gastric emptying caused by GIP and related to its enterogastrone activities (Brown & Otte, 1978; Pederson *et al.*, 1975). Furthermore, the chain length of fatty acids is directly related to the potency of GIP release; long chain/highly saturated fatty acids stimulated a more profound GIP secretory profile than either medium or short chain fatty acids (Lardinois *et al.*, 1988; Ross & Shaffer, 1981). The exact mechanism for triglyceride-stimulated IR-GIP release and differences in potency are thought to result from stimulation of the K-cell with/and possibly by metabolism of free fatty acids (FFA) that have been released by the prior action of gastric lipase (Wolfe *et al.*, 1999). As its name suggests, the insulinotropic

activity of GIP is strictly dependent on elevation of blood glucose, and GIP released by FFA has not been shown to be insulinotropic (Pederson & Brown, 1978; Pederson *et al.*, 1975).

Amino acids, peptone and proteins may also cause physiologic GIP release. It has been reported that a mixture of basic amino acids (I, L, K, T, R, H) but not a mixture of aromatic amino (M, P, Y, V) acids stimulates GIP release (Thomas *et al.*, 1978). However, protein meals consisting of cod or steak did not stimulate GIP release (Cleator & Gourlay, 1975; Sarson *et al.*, 1980). It was recently demonstrated that GIP release may be stimulated by protein, and this release was partially inhibited by omeprazole indicating that protein stimulated GIP release may be dependent on gastric acid secretion and subsequent acidification of the duodenum (Wolfe *et al.*, 2000). The mechanism for protein stimulated GIP release has been linked to sodium-dependent amino acid transport and/or K-cell membrane potential (Schulz *et al.*, 1982). GIP release may be inhibited by hyperinsulinemia. Bryer-Ash *et al.* (1994), demonstrated that under euglycemic conditions, hyperinsulinemia inhibited GIP release; however, at high glucose levels the effects of hyperinsulinemia were attenuated.

GIP gene expression is regulated in a parallel manner to that of GIP secretion. A number of groups have demonstrated that glucose increases the mRNA levels of GIP in both rat intestine and intestinal cell models (Higashimoto et al., 1994; Schieldrop et al., 1996; Tseng et al., 1995; 1994). Additionally, triglycerides or FFA may increase GIP expression in rats; although the fat induced effect is very short-lived (Wolfe *et al.*, 1999). Fasting has been shown to decrease both GIP mRNA and intestinal IR-GIP levels, (Higashimoto *et al.*, 1994) as well as to increase GIP mRNA expression (Sharma *et al.*,

1992). The effect of overall nutrition on GIP gene expression is unclear. The GIP promoter contains AP-1 and AP-2 consensus elements for gene regulation by PKA and PKC. In addition the GIP promoter contains 3 sequence elements which share similarity to cAMP response elements although, the exact roles of any of these sequences in controlling GIP expression have not been elucidated (Inagaki *et al.*, 1989).

1.4 The GIP Receptor

1.4.1 Discovery of Specific Binding Sites for GIP

The first demonstrations of specific binding sites for GIP were carried out using GIP radiolabelled with ^{125}I and bound to hamster In 111 cells, human insulinomas or mouse β-TC3 cells (Amiranoff *et al.*, 1984, 1985; Kieffer *et al.*, 1993; Maletti *et al.*, 1987; 1983). These studies all indicated that GIP binds to its receptor with an equilibrium dissociation constant (Kd) in the low nM range. Early studies used cross-linking techniques to irreversibly bind radiolabelled GIP to hamster β-cell membranes. These proteins were then run out on acrylamide gels with the majority of radiation running with an apparent molecular weight of 64 kDa: indicating that the receptor was approximately 59 kDa in size. Furthermore, these studies demonstrated that treatment with dithiothreitol reduced the electrophoretic mobility of the protein, indicating the presence of a disulfide bond (Amiranoff *et al.*, 1986). This group was the first to demonstrate that the GIP receptor was a glycoprotein containing N-acetylglucosamine, mannose and sialic acid: moieties often associated with asparagine-linked glycosylation.

1.4.2 Gene Structure and Homology

The GIP receptor (GIPR) was initially cloned from the rat insulinoma cell line, RINm5F (Usdin *et al.*, 1993). Following this, there were a number of studies reporting the cloning from other human, hamster and rat sources (Gremlich *et al.*, 1995; Volz *et al.*, 1995; Wheeler *et al.*, 1995; Yasuda *et al.*, 1994). Sequence analysis of the GIPR cDNAs isolated indicates that the human gene contains a 1389 base pair (bp) open reading frame (ORF) coding a 466 amino acid protein with a predicted molecular weight of approximately 50 kDa. The rat and hamster gene products are 455 and 462 amino acids respectively. Both the human and rat GIP receptor genes have been characterized. The human gene is composed of 14 exons spanning 13.8 kb: 13 of which encode protein sequences and the other encodes the 5' UTR; while the rat gene is comprised of 15 exons spanning 10.2 kb: with the extra exon encoding a 3' UTR (Boylan *et al.*, 1999; Wolfe *et al.*, 1999; Yamada *et al.*, 1995). Aside from the 3' UTR found in the rat gene, the human and rat genes are identical in structure. These studies collectively demonstrated that the GIP receptor had sufficient sequence identity (25-49 %) to be considered a member of the secretin/VIP family of serpentine, seven transmembrane domain G-protein-coupled receptors (GPCRs). The rat GIP receptor has the highest homology with members of the glucagon family of GPCRs: sharing 44 % sequence identity with the glucagon receptor and 40 % with the glucagon-like peptide–1 receptor. The transmembrane domains are the most highly conserved sequence elements within the GIP, GLP-1 and glucagon receptors, followed by the N-termini. The least conserved regions of the receptors are the C-termini with only 3 common amino acids between all three receptors (Gremlich *et al.*, 1995; Usdin *et al.*, 1993; Wheeler *et al.*, 1995).

The 5'-flanking promoter region of the rat GIPR gene has been sequenced and contains a number of transcription factor binding sites; including 3 SP-1 binding motifs, an octamer-1 (OCT-1) binding site, and a cAMP response element (CRE). However, binding of transcription factors (TF) to these sites has not been verified. The rat GIPR promoter does not contain a TATA box directly upstream of the transcription initiation site; although, there are TATA and CAAT motifs approximately 1kb upstream from the transcription start site. There is, however, an initiator element 10 bp upstream of the transcriptional start site that is identical to the Inr sequence in other genes. Inr elements are important for the binding of RNA polymerase II. Deletion analyses indicated that the first 100 bp upstream from the transcriptional initiation site are necessary for efficient transcription. Furthermore, deletion between −100 and −2500 bp upstream did not effect the ability of the promoter to stimulate luciferase transcription in RIN38 cells (Boylan *et al.*, 1999).

1.4.3 Binding of GIP and Signaling Pathways

Wheeler *et al* (1995) examined the affinity of different orthologs of GIP for the rat receptor following transfection in Chinese hamster ovary K1 (CHO) cells or COS-7 cells. They found that both synthetic porcine (sp) GIP and synthetic human (sh) GIP had comparable IC_{50} values for displacing radiolabelled spGIP from the GIP receptor. These IC_{50} values were approximately 3 nM and 8 nM in CHO cells and COS-7 cells respectively and were similar to those obtained for the hamster GIP receptor (Wheeler *et al.*, 1995; Yasuda *et al.*, 1994). Due to the sequence similarity in the N-terminus of the GIP receptor (the postulated binding site for GIP) with other members of the glucagon receptor family, it was hypothesized that the glucagon family of peptides, which share

homology with GIP, may be able to activate the GIP receptor (McIntosh *et al.*, 1996). However, when this was tested, only 1 μM exendin$_{9-39}$ or exendin-4$_{1-39}$, (GLP-1 receptor antagonist and agonist respectively), were able to displace ^{125}I labeled GIP from the receptor. Secretin, VIP, glucagon, GLP-1 and GLP-2 had no effect (Wheeler *et al.*, 1995).

Prior to the cloning of the GIP receptor it was demonstrated that GIP in the low nM range stimulated adenylyl cyclase in a hamster pancreatic tumor cell line (Amiranoff *et al.*, 1984; Lu *et al.*, 1993), as well as isolated islets (Siegel & Creutzfeldt, 1985) and in HGT-1 cells (Gespach *et al.*, 1984). Whether expressed in Chinese hamster ovary (CHO), lung, LVIP cells or COS cells, the human, rat and mouse forms of the receptor all respond to GIP by activation of adenylyl cyclase and subsequent elevation of cyclic adenosine monophosphate (cAMP). However, each group of authors reported slightly different EC$_{50}$ values ranging from 0.1 pM to approximately 15 nM (Gremlich *et al.*, 1995; Volz *et al.*, 1995; Wheeler *et al.*, 1995; Yasuda *et al.*, 1994). Studies indicate that there is not a glucose dependence for GIP-stimulated cAMP production in β-TC3 cells and in INS(832/13) cells; thus, glucose metabolism does not seem to affect this signal transduction module and the glucose dependence must come about at later steps in the exocytotic process (Ehses *et al.*, 2001; 2002; Hinke *et al.*, 2000a).

GIP has also been demonstrated to increase Ca^{2+} levels in isolated islets at elevated glucose levels (Wahl *et al.*, 1992), as well as in HIT-T15 insulinoma cells (Lu *et al.*, 1993) via influx through L-type voltage dependent calcium channels. Wheeler *et al.* (1995) demonstrated that activation of the GIP receptor in COS-7 cells led to an increase in intracellular calcium in a nifedipine-independent manner that could be inhibited by

thapsigargin: indicating that GIP was able to couple to other voltage-independent calcium channels. GIP, however, has never been shown to couple to phospholipase C (PLC) and stimulate the release of inositol trisphosphate (IP_3) (Lu *et al.*, 1993; Yasuda *et al.*, 1994). Furthermore, it was suggested by Ehses *et al.* (2001) that GIP could stimulate Ca^{2+} release from intracellular stores via activation of PLA_2 and the consequent release of arachidonic acid (AA). However, the exact pathway by which GIP increases intracellular Ca^{2+} has yet to be elucidated.

It has been previously speculated that GIP may exert its effects through both AA (Lardinois *et al.*, 1990) and through activation of MAP kinases (Kubota *et al.*, 1997). Recently, Ehses *et al.* (2001) demonstrated that GIP liberates AA via activation of a calcium independent form of PLA_2, suggesting that GIP may potentiate insulin secretion via this pathway. Furthermore, they demonstrated that PLA_2 is activated in these β-cell models (βTC-3 cells) by Gβγ dimers and that this activation is dependent on elevated cAMP. In another recent study, Ehses *et al.* (2002) demonstrated that GIP activates the ERK module in a cAMP and PKA dependent manner probably via activation of B-Raf in INS(832/13) β-cells. They hypothesized that GIP receptor activation could lead to proliferation/differentiation or gene transcription within the β-cell in response to activation of the ERK module. Thus, GIP signaling pathways in the β-cell are much more complicated than previously thought, and at present are not completely elucidated.

1.4.4 Structure-Function Relationships

The amino terminus of the receptor contains consensus sequences for N-type glycosylation; in addition, the third intracellular loop and the C-terminus of the receptor are rich in serine residues that could serve as potential phosphorylation sites (Usdin *et al.*,

1993; Wheeler *et al.*, 1999; 1995). Recent studies have begun to characterize the regions

of the GIP receptor which are important for binding of GIP, G-protein coupling,

desensitization and internalization. Studies utilising GIP/GLP-1 receptor chimeras have

indicated that the amino-terminal tail of the GIP receptor is important for high affinity

ligand binding and that the first transmembrane helix is important for coupling of the

receptor to the intracellular signal transduction machinery (Gelling *et al.*, 1997). Further

studies in which the carboxy-terminal tail of the receptor was truncated, demonstrated

that the C-terminus is not essential for binding or signaling but necessary for proper

expression and possibly orientation of the receptor within the cytoplasmic membrane

(Wheeler *et al.*, 1999). Also, it was recently shown that C-terminal receptor truncation

(at amino acid 425) did not greatly affect GIP-induced desensitization but may have

slowed initial receptor uptake (Wheeler *et al.*, 1999).

1.4.5 Tissue Distribution

GIP receptor mRNA is expressed in the pancreas, stomach, intestine, adipose

tissue, adrenal cortex, heart, lung, endothelium, telencephalon, diencephalon, brain stem,

cerebellum and the pituitary (Usdin *et al.*, 1993; Yasuda *et al.*, 1994; Zhong *et al.*, 2000);

although, the function of the receptor in some of these tissues is not known.

Radiolabelled GIP binding in the rat brain has been characterized using autoradiography.

Most of the brain sections that expressed GIPR mRNA also bound ^{125}I-GIP, with the

exception of the pituitary. High affinity binding sites were noted in the olfactory bulb

(Kaplan & Vigna, 1994). One enigmatic point is that GIP has never been detected in

brain extracts and GIP mRNA has never been detected in the brain. Therefore, it is

possible that an alternate molecule exists in the brain that activates the GIPR (McIntosh *et al.*, 1996).

Expression of the GIPR in the adrenal cortex may result in increased glucocorticoid metabolism in response to GIP release and has been shown to play a role in food-induced Cushing's syndrome (Croughs *et al.*, 2000; Lacroix *et al.*, 1992). Recently, it was shown that the GIPR was expressed in bone, and stimulation of SaOS2 cells (an osteoblast cell line) by GIP led to increased expression of collagen type I mRNA and increased alkaline phosphatase activity. Both of these effects are osteotrophic, and led Bollag *et al.* (2001; 2000) to propose the existence of an entero-osseous axis; whereby GIP could control bone density in response to nutrient intake.

1.5 Biological Actions of GIP

1.5.1 Gastric Secretion

GIP was initially isolated for its inhibitory effect on gastrin-stimulated gastric acid secretion in dogs (Pederson & Brown, 1972) and subsequent studies supported the role of GIP as an enterogastrone (Arnold *et al.*, 1978b; Villar *et al.*, 1976). However, some studies questioned the enterogastrone activity of GIP because they observed rather weak inhibition of gastric acid secretion and only with supraphysiological doses (Andersen *et al.*, 1978; Arnold *et al.*, 1978a; El Munshid *et al.*, 1980; Maxwell *et al.*, 1980; Soon-Shiong *et al.*, 1979; Yamagishi & Debas, 1980). However, during this time McIntosh *et al.* (1979) suggested that since the onset of acid inhibitory effects of GIP was slow, it was possible that GIP was causing the release of another substance that was inhibiting gastric acid secretion. Furthermore, there was ample evidence at this time that somatostatin secreting D-cells abutted on gastrin secreting G-cells (Larsson *et al.*, 1979)

and that somatostatin was capable of inhibiting acid secretion (Bloom *et al.*, 1974).

McIntosh *et al.* (1981b) demonstrated that IR-somatostatin was released from D-cells in response to GIP in the perfused rat stomach and that the release of somatostatin was inhibited by vagal activation or acetylcholine administration. This vagally mediated inhibition of GIP-stimulated somatostatin release was only partially blocked by atropine (McIntosh *et al.*, 1981b), indicating that other neurotransmitters may be involved (McIntosh *et al.*, 1983). The authors hypothesized that the putative processes from the D-cells in the stomach were in direct contact with gastrin releasing G-cells; with release of somatostatin having an inhibitory effect on gastrin secretion and a decreased acid output from the parietal cells (McIntosh *et al.*, 1981b). Another series of experiments demonstrated that sympathetic activation may also modulate GIP stimulated somatostatin secretion and thereby gastric acid secretion (McIntosh *et al.*, 1981a). Subsequently, Soon-Shiong *et al.* (1984) reported that GIP had no effect on acid secretion if it was co-administered with Bethanechol, a cholinergic agonist. This observation suggested that the parasympathetic nervous system also controlled the enterogastrone properties of GIP. Overall, the mechanism by which GIP exerts enterogastrone action is via stimulation of somatostatin secretion in the stomach with modulation from the autonomic nervous system.

1.5.2 Adipose Tissue and Fat Metabolism

Triglycerides are digested in the stomach and small intestine and the resultant FFA are absorbed by the K-cell. As previously described, these FFA are possibly the strongest stimulant of GIP release postprandially (Ebert & Creutzfeldt, 1980; Pederson, 1994; Ross & Shaffer, 1981; Yoshidome *et al.*, 1995). Additionally, GIP receptor mRNA

was found in adipose tissue as well as in differentiated 3T3-L1 cells and it has been demonstrated that GIP may be involved in the subsequent clearance of circulating triglycerides (McIntosh *et al.*, 1999). GIP has been shown to cause an increase in triglyceride clearance from the blood of dogs and rats (Ebert *et al.*, 1991; Wasada *et al.*, 1981), possibly by activation of lipoprotein lipase (Eckel *et al.*, 1979). GIP has also been shown to have discrete effects on lipid metabolism within adipose tissue. Although no systematic studies have been carried out, it has been demonstrated that GIP is capable of augmenting synthesis of fatty acids from both glucose and lipid sources (Hauner *et al.*, 1988). Furthermore, these authors and others (Dupre *et al.*, 1973) demonstrated that GIP also strongly inhibited glucagon-stimulated cAMP production and lypolysis and may have improved insulin binding affinity in adipose tissue; concluding that GIP has insulin-like effects in this tissue (Hauner *et al.*, 1988). In this vein, Miyawaki *et al.* (2002) recently demonstrated that GIPR -/- mice were protected from high fat induced obesity, while wild-type mice demonstrated "extreme visceral and subcutaneous fat deposition and insulin resistance." These authors also demonstrated that the *ob/ob* phenotype was partially rescued by crossing *ob/ob* mice (morbidly obese) with GIPR -/- mice. Thus, this group hypothesizes that the GIPR expressed on adipose tissue could be a potential target for anti-obesity therapy (Miyawaki *et al.*, 2002).

In contrast, GIP has also been shown to be lipolytic in some studies. Hauner *et al.* (1988) showed that GIP was weakly lipolytic and more recently a study demonstrated that the GIP receptor was expressed and signaled via cAMP in the differentiated 3T3-L1 adipocyte model (McIntosh *et al.*, 1999). Furthermore, this study demonstrated that GIP was able to stimulate glycerol release from these adipocytes in the physiological dose

range in a cAMP-dependent fashion, and that this could be inhibited by preincubation with insulin through a wortmannin-dependent pathway (McIntosh *et al.*, 1999). This paper concluded that GIP-induced lipolysis may be responsible for increasing FFA levels sufficiently, to optimize the insulin secretory response of the β-cell. In conclusion, it is clear that further studies need to be carried out to determine the effect of GIP on lipid metabolism; although, it could be the case that the exact effect of GIP on lipid metabolism is dependent on the ambient lipid levels and prevailing metabolic state of the organism.

1.5.3 GIP and Islet Hormone Secretion

Most of the evidence to date supports the fact that GIP is a potent incretin and acts via the enteroinsular axis to stimulate insulin secretion from the β-cell. The first studies indirectly showed that impure preparations of CCK stimulated insulin secretion, and that if the preparations of CCK were purified, the insulinotropic potency decreased (Rabinovitch & Dupre, 1972). These observations were similar to those made by Brown *et al.* (1970) on the effect of CCK preparations on gastric acid secretion and once isolated, provided the impetus for examining the role of GIP on insulin secretion. Later it was demonstrated that GIP stimulated insulin secretion in humans (Dupre *et al.*, 1973), dogs (Pederson *et al.*, 1975) and in rats (Ebert & Creutzfeldt, 1982; Pederson & Brown, 1976; 1978). Furthermore, GIP has been shown to be insulinotropic in isolated islets (Hinke *et al.*, 2000a; Lynn *et al.*, 2001) as wells as in many β-cell lines (Ehses *et al.*, 2001; 2002; Kieffer *et al.*, 1993; O'Harte *et al.*, 1998).

In vivo, GIP stimulates insulin secretion in the rat (Pederson & Brown, 1976; Tseng *et al.*, 1996b), in the human (Dupre *et al.*, 1973; Elahi *et al.*, 1979), and in dog

(Pederson *et al.*, 1975) only when glucose levels are elevated above approximately 5 mM. In the rat perfused pancreas model, the maximum co-stimulatory glucose concentration was determined to be around 16 mM; thus, many of the later experiments were carried out at this glucose concentration (Pederson & Brown, 1976). This property prompted Pederson *et al* (1976) to suggest an alternate name for GIP: **G**lucose-dependent **i**nsulinotropic **p**olypeptide. Aside from the glucose-dependence, the insulin secretory response to GIP is also dose dependent. It was demonstrated that GIP concentrations reached postprandially are able to stimulate insulin secretion in normal rats (Pederson & Brown, 1976; Pederson *et al.*, 1982).

The exact pathway by which GIP stimulates secretion of insulin has begun to be elucidated and it is believed that the hormone exerts the majority of its physiological effects on the β-cell via activation of adenylyl cyclase and stimulation of cAMP production. However, as previously mentioned, other signaling pathways have been implicated (Ehses *et al.*, 2001; Ehses *et al.*, 2002; Trumper *et al.*, 2002; 2001). Most of the studies carried out to date have demonstrated that glucose metabolism is a necessary prerequisite for GIP-stimulated insulin secretion. When D-glyceraldehyde was included in the perfusate, GIP was able to stimulate insulin secretion from the perfused pancreas in the absence of glucose (Dahl, 1983). Furthermore, mannoheptalose, a glycolysis inhibitor, abolished GIP stimulated insulin secretion in the perfused rat pancreas (Mueller *et al.*, 1982). A series of recent studies by our laboratory have indicated that GIP may also cause insulin secretion, in a K^+_{ATP} independent manner as well. These studies demonstrated that GIP was able to stimulate both cAMP production and insulin secretion

in clonal β-cells that had been depolarized with high external potassium and diazoxide; albeit in a Ca^{2+} dependent manner.

Recently, Béguin et al. (1999) demonstrated that stimulation of the β-cell by GIP caused phosphorylation of the Kir6.2 (K_{ATP}) channel on serine 372 via protein kinase A. Phosphorylation of this serine residue led to an increased open probability of the channel. This paper was the first demonstration that GIP stimulation of the β-cell leads to protein phosphorylation. However, the physiological basis for this phosphorylation event is still unclear since Béguin and colleagues believe that Kir6.2 is maximally phosphorylated in the basal state.

Additionally, GIP may have effects on β-cell proliferation and cell survival, and recent studies in our lab and others (Trumper et al., 2001) have demonstrated that GIP is an extremely potent anti-apoptotic agent; and that these effects are manifested via inhibition of the p38 stress activated kinase signaling module.

It has also been demonstrated that GIP has actions on the other cell-types within the islet. GIP-stimulated glucagon release from isolated, cultured islets (Fujimoto et al., 1978; Verchere, 1991), and from the perfused rat pancreas (Pederson & Brown, 1978). In addition, secretion of glucagon in response to GIP only occurs at glucose levels below a threshold of 5.5 mM in humans and rats (Elahi et al., 1979; Pederson & Brown, 1978); however, GIP is able to increase glucagon secretion in the face of high glucose in mice (Opara & Go, 1991). Thus, the effect of GIP on glucagon secretion is probably species dependent and may depend on the overall metabolic state of the organism. Finally, GIP has been demonstrated to stimulate somatostatin release from δ-cells in pancreatic islets; though, the physiological relevance of this is not clear because the direction of blood

flow is believed to be from β-cell to δ-cell and there is only a weak stimulation of somatostatin release produced by GIP (Schmid *et al.*, 1990; Verchere, 1991).

1.5.4 Other Biological Effects

There are many other examples of the effect of GIP on other tissues; however, none of these actions have been very well characterized. GIP has been shown to affect blood flow in the vascular beds of dogs: some beds are more highly perfused in the presence of GIP *e.g.* the superior mesenteric artery and portal vein while others are not affected *e.g.* the celiac artery and hepatic artery (Kogire *et al.*, 1988; Kogire *et al.*, 1992). Recently splice variants of the GIP receptor have been demonstrated in endothelial tissue that could be responsible for the disparities in GIP action in different vascular beds (Zhong *et al.*, 2000). Zhong *et al.* (2000) reported preliminary data that indicated that GIP can signal to different degrees via either increases in Ca^{2+} or PKA activation in different endothelial cell types. As previously mentioned, GIP has been implicated in bone metabolism, where it is believed to have an anabolic role (Bollag *et al.*, 2001; 2000). Thirdly, GIP receptors have been localized to various regions in the brain; however, GIP has never been localized to any areas of the brain. Interestingly, pharmacological doses of GIP injected into the 3rd ventricle reduced plasma follicle-stimulating hormone, and increased growth hormone levels but had no effect on luteinizing hormone, thyroid-stimulating hormone, or prolactin levels (Ottlecz *et al.*, 1985). Presently it has not been determined whether GIP is able to cross the blood-brain barrier or if another hormone or substance is able to activate GIP binding sites in the brain. GIP is also able to decrease lower esophageal sphincter pressure (Sinar *et al.*, 1978), decrease intestinal motility (Fara & Salazar, 1978), decrease water and electrolyte

uptake across the small intestine (Helman & Barbezat, 1977) and may play a role in skeletal muscle glucose utilization (Kahle *et al.*, 1986).

1.6 Evidence for Other Incretins

Early studies reported that infusion of GIP antibodies into rats did not completely block the differential insulin response between oral or intraduodenal (Ebert & Creutzfeldt, 1982) and IV glucose. More recently, Tseng *et al.* (1996b) infused GIP $_{7-30NH2}$, a specific antagonist at the GIP receptor, into rats and determined that the insulin reponse to oral glucose was decreased by 72 %. Taken together, these studies indicate that GIP contributes significantly to the enteroinsular axis: release of GIP causes 20-70 % of the response to oral vs IV glucose.

1.7 Glucagon-Like Peptide-1

GLP-1 is genetically encoded within the proglucagon gene. Posttranslational processing cleaves GLP-1, GLP-2 and glicentin from proglucagon in the L-cells of the intestine; whereas, different processing in the α-cells of the pancreas primarily produces glucagon (Fehmann *et al.*, 1995). GLP-1 is released from the L-cells of the ileum in response to ingested nutrients, primarily glucose and amino acids such as arginine (Elliott *et al.*, 1993). However, the mechanisms that control the postprandial secretion of GLP-1 are thought to be different from those that control GIP secretion. This is indicated by the fact that the majority of the L-cells are located in the distal small intestine, a site that is not directly stimulated by food prior to the rise in postprandial GLP-1 secretion (Elliott *et al.*, 1993; Fehmann *et al.*, 1995). A number of studies have indicated that GIP may exert a feed forward effect on the L-cells to stimulate postprandial GLP-1 secretion (Damholt

et al., 1999; Elliott *et al.*, 1993; Herrmann-Rinke *et al.*, 1995; Roberge & Brubaker, 1993).

The GLP-1 receptor has a wide tissue distribution including: brain, lung, stomach pancreatic islet, hypothalamus, heart, intestine, and kidney. Upon binding to its receptor, GLP-1 activates a seven transmembrane domain G-protein-coupled receptor that has sufficient identity to be considered a member of the secretin/VIP family of GPCRs. Activation of the GLP-1 receptor involves many signaling pathways that appear to be similar to those activated by GIP, at least in the proximal, or receptor associated events. These include activation of adenylyl cyclase and an increase in cAMP, increase in intracellular Ca^{2+} via extrusion from intracellular stores as well as opening of VDCC, and activation of PLC (Kieffer & Habener, 1999).

The primary biological action of GLP-1 is believed to be potentiation of insulin secretion from the β-cell. Schmidt *et al.* (1985) demonstrated that GLP-1, but not GLP-2, was capable of potently stimulating insulin secretion from the perfused rat pancreas. More than 80 % of circulating GLP-1 is in the $7\text{-}36_{NH2}$ form, and it was hypothesized that due to the shared sequence identity between glucagon and GLP-1, this was probably the biologically active, highly insulinotropic form (Fehmann *et al.*, 1995). GLP-1 is insulinotropic in the presence of high glucose in human (Kreymann *et al.*, 1987), pig (Holst *et al.*, 1987), and rat (Mojsov *et al.*, 1987), as well as in a number of β-cell lines (Lu *et al.*, 1993; Montrose-Rafizadeh *et al.*, 1994; Susini *et al.*, 1998), and isolated islets (Siegel *et al.*, 1992; Suzuki *et al.*, 1992).

GLP-1 also inhibits pancreatic glucagon secretion in humans (Nauck *et al.*, 1993b), the rat (Matsuyama *et al.*, 1988), the dog (Kawai *et al.*, 1989) and in isolated

islets (Fehmann *et al.*, 1995) thus enhancing its glucose lowering effects. Additionally, GLP-1 stimulates pancreatic δ-cells causing profound somatostatin release (Fehmann *et al.*, 1995). It is still unclear whether GLP-1 inhibits glucagon secretion by causing somatostatin release or if there are GLP-1 receptors located on α-cells (Heller *et al.*, 1997; Moens *et al.*, 1996).

One of the other physiological actions of GLP-1 that may contribute to its glucose lowering effect is its inhibitory effect on gastric emptying (Nauck *et al.*, 1997). Since fats and chyme are potent stimulators of GLP-1 release, it has been proposed that GLP-1 might be the major hormone acting as an 'ileal brake' and have a more minor role as an incretin (Kieffer & Habener, 1999). In support of this hypothesis, GLP-1 does inhibit both gastric acid secretion and gastric emptying when infused in physiological concentrations in many models (Nauck *et al.*, 1997; O'Halloran *et al.*, 1990; Schjoldager *et al.*, 1989; Willms *et al.*, 1996)

Glucagon-like peptides and GLP-1 receptors are expressed in the hypothalamus where it is believed that binding of agonist can exert anorexic effects. In fact, GLP-1 injection into the 3rd ventricle leads to large decreases in food and water intake that can be inhibited with exendin$_{9-39}$; indicating specificity for GLP-1 receptors (Turton *et al.*, 1996). It is still not clear whether GLP-1 leads to satiety or food aversion and whether or not GLP-1 is able to cross the blood-brain barrier or if locally produced GLP-1 acts on these neurons (Kieffer & Habener, 1999).

1.8 GIP and GLP-1 Metabolism

Upon release into the circulation GIP_{1-42} is rapidly (1-2 min) degraded to GIP_{3-42} which renders the peptide biologically inactive (Jornvall *et al.*, 1981; Kieffer *et al.*,

1995b; Pederson *et al.*, 1996; Suzuki *et al.*, 1989). This degradative process is catalysed by the aminopeptidase, dipeptidyl peptidase IV (DP IV), which preferentially cleaves peptides containing a penultimate N-terminal proline or alanine residue (*e.g.* GIP, GLP-1, GHRH, NPY) but can also degrade peptides containing a serine in the penultimate position, such as glucagon (Hinke *et al.*, 2000b; Pospisilik *et al.*, 2001; Yaron & Naider, 1993). DP IV is ubiquitously distributed; however, the highest concentrations are found in the brush borders of both the kidney and the intestinal epithelia (Yaron & Naider, 1993). Recent studies have shown that inhibition of circulating DP IV, by unhydrolyzable analogue substrates, such as isoleucine thiazolidide, improves the glucose tolerance in the VDF model of T2D (Pederson *et al.*, 1998b) and further that these inhibitors can alleviate the hyperglycemia associated with T2D (Pospisilik *et al.*, 2002). These findings suggest that inhibition of DP IV is effective in increasing the biological half-life of GIP (and GLP-1) within the circulation and thereby, augmenting the role of the incretins within the entero-insular axis and that DP IV is the primary means of modulating incretin bioactivity *in vivo* (Deacon *et al.*, 2000; Hansen *et al.*, 1999; Pauly *et al.*, 1996).

1.9 Pathophysiology of GIP release and Actions

Because GIP is an important incretin, the role of this hormone in T2D has been extensively studied. No consensus exists regarding changes in circulating GIP levels in T2D. It has been reported that GIP levels are increased (Elahi *et al.*, 1984; Jones *et al.*, 1989b; Ross *et al.*, 1977); although, there has been some research indicating GIP levels decrease (Groop, 1989) or remain unchanged (Levitt *et al.*, 1980; Service *et al.*, 1984) in T2D. Another defect in T2D patients is a reduced incretin effect; consequently, oral

glucose does not produce a markedly greater insulin response than an isoglycaemic intravenous infusion as described by Perley and Kipnis (1967). Furthermore, studies indicate that the pancreas is responsive to GIP in T2D (Jones *et al.*, 1989b; 1987); however, there is a marked attenuation of GIP induced insulin secretion (Meneilly *et al.*, 1993). Nauck *et al.* (1993b) and others have shown that there is little or no pancreatic response to natural or synthetic human or porcine GIP in some type 2 diabetic groups (Elahi *et al.*, 1994; Krarup *et al.*, 1987). In contrast, numerous investigations have shown that T2D patients are fully responsive to exogenous GLP-1 and additionally, the pancreata of those patients that are unresponsive to GIP are responsive to GLP-1 (Elahi *et al.*, 1994; Nauck *et al.*, 1993b). Both hormones signal via seven transmembrane domain G-protein coupled receptors (of the same family) to increase adenylyl cyclase activity and intracellular cAMP concentrations (Thorens, 1995; Usdin *et al.*, 1993; Wheeler *et al.*, 1995) and therefore, it is interesting that the sensitivity of the diabetic β-cell to the two hormones is so distinct (Holst *et al.*, 1997). A complicating factor in assessing the glucose lowering actions of GLP-1 is that this hormone has physiologically important insulin-independent glucose lowering actions such as decreasing hepatic glucose output, increasing muscle and adipose glucose uptake, decreasing gastric emptying, and suppressing glucagon secretion (Drucker, 1998).

One explanation that could be given for the lack of GIP effect on the diabetic β-cell is that these cells either do not express a GIP receptor or express a defective form. In fact, Kubota *et al.* (1996) identified two missense mutations in the GIP receptor gene (G198C, Q354E) in Japanese T2D subjects. One of these mutations (G198C) was shown to dramatically affect GIP stimulated cAMP production; however, association studies

were unable to conclusively provide a relationship between T2D and either of these mutations. Thus, it does not seem probable that a mutant GIP receptor is a causative factor in T2D; however, it is possible that a mutation in the 5' flanking/promoter sequence of the GIP receptor gene could cause inefficient receptor transcription (Holst *et al.*, 1997). This in turn, could decrease receptor expression level and potentially predispose an individual to T2D. Furthermore, it has been shown by Tseng *et al.* (1996a) that rats rendered diabetic by streptozotocin treatment had markedly increased GIP mRNA levels. Furthermore, these experiments demonstrated that when GIP was infused over 6 hours in anaesthetized animals there was a lack of insulinotropic activity at approximately 4 hours, indicating GIP receptor desensitization. Additional studies in the LGIPR2 cell line indicated that the GIP receptor was desensitized in a ligand specific manner, as the cAMP response to other substances was unaffected (Tseng *et al.*, 1996a). Thus, it is also possible that the insensitivity of the islet to GIP in T2D is a result of chronic desensitization of the GIP receptor by the high ambient GIP levels.

Chan *et al.* (1984) found that the insulin secretory response to GIP was enhanced in fatty Zucker rats and additionally, that the glucose threshold for GIP actions was lower than fasting glucose levels. It has also been shown that postprandial GIP levels in obese subjects are much higher than in normal subjects (Brown & Otte, 1978). Thus, it appears that in the obese state the insulinotropic activity of GIP may become uncontrolled.

A substrain of the Zucker (*fa/fa*) rat, the Zucker Diabetic Fatty rat (ZDF) has recently been described (Friedman *et al.*, 1991). In this strain, obese animals (males more pronounced) develop severe glucose intolerance and an impaired ability of the β-cell to respond to glucose. This decreased responsiveness to glucose including the loss of

the first phase insulin secretory response to glucose is characteristic of T2D in humans (Sturis *et al.*, 1994). As noted above, this is in contrast to the Zucker *fa/fa* rat which remains hyperresponsive to all insulin secretagogues (Chan *et al.*, 1984). The Zucker *fa/fa* colony maintained by our laboratory (Vancouver Zucker Fatty, VDF) has developed a milder form of the glucose intolerance and insulin secretory defects exhibited by the ZDF rat, including fasting hyperglycaemia, and glucose intolerance as well as the loss of first phase of insulin secretion. Preliminary results also indicate a decreased responsiveness of the isolated perfused pancreas to GIP compared to lean littermates. Thus, these animals provide a model to investigate possible changes in GIP and the GIP receptor at the time of onset of the diabetic state (10-12 weeks).

1.10 Nutrient Regulation of Gene Expression

1.10.1 Glucose Regulation of Gene Expression

The regulation of gene expression by glucose allows organisms to adapt to their internal nutritional load usually by regulation of genes involved in lipid or glucose metabolism. Genes that are regulated by glucose can fall into two categories; those that are regulated by glucose levels greater than 5 mM and are regulated to improve the response during nutritional abundance or those which are strongly regulated in the 0-5 mM range and offer protection/adaptation to energy/glucose starvation (Foufelle *et al.*, 1998). Most of the genes identified to date have been of the first category and are those that are induced at the transcriptional level by high glucose, for example glucose induces expression of fatty acid sythase (FAS) in adipose tissue as well as pyruvate kinase (PK) in the liver and pancreatic β-cell (Towle, 1995; Vaulont & Kahn, 1994). It is believed

that phosphorylation of glucose is a prerequisite for its regulatory effects on gene transcription. Studies in both adipose tissue and INS-1 cells have demonstrated that 2-deoxyglucose (which is phosphorylated to 2-deoxyglucose-6-phosphate but then not further metabolized) is able to regulate expression of PK in a manner similar to glucose. Furthermore, the cellular concentrations of glucose-6-phosphate are regulated in a manner similar to the PK gene and finally the kinetics of the upregulation match those of glucose phosphorylation (Foufelle *et al.*, 1998). Some groups have also proposed that this regulation could occur via xylulose-5-phosphate, which is found in some cells and is an intermediate in the pentose-phosphate pathway of non-oxidative glucose metabolism (Doiron *et al.*, 1996)

There have been two glucose response elements (GRE) identified: the first from the PK gene promoter (Thompson & Towle, 1991) and the second from the S14 gene promoter (Shih & Towle, 1992). Sequence comparison between these two GREs revealed that the canonical sequence for a GRE is two E-box-like sequences of CANNTG separated by 5 nucleotides. Two trans-acting factors have been proposed to have roles in the control of gene expression by glucose. First, the upstream stimulatory factor/major late transcription factor (USF/MLTF) family has been implicated in binding to the PK GRE in β-cells (Kennedy *et al.*, 1997). Secondly, the sterol regulatory element binding protein/adipocyte determination differentiation-dependent factor 1 (SREBP/ADD1) has been shown to activate the S14 GRE in response to glucose (Kim *et al.*, 1995) and in another study found to bind an E-box motif in the FAS promoter and activates transcription of FAS (Kim *et al.*, 1998). The exact pathway by which glucose-6-phosphate stimulates activation or repression of either SREBP/ADD1 or USF is not clear

but involves regulating the amount of active transcription factor within the cell by any of a number of mechanisms, for example phosphorylation, allosteric modification by binding of glucose-6-phosphate, or a combination of the two (Foufelle *et al.*, 1998).

There have been two reports of genes that are suppressed by high glucose. The phosphoenolpyruvate carboxykinase gene is downregulated in hepatocytes (Cournarie *et al.*, 1999) and the peroxisome proliferator activated receptor α gene is downregulated in the pancreatic β-cell by high glucose (Roduit *et al.*, 2000). Both of these studies reported that glucose phosphorylation was necessary for the downregulation to occur.

1.10.2 Fat Regulation of Gene Expression – Peroxisome Proliferator Activated Receptors

FFA regulation of gene transcription is also a relatively new field; however, the last 10 years have yielded significant developments in understanding the effects of fatty acids on gene transcription. One of the most studied pathways by which polyunsaturated fatty acids (PUFA) negatively regulate gene expression is that of the hepatic lipogenic enzymes including: FAS, acetyl CoA carboxylase (ACC), Liver PK, ATP citrate-lyase, malic enzyme, stearoyl CoA desaturase (SCD1), apolipoprotein A-1 (apo-A1), the S14 protein (S14), and Δ5- and Δ6-desaturases (Duplus *et al.*, 2000). Most of these genes are negatively regulated by decreasing mRNA transcription; however, as yet it is unclear whether this is a direct effect of PUFA or of their peroxidative products on gene promoters (Foretz *et al.*, 1999). Fatty acids also regulate genes in a positive manner, mostly in adipocytes. This was first demonstrated by Amri *et al* when they demonstrated FA induced adipocyte lipid-binding protein (aP2) gene transcription in pre-adipocytes through a cycloheximide-dependent mechanism; indicating that FFA are not having a

direct effect on gene transcription but may be acting via a transcription factor (Amri *et al.*, 1991a; 1991b). Phosphoenolpyruvate carboxykinase (PEPCK) is also regulated positively by FFA in adipocytes (Antras-Ferry *et al.*, 1994; 1995) and many hepatic genes are upregulated including: acyl-CoA oxidase (AOX), carnitine palmitoyl transferase-1 (CPT-1), the liver fatty acid binding protein (L-FABP), cytochrome P4504A1, acyl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA synthase, and cholesterol 7α-hydroxylase. CPT-1 expression is regulated at both the transcriptional and translational level and it appears that oxidation of fatty acids are not necessary for induction (Duplus *et al.*, 2000).

There are a number of proposed mechanisms by which fatty acids can affect gene transcription, including: phosphorylation of TF via a kinase cascade, direct binding of FA to TF, change of transcription rate of target gene or TF, change in mRNA stability of target gene or TF (Duplus *et al.*, 2000). One of the only families of TF that fulfill the requirement of being FA activated receptors are the peroxisome proliferator activated receptors (PPARs). The first member of this family of TFs was cloned in the 1990 on the basis that it was activated by 'hepatocarcinogens' that caused proliferation of peroxisomes in the hepatocytes of mice and subsequent hypolipidemia due to an increase in peroxisomal β-oxidation (Issemann & Green, 1990). Subsequently, PPARs were cloned from other species including, hamsters (Aperlo *et al.*, 1995), humans (Sher *et al.*, 1993) and *Xenopus* (Dreyer *et al.*, 1992). The study done in *Xenopus* indicated that there are at least 3 PPAR isoforms: they were designated PPARα, PPARβ/δ and PPARγ; subsequently the nomenclature was revised for the superfamily and now they are all included as members of group C in the subfamily 1 of nuclear receptors or NR1C1,

NR1C2 and NR1C3 respectively (Escher & Wahli, 2000). Furthermore, the three paralogs have now been cloned from rodents and humans and have been found to share considerable sequence identity. The PPAR genes that have been analyzed show considerable conservation in exon structure. Six exons are common to all of the PPAR genes: one exon encodes the N-terminal A/B domain, two exons encode the DNA-binding domain (one exon for each of the two zinc fingers), one exon encodes the hinge region and two exons encode the ligand binding domain (Beamer *et al.*, 1997; Gearing *et al.*, 1994; Krey *et al.*, 1993; Zhu *et al.*, 1995). However, there is some variation in the 5' UTR structure in some of the PPARs, specificially with PPARγ which, in humans can have 3 different splice variants (Fajas *et al.*, 1998; Zhu *et al.*, 1995).

Using transactivation assays, it was demonstrated that PPARα could activate transcription of the AOX promoter in cooperation with the retinoid X receptor (RXR) in response to PUFA as well as saturated FA with chain lengths > 6 carbons (Gottlicher *et al.*, 1992; Keller *et al.*, 1993). Using similar techniques it has been demonstrated that PUFA and thiazolidiones are higher affinity ligands for PPARβ and PPARγ while saturated FA and fibrate drugs bind PPARα with high affinity (Desvergne & Wahli, 1999). Additionally, it has been demonstrated that various fatty acid metabolites, particularly leukotrienes (B4), prostaglandins (15-deoxy-$\Delta^{12,14}$-prostaglandin J2) and arachidonate (8S-hydroxeicosatetraenoic acid) derivatives, are able to bind to and potently activate PPARs. These molecules could act as second messengers in the FA control of gene transcription; however, the transduction cascades by which these molecules are produced are still unclear (Duplus *et al.*, 2000).

Upon binding to ligands, PPARs heterodimerize with the RXR via the

'D-box' domain and are transported to the nucleus where they influence gene transcription by binding to specific sequence elements, known as peroxisome proliferator response elements (PPREs), within gene promoters. Using transactivation assays for a number of genes that are responsive to PPAR activation, the exact sequence element that makes up the PPRE has been determined (Tugwood *et al.*, 1992). The canonical sequence is a direct repeat of the sequence AGGTCA separated by one nucleotide. On the 5' end of the sequence there is an extended AACT that is important for specificity and polarity of PPAR binding with the PPAR and RXR moieties binding to the up and downstream repeats respectively (Desvergne & Wahli, 1999). Genes that are either regulated in a positive or negative manner by PPAR/PPREs include: AOX, apoA-1, aP2, CPT-1, L-FABP, SCD1, S14, malic enzyme, uncoupling protein 1 (UCP1), and acyl-CoA synthase (ACS) (Desvergne & Wahli, 1999).

The three PPAR paralogs are expressed in a distinct pattern within various tissues. In general, PPARα is expressed in tissues where catabolism of fatty acids usually occurs. In the mature rodent, mRNA for PPARα has been found in hepatocytes, cardiomyocytes, proximal tubules of the nephron, intestinal mucosa, brown adipose tissue and in pancreatic β-cells (Braissant *et al.*, 1996; Ouali *et al.*, 1998; Zhou *et al.*, 1998). PPARβ is ubiquitously expressed and in most tissues has a higher expression level than either of the other paralogs (Braissant *et al.*, 1996; Kliewer *et al.*, 1994). PPARγ is highly expressed in adipose tissue with some expression in the large intestine, jejunum and spleen (Braissant *et al.*, 1996; Kliewer *et al.*, 1994). The expression and tissue distribution of the PPARs in humans is similar to that in rodents with the exception that

PPARα may not be as highly expressed in hepatocytes (Auboeuf *et al.*, 1997; Mukherjee *et al.*, 1997; Palmer *et al.*, 1998).

The physiological roles of PPARs can be divided into three broad categories. First, the main role of PPARα seems to be upregulation of enzymes involved in lipid oxidation in tissues of the body where this is important, primarily the liver but including the pancreatic β-cell and the cardiomyocyte (Desvergne & Wahli, 1999). PPARα stimulates expression of lipoprotein lipase (LPL) (Schoonjans *et al.*, 1996), as well as proteins involved in the translocation of fatty acids across the cell membrane e.g. fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36) (Motojima *et al.*, 1998) and L-FABP (Issemann *et al.*, 1992). The physiological role of the upregulation of these proteins is to aid in the absorption of fatty acids into cells. Once in the cells, FA become activated as acyl-CoA thioesters by ACS (Schoonjans *et al.*, 1995) and then may be catabolized in the peroxisomal β-oxidation pathway by AOX (Tugwood *et al.*, 1992), bifunctional enzyme (Zhang *et al.*, 1992), and thiolase (Lee *et al.*, 1995), all of which are target genes for PPARα. Acyl-CoA esters may also be shuttled into the mitochondrial oxidative pathway. PPARα is important in regulating mitochondrial β-oxidation by controlling the expression of CPT-1 (Brandt *et al.*, 1998), acyl-CoA dehydrogenase (Gulick *et al.*, 1994), and 3-hydroxy-3-methylglutaryl-CoA synthase (Rodriguez *et al.*, 1994). Furthermore, PPARα controls the expression of the CYP4A family of genes that are involved in microsomal ω-hydroxylation (Kroetz *et al.*, 1998), the lipogenic malic enzyme (Castelein *et al.*, 1994), and apolipoproteins (ApoA-2, and ApoC-3) (Staels *et al.*, 1995; Vu-Dac *et al.*, 1995) as well as UCP1 (Sears *et al.*, 1996). In addition, the PPARα knockout mouse has outlined the importance of PPARα expression in the control of fatty

acid metabolism and lipid homeostasis (Lee *et al.*, 1995). These animals have defective fatty acid catabolism and thus mice fed a high fat diet develop lipid accumulation in liver and heart tissue (Aoyama *et al.*, 1998; Djouadi *et al.*, 1998).

Secondly, the main functions of PPARβ is believed to be the control of gene expression during development, particularly in the central nervous system where it is believed to have a positive effect on cell proliferation/differentiation (Braissant & Wahli, 1998). Because of its ubiquitous expression pattern, PPARβ may also be involved in the control of basic cellular functions including lipid synthesis and turnover (Braissant & Wahli, 1998). Overall, PPARβ has not been highly studied because of the lack of specific agonists for this isoform (Desvergne & Wahli, 1999).

Thirdly, PPARγ is highly expressed in adipose tissue and is an important transcription factor for differentiation of white and brown adipose tissue from preadipocytes (Dreyer *et al.*, 1992; Tontonoz *et al.*, 1994b). In the adipocyte, PPARγ regulates expression of: the adipocyte fatty acid binding protein 2 (aP2) (Tontonoz *et al.*, 1994a), PEPCK, LPL, FATP and FAT/CD36 (Motojima *et al.*, 1998; Schoonjans *et al.*, 1996). All of these proteins are involved in fat storage and movement within adipocytes.

1.10.3 Other Fat-Activated Transcription Factors

There is a substantial body of evidence proving the important role of PPARs in gene regulation in response to fat. However, recently it has become apparent that other TF are able to bind fatty acids and as a consequence modulate gene expression. The hepatic receptor HNF4 binds FA-CoA and is able to regulate the human ApoC3 gene promoter (Hertz *et al.*, 1998). Fatty acids may also be able to regulate the synthesis of some TF. One example of this is the fatty acid regulation of genes with a sterol

regulatory element in their promoters. It has been shown that PUFA can inhibit transcription of genes with a SRE in their promoter (Worgall *et al.*, 1998). This has been attributed to downregulation of SRE binding protein 1 (SREBP1) by PUFA, which is a post-transcriptional event (Mater *et al.*, 1999; Shimano *et al.*, 1999; Yahagi *et al.*, 1999). Finally, it has been demonstrated that palmitate and oleate can positively regulate expression of the immediate-early response genes nur-77 and c-fos in pancreatic β-cells (INS-1) by a mechanism that changes the transcription rate and is dependent on Ca^{2+}, PKC and metabolism of the fatty acid (Roche *et al.*, 1999). Thus, in these (SREBP, c-fos, nur-77) and probably other cases, FA can regulate the expression of TF themselves and do not seem to affect the transactivation properties of the TF.

1.11 Rationale

Much of the present research in the incretin field is driven by the ultimate goal of developing treatments for T2D (Holst *et al.*, 1997). However, at present it is not understood why GIP is unable to adequately stimulate insulin secretion in T2D, considering the effectiveness of the "partner" incretin GLP-1. As previously discussed there are a number of factors that could cause the diabetic β-cell to be unresponsive to GIP; specifically, it could be hypothesized that three pathways are involved. First, but not foremost as previously discussed, there could be a defect in the receptor; which could result from either a mutation in the promoter or within the gene itself (Almind *et al.*, 1998; Holst *et al.*, 1997; Kubota *et al.*, 1996). Secondly, there could be a defect in GIP-mediated signal transduction. Thirdly, there could be a defect in GIP receptor expression, leading to desensitization and/or down regulation of the GIP receptor within the β-cell and elsewhere (Tokuyama *et al.*, 1995; Tseng *et al.*, 1996a). All of these pathways could

contribute to a decreased incretin effect, much like that observed in T2D. Here we set out

to determine if the GIP receptor is downregulated and if so, how this downregulation

might occur. We hypothesized that a defect in receptor expression was responsible for

the decreased effectiveness of GIP in T2D. Furthermore, we believed that this

downregulation may be a result of hyperglycemia, hyperlipidemia or hyperinsulinemia.

One consequence of the hyperglycemia associated with T2D is abnormal glycosylation of

proteins. Furthermore, it has been demonstrated that the expression of many G-protein

coupled receptors relies on correct glycosylation. These observations led us to

hypothesize that correct glycosylation of the GIP receptor may affect cell surface

expression and thereby GIP responsiveness of the β-cell. By elucidating if these

pathways alter the insulinotropic effects of GIP in T2D, it would become much easier to

test if alteration of these pathways in a normal animal yields glucose intolerance and β-

cell defects that would predispose it to a T2D -like condition.

Chapter 2 - Methods

2.1 Chemicals

Synthetic human GIP (shGIP) and GLP-1 were purchased from Bachem

California, Inc (Torrance, CA, USA) and 3-isobutyl-1-methylxanthine (IBMX) was

purchased from Research Biochemicals International (Natick, MA, USA). All chemicals,

of reagent or molecular biology grade were from Sigma (Oakville, ON, Canada) or Fisher

Scientific International (Pittsburgh, PA, USA). All tissue culture disposables were from

BD Falcon (San Jose, CA, USA).

2.2 Animals

Diabetic fatty Zucker (VDF) rats spontaneously developed from a Zucker strain maintained by our laboratory. These diabetic rats are homozygous recessive for a mutation in the leptin receptor gene, fa, ($Gln_{269}Pro$). Rats carrying one normal Fa allele are phenotypically lean and display normal glucose tolerance. Male animals age of 4 and 14-16 weeks of age were used in these studies. All animals tested displayed glucose intolerance and decreased first phase insulin response, characteristic of VDF rats. Lean littermates were used as control animals in these experiments.

2.3 Intraperitoneal Glucose Tolerance Test (IPGTT)

Zucker rats were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (65 mg/kg) (Somnotol®, MTC Pharmaceuticals, Cambridge, ON, Canada). The right jugular vein was then exposed and cannulated with heparinized polyethylene tubing (PE50, Becton-Dickinson Co, Sparks MD, USA). GIP (4 pmol/min/kg) or physiological saline was infused (30 µl/min) via the cannula using an infusion pump (Harvard Apparatus, South Natick, MA, USA), for five minutes prior to an IP glucose injection (40 %, 1g/kg). Blood samples (0.5ml) were collected from the tail vein into heparinized Caraway/Natelson collecting tubes (Fisher Scientific, Pittsburgh, PA, USA) 5 minutes prior to (basal) and 10, 20, 30 and 60 minutes following glucose injection. Concomitantly, blood glucose measurements were taken before the infusion started (basal) and then every 10 minutes following glucose administration, using a handheld blood glucose meter (SureStep®, Lifescan Inc., Burnaby BC, Canada). Plasma was then separated from red cells by centrifugation at 10 000 xg for 20 minutes at 4 °C, and then

stored at −20 °C until GIP and insulin radioimmunoassays could be carried out (Pederson *et al.*, 1982).

2.4 Measurement of Immunoreactive GIP

Samples were diluted in assay buffer containing 5% charcoal extracted human plasma, 2 % Trasylol and 0.04 M PO_4 buffer (pH 6.5). shGIP standards were diluted from 7.8 pg to 2000 pg and used for the standard curve. Samples and standards were incubated at 4 °C with GIP antiserum RK343F (1:30 000; Linda Morgan, University of Surrey, Guilford, Surrey) for 24 hours before radiolabelled ^{125}I-GIP (5000 cpm/tube, >350 mCi/mg) was added. The samples were then allowed to equilibrate for a further 24 hours before the antisera-bound GIP was separated from the unbound GIP with 25 % polyethylene glycol 8000 (PEG 8000). Antisera bound ^{125}I-GIP was then counted for radiolabel using a gamma counter (LKB/ Wallac 1277).

2.5 *In Vitro* Pancreatic Perfusion

Anesthesia was established using Somnotol® and pancreata were isolated as previously described (Pederson & Brown, 1976). The perfusate consisted of a modified Krebs-Ringer bicarbonate buffer containing 3 % dextran and 0.2 % bovine serum albumin (BSA, Fraction V, RIA grade, Sigma) gassed with 95 % O_2 / 5 % CO_2 to achieve pH 7.4. The abdominal aorta was perfused at a rate of 4 ml/min and portal venous outflow was collected at one minute intervals. Following a 10 minute equilibration period, the pancreatic perfusion continued with 4.4 mM glucose for 4 minutes followed by 8.8 mM glucose for the remainder of the experiment. GIP (10 pM), GLP-1 (50 pM) or saline were introduced into the perfusion system from 20-40 minutes.

2.6 Isolation and Culture of Rat Pancreatic Islets

Rat pancreatic islets were isolated as previously described (Van der Vliet *et al.*, 1988). Briefly, the rat was anesthetized and a midline incision was made. The common bile duct was cannulated and the pancreas was inflated with collagenase (320 mg/l, Type XI, Sigma) in Hank's Balanced Salt Solution supplemented with 10 mM HEPES, 2 mM L-glutamine and 0.2 % BSA (HBSS+) (Invitrogen, Burlington, ON Canada). The pancreas was then removed from the rat and macerated with scissors prior to collagenase digestion. The pancreatic tissue was initially digested in a shaking 37 °C water bath for 20 minutes and 10 minutes for pancreata from lean and fat rats respectively; a second digestion was then carried out for 10 and 7 minutes for the lean and fat rats respectively. Following collagenase digestions, the islets were passed through a 1 mm nylon mesh and separated from exocrine tissue via centrifugation (1000 xg/ 4 °C) through a discontinuous dextran gradient. Finally, islets were picked under a dissecting microscope, washed in HBSS+ and used for mRNA isolation or cultured in RPMI 1640 with 8.8 mM glucose, 10 % fetal calf serum (Cansera, Rexdale Ontario, Canada), antibiotics (50 U/ml each penicillin G and streptomycin), 0.07 % human serum albumin, 0.0025 % human apotransferrin, 25 pM sodium selenite and 20 μM ethanolamine hydrochloride for 20-24 hours in 10 cm plastic culture dishes (Falcon, Beckton Dickinson, Sparks, MD, USA) in a humidified, 5 % CO_2 environment.

2.7 Perifusion of Pancreatic Islets

After the culture period, 40 healthy islets (healthy refers to islets that retained a characteristic pink colour when viewed with a dissecting microscope) were selected and sandwiched between two layers of Cytodex-3 beads (Amersham-Pharmacia, Baie d'Urfé,

PQ, Canada) in 0.2 ml polyethylene perifusion chambers (Endotronics Inc., Coon Rapids, MN, USA). The chambers were then perifused in an Acusyst-s perifusion apparatus (Endotronics) under a humid 37 °C, 5 % CO_2 environment at a flow-rate of 0.5 ml/min with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (KRBH) supplemented with 0.2 % BSA. Perifusion experiments were carried out for 80 minutes following a 60 minute equilibration period in 2.8 mM glucose (low glucose) perifusate. After 20 minutes, the perifusate glucose concentration was switched to 16 mM with or without GIP. Samples were collected every 2 minutes and insulin levels determined by radioimmunoassay as previously described (Pederson *et al.*, 1982).

2.8 Measurement of Insulin and cyclic AMP Production by Islets

After overnight culture, 40 healthy islets were selected, washed twice with 0.5 ml of KRBH supplemented with 0.2 % BSA and allowed to equilibrate for 30 minutes in a humidified, 5 % CO_2 environment. The islets were then stimulated with either vehicle, 10 μM forskolin, or 10 nM GIP for 30 minutes in the presence of 0.5 mM IBMX. The islets were then lysed by boiling for 5 minutes in 0.05 N hydrochloric acid. Samples were then dried by vacuum centrifugation (Speed-Vac, Sorvall, Farmingdale, NY, USA) and stored at –20 °C for cAMP radioimmunoassay (Biomedical Technologies, Stoughton, MA, USA). For insulin secretion experiments, 40 islets were selected, washed with KRBH containing 2 mM glucose and allowed to equilibrate for 60 minutes. Following equilibration, islets were incubated with 10 nM GIP for 30 minutes in 16 mM glucose KRBH. The supernatant was then collected and analyzed for insulin content by radioimmunoassay as previously described. Total insulin was measured by lysing the

islets in 0.2 M acetic acid followed by boiling, centrifugation, dilution and radioimmunoassay (Pederson et al., 1982).

2.9 Isolation and Measurement of Islet GIP Receptor messenger RNA by Real-Time Reverse Transcription Polymerase Chain Reaction

Rat islet RNA was isolated immediately following islet isolation using Trizol® and the standard protocol supplied by the manufacturer (Invitrogen). Specifically, 1 ml of Trizol® reagent was utilized per 100 islets and the A_{260}/A_{280} ratios of isolated RNA were > 1.80. Following RNA isolation, 1 μg of islet RNA was subjected to reverse-transcription (RT). Total RNA was reverse transcribed in a volume of 10 μl containing, 0.5 mM deoxynucleotide triphosphates, 15 pmol gene specific primer targeted at the carboxy terminus of the rat GIP receptor (5'- GTT CTG GAG TAG AGG TCC GTG TA-3'), 75 pmol of random hexamers (Amersham-Pharmacia), 100 U Superscript II® RNAse H⁻ Reverse Transcriptase (Invitrogen), 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, 100 ng of rat islet tissue cDNA was used in the real-time PCR reaction to measure GIP receptor expression; whereas, 10 ng cDNA was used in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control PCR reaction. The PCR reaction mix consisted of 1x TaqMan Buffer A® (PE Applied Biosystems, Foster City, CA, USA), 10mM $MgCl_2$, 200 μM dATP, dCTP, dGTP and 400 μM dUTP, 200 nM rat GIP receptor 5' forward primer (5'- CCG CGC TTT TCG TCA TCC -3'), 200 nM rat GIP receptor 3' reverse primer (5'- CCA CCA AAT GGC TTT GAC TT -3'), 200 nM GIP receptor probe co-labelled with the fluorescent dyes FAM and TAMRA (5'- CCC AGC ACT GCG TGT TCT CGT ACA GG -3'), 0.01 U/μl AmpErase® uracil N-glycosylase

(UNG, PE-Applied Biosystems), and 0.025 U/µl of AmpliTaq Gold® (PE Applied

Biosystems). The GAPDH reactions included the above reaction conditions with the

exception of the primers and probe which were purchased from PE Applied Biosystems

and were directed towards rodent GAPDH. 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 *et al.*, 1999).

2.10 Western Blot Analysis of Islet GIP Receptor Protein

Islets were isolated as previously described. Following isolation, islet GIP

receptor protein was analyzed as previously described (Lewis *et al.*, 2000). Briefly, islets

were lysed in ice cold RIPA buffer (150 mM NaCl, 20 mM Tris-Cl pH 7.5, 1 mM EDTA,

1 % Nonidet P-40, 1 % deoxycholate, 0.1 % SDS, 5 mM NaF, 1 mM

phenymethylsulfonyl fluoride, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10

µg/ml bestatin and 1 % Trasylol (Bayer Pharmaceuticals, Etobicoke, ON, Canada)) for 30

minutes on ice. Protein concentration was determined using Bicinchoninic Acid (BCA)

kit (Pierce, Rockford, IL, USA). Fifty micrograms of total islet protein were denatured

under reducing conditions (100 mM DTT) at 100 °C for 5 minutes and run by SDS-

PAGE. Proteins were then transferred to nitrocellulose membrane, blocked with 5 %

skim milk (in tris-buffered saline with 0.5 % Tween 20 (TBST)) and then incubated with

a well-characterized polyclonal anti-GIP receptor antibody (Lewis *et al.*, 2000).

Membranes were then washed three times in TBST and then incubated with horseradish

peroxidase conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch

Laboratories, West Grove, PA, USA). Following further washing, the immunoreactive

bands were visualized using enhanced chemi-luminescence (ECL) (Amersham-

Pharmacia). Finally, bands were subjected to densitometry using Eagle Eye II software

(Stratagene, La Jolla, CA, USA) and molecular weight was determined using R_f analysis.

2.11 Culture of BRIN-D11 and INS(832/13) Cells

BRIN-D11 cells were obtained from Dr. P.B. Flatt (University of Ulster, Belfast,

N. Ireland), and INS(832/13) cells were obtained from Dr. C.B. Newgard (University of

Texas, USA) (Hohmeier *et al.*, 2000; McClenaghan *et al.*, 1996). Cell lines were

maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C. Both cell lines were

grown in RPMI-1640 medium containing 11 mM glucose, supplemented with 10 % fetal

bovine serum (Cansera; Rexdale ON), and penicillin/streptomycin. The media in which

the INS(832/13) cells were grown was supplemented with 10 mM HEPES (pH 7.4), 1

mM sodium pyruvate, 2 mM glutamine and 50 μM β-mercaptoethanol.

2.12 Transfection of INS(832/13) Cells.

The mPPARα-G (a mutant (G282E) form of mouse PPARα with low intrinsic

transactivation properties but a higher affinity for WY 14643 and other fibrates than the

wild-type form) construct was obtained from Dr. E.F. Johnson (Scripps Research Institute, La Jolla, CA, USA) and a dominant negative form of human PPARα (hPPARα$_{tr}$) was obtained from Dr. B. Staels (Institut Pasteur de Lille, France) (Gervois *et al.*, 1999; Hsu *et al.*, 1995). Cells were seeded at 6×10^6 cells/plate in 10 cm dishes. After two days of growth or when the cells were 90 % confluent, transfection was carried out using Lipofectamine 2000™ (Invitrogen) using the manufacturer's protocol. The day after transfection the cells were transferred to 12 well plates with a seeding density of 1×10^6 cells/well and then allowed to grow for 24 hours before the media was replaced and the experiment was started. Transfection efficiencies were determined by co-transfection with the jellyfish green fluorescent protein containing plasmid (pGFPN2; Invitrogen): typically the transfection efficiency was 40 %.

2.13 Isolation and Measurement of GIP Receptor mRNA from Isolated Islets and Cultured Cells

For mRNA experiments, cells were seeded into 12 well plates at a density of 1×10^6 cells/well in RPMI or INS(832/13) media containing 5.5 mM glucose. Cells were grown in these media for 24 hours before media were changed and experimental agents were applied. These included 100 µM WY 14643, a specific PPARα activator, 10 µM MK-886, a PPARα antagonist (Biomol Research Laboratories, Plymouth Meeting, PA, USA), 5 µM H89, a specific PKA inhibitor, 100 µM PD 98059, a MEK inhibitor, 2 µM bisindolylmaleimide, a general PKC inhibitor (Bis) (Calbiochem, La Jolla, CA, USA), 100 nM wortmannin, a PI-3 kinase inhibitor (RBI/Sigma, Natick, MA, USA), 1 µM insulin or 2 mM palmitate (Sigma). After a further 24 hours incubation, messenger RNA

was isolated using 0.5 ml/well Trizol® and the standard protocol supplied by the manufacturer (Invitrogen).

Palmitate solution was made by complexing sodium palmitate to BSA. This was accomplished by first emulsifying the sodium palmitate in water at 60 °C. This palmitate mixture was then complexed to a solution of RPMI (0 mM glucose) containing 20 % fatty acid free BSA. This mixture was then diluted in growth medium to a final concentration of 2 mM palmitate and 2 % BSA and filter sterilized. Control conditions for experiments in which palmitate was used contained the 2 % BSA without the palmitate.

Islets were isolated from lean Zucker rats as previously described and grown overnight in supplemented RPMI-1640 media as described in section 2.6. Groups of 50 islets were then incubated for 8 hours with either 2 mM palmitate or 100 μM WY 14643. Following stimulation, RNA was isolated by addition of 1 ml of Trizol as described.

RNA was then quantified using the fluorescent Ribogreen reagent (Molecular Probes; Eugene OR). Following RNA isolation and quantification, 125 ng of RNA was subjected to reverse-transcription.

2.14 mRNA Degradation and Half-Life Analyses

These studies were carried out by applying actinomycin D (5 μg/ml) to cells at various times following the beginning of the experiment and then measuring the amount of GIPR mRNA remaining using real-time RT-PCR (Roduit *et al.*, 2000).

2.15 Iodination of GIP and Saturation Binding Studies

As previously described, synthetic porcine GIP (5 μg) was iodinated by the chloramine-T method and the ^{125}I-GIP was purified using reverse phase HPLC to a specific activity of 350 μCi/μg (Kieffer *et al.*, 1995b). Aliquots of the tracer were lyophilized and stored at −20 °C until needed. Cells were seeded into 24 well plates at a density of 5 x 10^5 cells/well in 5.5 mM glucose containing medium. Following 24 hours of culture the medium was changed and experimental conditions were applied. After a further 24 hours of culture the cells were washed twice with ice-cold KRBH containing 0.2 % BSA. The saturation binding experiment was carried out at 4 °C in KRBH containing 5.5 mM glucose and 1 % Trasylol (aprotinin: Bayer, Etobicoke, ON, Canada) and varying amounts of radiolabelled ^{125}I-GIP (12.5-112 fmol). Cells were washed twice with ice cold KRBH and radioactivity bound to cells was measured using a gamma counter. Non-specific binding was defined as that measured in the presence of 1 μM non-labeled shGIP. All binding data are expressed as specific binding of ^{125}I-GIP to cells.

2.16 Cloning of the Rat 5' GIP Receptor Promoter

The proximal 2 Kb of the rat GIPR promoter was cloned from rat liver using PCR and primers generated from the published sequence (Boylan *et al.*, 1999). Briefly, a male Wister rat was anesthetized using Somnotol®, as previously described, and a laporotomy performed to expose the liver. The animal was then sacrificed by pneumothoraectomy and a ~ 250 mg portion of the liver was excised. Genomic DNA was then isolated from the liver tissue by lysing the cells in a buffer containing 500 μl of 10 mM Tris HCl, 0.5 mM EDTA, 0.2 % SDS, 0.2 M NaCl, and 0.1 mg/ml Proteinase K. Cell lysis and digestion was allowed to proceed for 3 hours at 55 °C. Following lysis, one volume of

isopropanol was added to the lysate and samples were mixed on a rotator for 10 minutes. Genomic DNA was then recovered by lifting it from the microcentrifuge tube with a 200 μl pipette tip. DNA was placed in a fresh 1.5 ml microcentrifuge tube, washed twice with 70 % ethanol and dissolved in an equal volume of 10 mM Tris HCl overnight at 37 °C. PCR was then carried out on the genomic DNA in the following manner: 1 μg of the genomic DNA, 100 μM each dNTP, 200 nM of each primer (GIPRP1 5'-GAATCCCCAGTGAGGGGC-3', GIPRP2 5'-CTGTACCGAGTCCTGCTC-3'), 2.5 U of Expand high fidelity polymerase all in the proprietary buffer mix supplied with the enzyme. PCR was carried out by using a hot start for 5 minutes at 95 °C, followed by 35 cycles of 95 °C for 30s, 56 °C for 1 minute, and 1 minute at 72 °C and then a 10 minute final extension at 72 °C. The PCR was then run out on an agarose gel using standard methods and the 2 kb band was excised and purified using the GeneClean kit (Q Biogene, Carlsbad, CA) and the provided protocol. The 2 Kb PCR product was then cloned into the PCR 2.1 TOPO TA cloning vector and transformed into TOP10 F' cells using the manufacturers protocol (Invitrogen). To ensure that the correct sequence had been obtained, fluorescent sequencing was carried out using the NAPS unit at the University of British Columbia. The sequence matched the previously published sequence exactly (Boylan *et al.*, 1999).

2.17 GIP Receptor 5'-Promoter Stimulated Gene Transcription and Luciferase Assay

The cloned portion of the GIP receptor promoter corresponded to the 2 kb directly upstream of the transcriptional start site. To measure transcriptional activity of this promoter region, it was subcloned into the *Eco* RI site of PGL3 (Promega, Madison WI,

USA) and two clones containing the construct in both orientations were obtained

(pGL3GP+ and pGL3GP-). PGL3GP constructs were then transfected into INS(832/13)

cells and analyzed for promoter activity using the Bright Glo Luciferase assay (Promega).

First, 5×10^6 cells were plated into 10 cm dishes and allowed to grow for 24 hours.

Secondly, the cells were washed two times with low glucose (11 mM) DMEM

(Invitrogen) and transfected with 2.5 µg of either pGL3GP+, pGL3GP-, pGL3, or pGL3

Control; the final two being negative and positive controls respectively. The transfection

consisted of mixing 2.5 µg of pGL3 plasmid DNA plus 1 µg of pGFPN2 (BD-Clonetech,

Palo Alto, CA, USA) (a green fluorescent protein containing vector co-transfected and

used as a measure of tranfection efficiency) DNA in 250 µl of low glucose DMEM.

Concomitantly, 7 µl of Lipofectamine 2000 (Invitrogen) were mixed in an additional 250

µl of low glucose DMEM (Invitrogen). The two, 250 µl, portions were mixed and the

cationic lipid was allowed to complex with the DNA for 30 minutes. The complexed

DNA was then added to the cells in a total volume of 3 ml and the tranfection was

allowed to proceed for 6 hours. Following the 6 hour incubation period, 15 ml of growth

media was added to the cells and they were grown overnight. Thirdly, cells were then

plated at a density of 5×10^4 cells in 96 well plates and allowed to grow for a further 24

hours. Fourth, the medium was changed and experimental stimuli were applied (e.g.

stimulation with 2 mM palmitate or 100 µM WY 14643) and the cells were allowed to

grow for a further 24 hours. Finally, the luciferase activity of the samples was measured

using the BrightGlo Luciferase reagent kit (Promega) and in a Turner Designs

(Sunnyvale CA, USA) 96 well luminometer, using the manufacturer's suggested

protocols (Promega). Cells were counted under a fluorescent microscope and the percent

of cells that fluoresced and therefore contained green fluorescent protein (GFP) was used as a measure of transfection efficiency.

2.18 *In Vivo* Hyperglycemic Clamp Experiments

Lean, 16 week old Zucker rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg) (Somnotol; MTC Pharmaceuticals). The right jugular vein was then exposed and cannulated with heparinized polyethylene tubing (PE50, Becton-Dickinson). Blood glucose measurements were taken every 10 minutes from the tail vein using a handheld blood glucose meter (SureStep®, Lifescan Inc.) and 50 % glucose or saline was infused (0.5-3 ml/hr) via the cannula using an infusion pump (Harvard Apparatus), and the infusion rate was adjusted to maintain blood glucose levels of 5.5, 10, or 25 mM. Following 6 hours of glucose clamp, the islets were isolated and GIP receptor mRNA levels were determined as described using real-time RT-PCR.

2.19 Pancreatic Perfusions of Hyperglycemic-Clamped Rat Pancreata

Circulating glucose concentrations in rats were clamped as described previously however, following 6 hours of clamp the pancreata of the rats were perfused as previously described with 25 pM human GIP in the presence of 8.8 mM glucose (Pederson & Brown, 1976). Samples were collected every minute and insulin secretion was determined using radioimmunoassay as previously described (Pederson *et al.*, 1982).

2.20 Site-Directed Mutagensis

Site-directed mutagenesis was carried out using modifications on the Quickchange method that was developed by Stratagene Corp (La Jolla, Ca, USA). Briefly, megaprimers were synthesized (table 1) that contained the desired mutations and

were complementary to one another. PCR was carried out using these primers and the WT rat[1] GIP receptor that had previously been subcloned, in frame, into the pcDNA 3.1(b) V5-HIS vector (Invitrogen). This allowed characterization of protein using both the poly-His tag as well as the V5 epitope. The mutagenesis was done by combining 200 pg of the vector, 125 ng of each of the megaprimers, 125 μM each dNTP, 2.5 U of either Pfu polymerase (Fermentas, Ma, USA) or Expand High fidelity DNA polymerase (Roche Diagnostics, Laval Quebec, Canada), and each of the manufacturer's PCR buffers including 2.5 mM $MgCl_2$ in a final volume of 50 μl. PCR reactions were then overlayed with oil and PCR was carried out over 16 cycles in a Robocycler (Stratagene) using the following reaction profile: 30s at 95 °C, 1 minute at 55 °C and 16 minutes at 68 °C. Following the PCR reaction the product was treated with 10 Units of *Dpn* I (New England Biolabs, Beverly, MA, USA) and restriction digestion was allowed to proceed for 1 hour at 37 °C. *Dpn* I digests only methylated DNA, allowing only non-mutated DNA to be restriction digested. One microliter of the resulting mutated DNA was then transformed into competent DH5α cells via heat shock. Colonies were picked from the plates and sequenced using both radiolabelled dideoxynucleotide sequencing followed by TBE-acrylamide gel and BigDye cycle sequencing followed by analysis on a PE310 genetic analyzer. Double and triple mutants were made by subcloning portions of the receptor or by mutating single site mutants using the same technique. All mutations were subcloned out of the vector in which they were mutated and inserted into a wild-type

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vector to ensure that mutations to the vector sequence would not affect the phenotype. They were then fully sequenced through the areas that were mutated.

Table 1: Megaprimers used for glycosylation site mutation

N59T1	5'-CGA AGG AAC CAG TAC AGG CC-3'
N59T2	5'-GGC CTG TAC TGG TTC CTT GG-3'
N69T1	5'-GGC AGC CGT GTA GGT CCA GCA GGC-3'
N69T1	5'-GCC TGC TGG ACC TAC ACG GCT GCC-3'
N74T1	5'-CGG CTG CCA CCA CCA CTG CCC GG-3'
N74T2	5'-CCG GGC AGT GGT GGT GGC AGC CG-3'
N200T1	5'-GGG TCC CTA CAC GGG AAA CCA GAC CCC TAC CC-3'
N200T2	5'-GGG TAG GGG TCT GGT TTC CCG TGT AGG GAC CC-3'

2.21 Transfection, Affinity Purification of GIP Receptor Protein and Western Analyses

Following construction of the glycosylation mutants, they were transfected in HEK 293 cells, and receptor protein was isolated by affinity purification. This was accomplished by transfecting 2.5 µg of DNA into HEK 293 cells with Lipofectamine 2000 after 24 hours of growth from a plating density of 2×10^6 cells/10 cm dish as previously described. Cells were then allowed to grow for 24 hours before protein was harvested for affinity purification. Cells were lysed in 500 µl of buffer consisting of 0.5 % Triton X100, 60 mM β-glycerophosphate, 20 mM MOPS pH 7.2, 1 mM Na_3VO_4, 20 mM NaF, 1 % Trasylol 1 mM PMSF and 2x EDTA-Free Protein Inhibitor cocktail

(Protein Inhibitor tablets, Roche Diagnostics). Cells were scraped from the 10 cm dishes

and extracts were put in 1.5 ml microcentrifuge tubes and sonicated with a needle-tip

sonicator for 20 s on ice. Cell extracts were then centrifuged at 14 000 x g for 30 minutes

at 4 °C and supernatent was quickly added to 50 μl of Talon resin that had been pre-

equilibrated in lysis buffer (Clonetech). The poly-histidine tagged GIPR protein in the

cell extracts was allowed to bind to the Talon resin during 15 minutes of gentle agitation.

The supernatant was then removed and the resin was washed 3 times with 1 ml volumes

of lysis buffer. Finally, the poly-histidine tagged protein was eluted from the Talon resin

by using a 0.5 M imidazole buffer. Protein concentrations were analyzed by BCA kit

(Pierce).

Affinity purified protein was then analyzed for glycosylation using PNGase F and

Western blotting. First, purified protein extracts were denatured and digestion was

carried out on 5 μg of protein with peptide:N-glycosidase F (PNGase F, New England

Biolabs) using the suggested reaction conditions, at 37 °C for 1 hour. Following

digestion, proteins were loaded onto a 12 % acrylamide gel and then Western blotted

using conventional techniques. The blots were blocked overnight with 5 % skim milk in

tris-buffered saline containing 0.1 % Tween 20 (TBST). Following blocking, blots were

incubated with a monoclonal Anti-V5 antibody (Invitrogen) at a dilution of 1:1250 for 3

hours at room temperature. Membranes were then thoroughly washed and incubated with

horseradish peroxidase conjugated goat anti-mouse secondary antibody (Jackson

Laboratories) for one hour at room temperature. Protein bands were visualized using the

enhanced chemiluminescence (ECL) reagent (Amersham-Pharmacia) followed by film

(Kodak, Rochester NY, USA) exposure for up to five minutes. Molecular weights of the proteins were determined using standard R_f analysis.

2.22 Competitive Binding and cAMP Production Analyses in HEK 293 and INS(832/13) Cells

Competitive binding analyses were carried out as previously described, with minor modifications (Wheeler *et al.*, 1995). Briefly, transfected HEK 293 and INS(832/13) cells were plated in 24 well plates at a density of 6 x 10^4 and 5 x 10^5 cells/well respectively and allowed to grow for 48 hours. Cells were then carefully washed twice with 1 ml of ice-cold KRBH. Cells were incubated for 4 hours at 4 °C with various amounts of unlabelled GIP (10^{-6}-10^{-12}M) in the presence of 5 x 10^4 cpm of purified ^{125}I-GIP. Then the cells were washed two more times with ice-cold KRBH and solubilized with 0.1 M NaOH. The solubilized cells were transferred to test tubes and radioactivity was counted on a gamma counter (LKB-Wallace). Non-specific binding was taken as the amount of label bound to cells in the presence of 1 μM non-labelled GIP and specific binding was expressed as a percent of total binding.

For cAMP studies, cells were plated in 24 well plates as above. Cells were then washed twice with 37 °C KRBH and then allowed to preincubate for one hour at 37 °C. Following the preincubation, cells were incubated with GIP (10^{-6}-10^{-12}M) in the presence of 0.5 mM isobutylmethylxanthine (IBMX): a phosphodiesterase inhibitor. After 30 minutes of stimulation, cAMP production was arrested by addition of 1 ml of ice cold 70 % ethanol. Cells were scraped from the plates and transferred to 1.5 ml microcentrifuge tubes; followed by centrifugation and recovery of the supernatant to fresh tubes. The

supernatant was then dried by vacuum centrifugation before cAMP quantification by RIA (Biomedical Technologies, Stouton, MA, USA).

2.23 Insulin Release from INS(832/13) cells

INS(832/13) cells β-cells were seeded into 24 well plates at a density of 5 x 10^5 cells/well. Following plating, cells were allowed to grow for 2 days. On the second day, cells were washed twice with KRBH and preincubated for 1 hour in KRBH containing 2 mM glucose. Following preincubation, cells were stimulated to release insulin in varying glucose concentrations for 30 minutes in a total volume of 200 μl. After the stimulation period, the medium was removed and centrifuged at 12 000 xg for 5 minutes at 4 °C. Concomitantly, total insulin was extracted from the cells using 2 M acetic acid. This was accomplished by adding 200 μl of acetic acid to the cells, scraping the surface of the plate and boiling the cells for 5 minutes. Samples were then stored at –20 °C for insulin RIA as previously described (Pederson et al., 1982).

2.24 Fatty Acid Oxidation in BRIN-D11 Cells.

Fatty acid oxidation experiments were carried out as previously described (Shimabukuro et al., 1998). BRIN-D11 cells were used because these experiments were carried out prior to the availability of the INS(832/13) cells. Cells were plated in 24 well plates at a density of 1 x 10^5 cells/well and allowed to grow for 48 hours in growth medium containing 2 μCi/ml 9,10-[^{3}H]Palmitic acid (PE-Applied biosystems). Medium was then removed, cells were washed 4 times to remove extracellular radioactivity and preincubated for 2 hours in 11 mM glucose KRBH. Following preincubation, medium was changed and 200 μl of fresh medium containing various concentrations of GIP

(1nM-1μM) was added and incubated at 37 °C for 4 hours. Medium was then removed and extracted twice with an equal volume of 10 % trichloroacetic acid to remove any excess 9,10-[^{3}H]Palmitic acid. The supernatant was placed in a 1.5 ml microcentrifuge tube and this tube was transferred, uncapped to a 10 ml scintillation vial containing 0.5 ml of ddH$_2$O. The scintillation vials were then incubated for 24 hours at 60 °C to allow the ^{3}H$_2$O to equilibrate with the non-labelled water before 10 ml of Econo 2 scintillation fluid (Fisher) was added, and the radioactivity was determined by liquid scintillation spectrometry. A standard ^{3}H$_2$O solution was equilibrated along with the samples to control for the equilibration step between different experiments.

2.25 Tunicamycin Treatment of INS(832/13) Cells

Cells were plated into 24 well plates as previously described. Following 1 day of culture, the medium was changed and tunicamycin was added at a final concentration of 1μg/ml. Cells were then allowed to grow for a further 24 hours before insulin release studies were carried out.

2.26 Data Analysis

Where applicable, data are expressed as mean ± standard error of the mean, with the sample size indicated in the appropriate figure legend. In general, for animal studies an n=1 is one animal, and individual measurements were done in at least duplicate. For cell culture experiments an n=1 means one plate, on this plate there were individual conditions were carried out in triplicate and each well was analyzed in at least duplicate. Unpaired, two-tailed t-tests were carried out to compare groups of animals. The means from larger groups were compared using two-tailed ANOVA and either the Dunnet or the

Tukey post hoc test. P values ≤ 0.05 were considered statistically significant. Area under the curve was determined using curve analysis software (Graphpad, Prism, San Diego, CA, USA).

Competitive and saturation binding data were analyzed using Prism (Graphpad) and the non-linear regression software included in this software bundle. A one site model for binding was previously determined to be sufficient for describing the binding of GIP to its receptor (Gelling, 1998). Thus for saturation binding studies, the specific binding was determined and plotted against the concentration of radiolabelled GIP that was added to the cells. The data were then fit to a curve with the following equation:

$$Y=B_{max} \cdot X/(Kd+X)$$

Where B_{max} is the maximal binding, or the binding attained at saturation of the cells with ^{125}I-GIP, and Kd is the concentration of ^{125}I-GIP required to reach half-maximal binding at equilibrium. Thus, both B_{max} and Kd values were determined by Prism during the regression analysis. Once the B_{max} value was calculated using regression, the number of receptors on each cell could be determined using the specific activity of the radiolabel and Avagadro's number.

Competitive binding analyses were carried using a one site competition model. In these studies the amount of non-labelled GIP was varied to compete for binding sites (GIPR) with ^{125}I-GIP. Non–specific binding was defined as the amount of binding observed in the presence of 1 μM non-labelled GIP. This amount of binding was subtracted from all the other binding values to yield specific binding. The specific binding was then plotted against the concentration of non-labelled GIP and non-linear regression was carried out using the following one site competitive binding equation:

$$Y = \text{Bottom} + (\text{Top-Bottom})/(1+10^{\wedge}(X-\text{LogIC}_{50}))$$

Where Y is the specific binding, X is the Log_{10}[cold GIP], Top is the top plateau, Bottom is the bottom plateau and IC_{50} value is the concentration of cold GIP at which half of the maximal binding is displaced.

Chapter 3 – Development of Competitive RT-PCR and TaqMan Real Time RT-PCR Methodologies

3.1 Competitive RT-PCR

Initially a competitive RT-PCR strategy was developed and utilized to measure the expression of GIP receptor message in the islets of lean and fatty Zucker rats. This methodology was utilized because the Taqman, real-time PCR methodology that has now become standard for measuring RNA abundance was not yet readily available. Furthermore, there are strong points to both of these methodologies and therefore, in retrospect it proved advantageous to employ both technologies.

Competitive RT-PCR (cPCR) uses an RNA competitor, which binds both the primer used during the reverse transcription step as well as the primers used in the PCR reaction. The competitor that was utilized in these studies was constructed by inserting a portion (EcoRV-T7) of the polylinker from bluescript (pBKS) into the Sma I site in the carboxy (C)-terminus of the GIP receptor cDNA. This manipulation resulted in a 74 bp insertion in the GIP receptor carboxy terminus, and allowed a differentiation on size basis from the wild-type receptor DNA. Competitor RNA was synthesized from this mutant GIP receptor DNA using and a Megascript kit (Ambion Inc, Austin, Tx). The synthetic mutant GIP receptor RNA was gel purified using conventional acrylamide/urea gel electrophoresis and quantified using spectrophotometry.

RNA was isolated using Trizol as described in Chapter 2. Following RNA isolation, RNA was quantified using spectrophotometry and then 1.5 µg of total RNA was reverse transcribed. This was accomplished over 1 hour at 50 °C in a 20 µl reaction

volume containing: 0.5 mM dNTPs, 30 pmol 3' gene specific primer (FCL23':CAA GAC CTC ATC TCC AGG CAC AT), 200 U Superscript II Rnase H⁻ Reverse Transcriptase (Invitrogen), 10 U RNAse Inhibitor (RNA Guard; Pharmacia), 1 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$. After RT, 2 µl of the RT mix was amplified in a 50 µl PCR reaction containing 67 mM Tris HCl, 3.0 mM $MgSO_4$, 166 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, pH 8.3, with 200 mM dNTPs, 10 pmol of each primer (FCL5': 5'-ACC TGT ACG AGA ACA CGC AGT GC-3' and FCL23':CAA GAC CTC ATC TCC AGG CAC AT), and 1 U of Taq DNA Polymerase. The PCR reaction profile included a 5 minute intial denaturation step at 94 °C, followed by 40 cycles of 94 °C (45 s), 59 °C (60 s), 72 °C (60 s), with a final extension step at 72 °C for 5 minutes. Twenty microlitre samples were then run out on 1.5 percent agarose gels and imaged using ethidium bromide fluorescence.

Trial RT-PCR runs were carried out to determine the range of GIP receptor mRNA concentrations in pancreatic islets and pancreatic β-cell lines, from which standard GIP receptor concentrations could be derived. The standard curve values that were employed used GIP receptor competitor concentrations in the range of 0.24 to 20 amol of GIP receptor competitor RNA per µg of total cellular RNA (figure 1). The amount of wild-type GIP receptor mRNA in the original RNA sample was determined by finding the equivalence point between competitor and GIP wild-type RNA. Figure 1 illustrates that the competitor concentration decreases from 2 amols to 0.25 amols as the amplification of wild-type GIP receptor increases. The point at which this amplification is equal or identical is the point at which there are equal molar amounts of wild-type and competitor RNA in the sample. To determine this point, the density of each of the bands

was determined using the gel imager and accompanying software (Eagle Eye II, Stratagene). Then, since ethidium bromide binding and subsequent fluorescence is dependent on fragment size, the densities were divided by their fragment sizes (ie 323 bp for competitor and 249 for the WT). The corrected densities of the competitor were then divided by the corrected densities for the wild-type DNA and the Log_{10} values of these numbers were calculated. This was plotted against the Log_{10} values of competitor RNA that was added to the tubes. When the amount of competitor equals the amount of wild-type DNA the Log_{10} will equal 0. Therefore, the X-intercept on the resulting curve will give the amount of wild-type GIP receptor RNA (figure 2). Overall, this method was quite labor intensive; thus, real time RT-PCR was employed in further studies.

3.2 Real Time RT-PCR – The Taqman System

Due to the labor-intensive nature of competitive PCR, high sample throughput is impossible. Thus, when the Taqman system became available it was utilized for determination of GIP receptor mRNA content of total RNA samples. This system relies on the 5'-3' exonuclease activity of Taq polymerase, as well as a dual labeled fluorescent probe for quantitation of RNA. In these studies, RNA was isolated and reverse transcription was carried out as previously described; however, the PCR reactions were different, as described in detail in chapter 2

One major difference between the PCR reactions carried out for real time PCR (rPCR) and competitive PCR is the inclusion of a fluorogenic probe. The probe used in these studies contained two dyes: 1-Dimethoxytrityloxy-3-[O-(N-carboxy-(di-O-pivaloyl-fluorescein)-3-aminopropyl)]-propyl-2-O-succinoyl-long chain alkylamino

Figure 1: A typical gel obtained during competitive RT-PCR of GIP receptor RNA. Varying amounts of competitor GIP receptor RNA were added to the reverse transcription reactions that containing 1.5 µg of total RNA extracted from BRIN-D11 cells. The reverse transcription was carried out, followed by 40 cycles of PCR. Twenty microlitres of the PCR reaction were run on a 1.5 % agarose gel and visualized using ethidium bromide fluorescence.
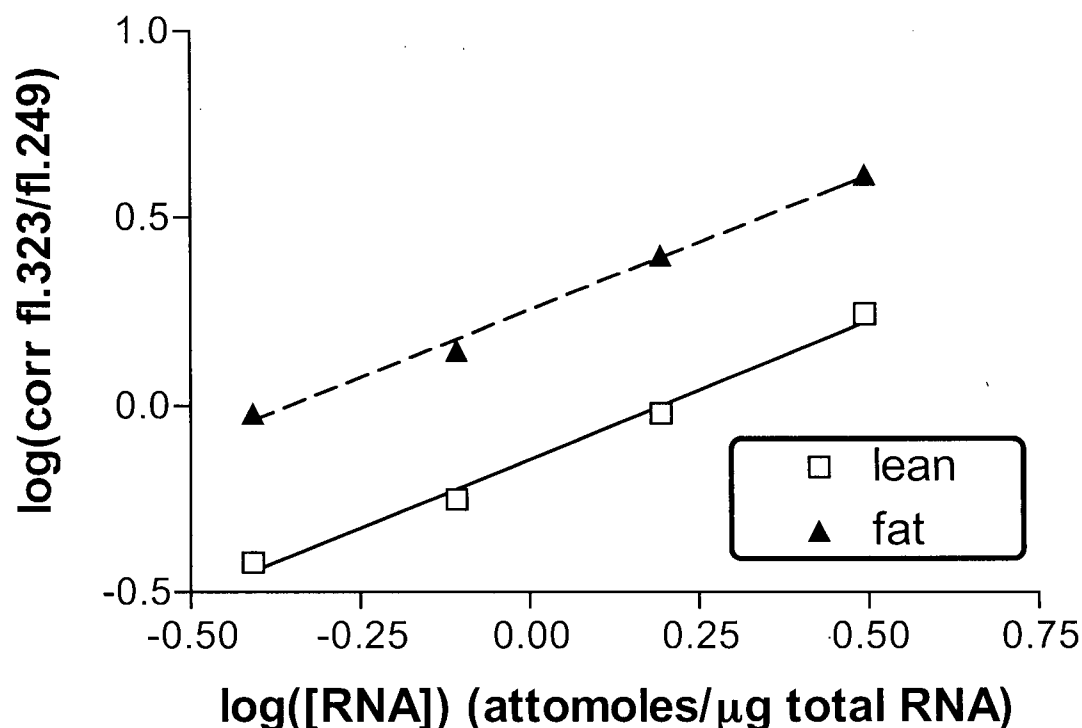
Figure 2: Standard curve derived from GIP receptor competitive PCR band density data. The band densities for each agarose gel lane were determined using the desitometry function in the Eagle Eye II gel analysis system (Stratagene). The densities were then corrected for the oligonucleotide band size (323 and 249: WT and competitor respectively) and the Log_{10} (log) of the ratio of band densities was plotted on the Y-axis. The Log_{10} of the starting concentration of competitor RNA was plotted on the X-axis. The two lines represent competitive PCR data for RNA collected from either the islets of a lean Zucker rat (open squares) or a fatty Zucker rat (closed triangles). The X-intercept (or when the Log_{10} of competitor/WT RNA = 0) is the equivalence point, and thus the point at which the amount of competitor equals the amount of GIP receptor mRNA in the sample. In this case the lean rat islets contained approximately 3 times more GIP receptor mRNA than the fatty rat islets.

(FAM) and 1-Dimethoxytrityloxy-3-[O-(N-carboxy-(Tetramethyl-rhodamine)-3-

aminopropyl)]-propyl-2-O-succinoyl-long chain alkylamino (TAMRA). When these

dyes are attached to the probe in its native conformation, energy is transferred from FAM

to TAMRA and the FAM fluorescent signal is quenched. However, when the probe

binds to DNA and Taq polymerase reads through the probe sequence, the 5'-3'

exonuclease activity of Taq removes the dyes from the probe and FAM is able to

fluoresce. Thus with each cycle, more FAM is released and fluoresces. This signal can

be detected and measured using the PE-Applied Biosystems Sequence Detector System

7700 (figure 3). To determine the amount of GIP receptor cDNA in a sample, a threshold

is set at which point the PCR reaction is still in its exponential phase. This ensures that

with each passing cycle there is an exact doubling of PCR product. The point at which

any of the reactions pass through this threshold value is known as the cycle threshold and

is directly proportional to the amount of starting receptor cDNA (figure 3). To compare

the amount of GIP receptor in samples between PCR runs, a standard curve was used in

real-time PCR. Wild type GIP receptor mRNA standard was synthesized *in vitro*

(Megascript Kit; Ambion Inc.), followed by electrophoretic acrylamide/urea gel

purification and spectrophotometric quantification. The standard curve used in all

experiments consisted of 1000, 100, 10, 1, 0.1 amol GIP receptor RNA/reaction tube

(figure 4). The amount of GIP receptor that was contained in each sample was then

determined automatically by the sequence detection system software from a standard

curve and could then be easily plotted (figure 4). Samples were analyzed in triplicate,

and each PCR run contained 24 separate RNA extractions.
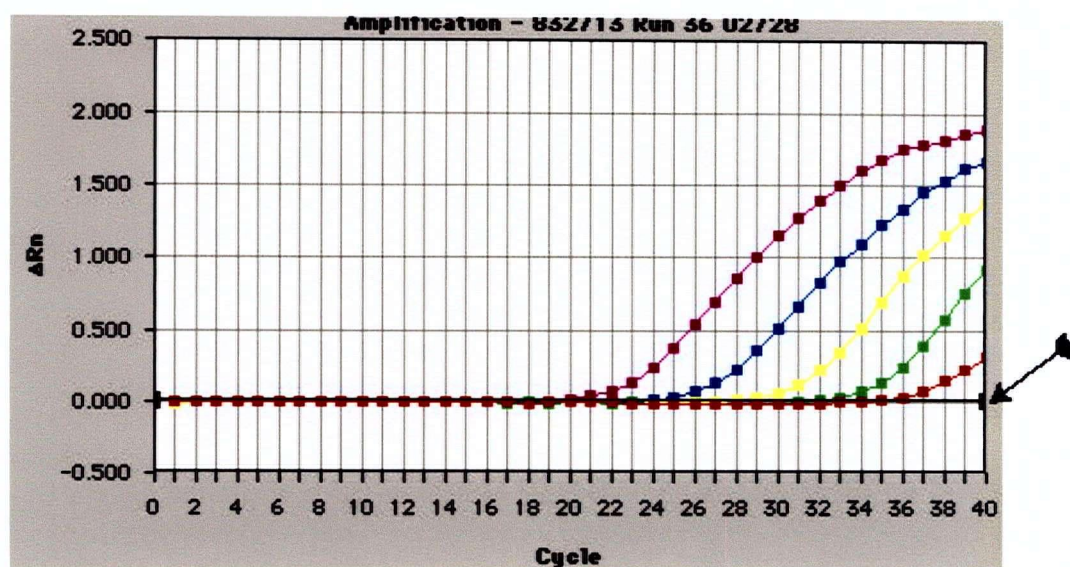
Figure 3: Raw, standard curve data obtained from the PCR amplification of synthetic standard GIP receptor cDNA. Varying amounts: 0.1 amol (red), 1 amol (green), 10 amol (yellow), 100 amol (blue) and 1000 amol (mauve) of synthetic GIP receptor mRNA were reverse transcribed and then amplified as described in Chapter 2. The fluorescence in each tube was measured at the end of the extension phase of each PCR cycle and the change of fluorescence from a baseline value was plotted against the specific cycle number. The cycle threshold is the indicated by an arrow.
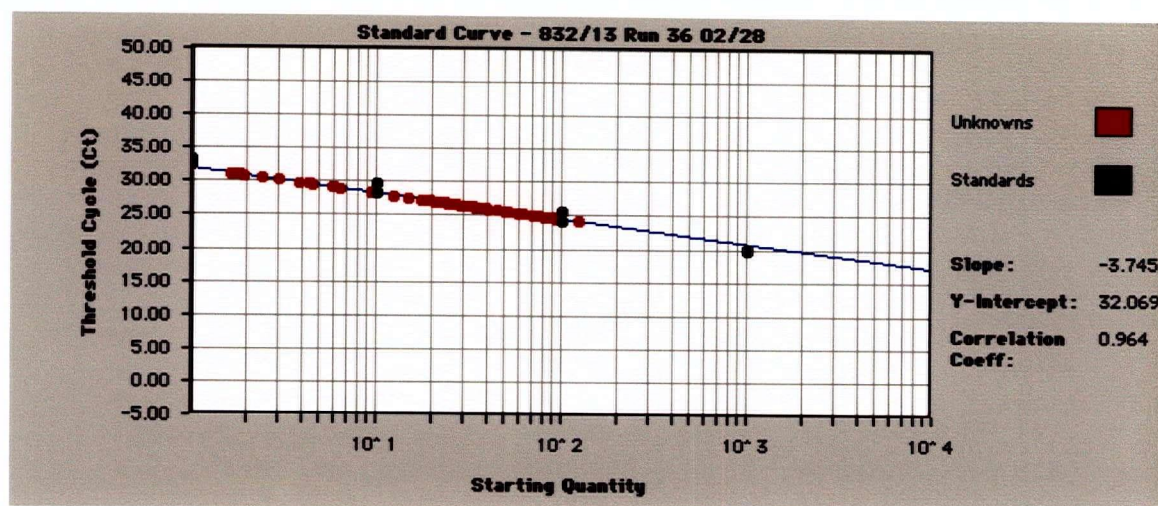
Figure 4: Real time RT-PCR standard curve. RNA was extracted from INS(832/13) cells (unknowns) or synthetically synthesized (standard) and RT-PCR was carried out as outlined in Chapter 2. Fluorescence was measured at the end of each extension phase and standard curve was created by the AB-PE 7700 SDS analysis software using the cycle thresholds. Linear regression was carried out and the unknowns plotted on the standard curve in red.

3.3 A Comparison of the Two Methodologies

Competitive RT-PCR and rPCR both have strong points and the two techniques complement each other well (Freeman *et al.*, 1999). For instance, the major drawback of rPCR is that it is not able to correct for differences in the efficiencies of specific reverse transcription reactions. On the other hand, because the competitor is within the same tube as the target WT mRNA in cPCR, the reverse transcription efficiency is always accounted for.

Competitive RT-PCR also has a number of problems associated with it. The greatest problem with cPCR is that it is impossible to know if the PCR reaction is in the exponential phase of the PCR (when the reaction is not limited) or if the reaction has reached the plateau phase (where primer concentrations may be limited or where *Taq* Polymerase may not be as efficient). Others have demonstrated that there can be a great deal of variability between reaction tubes containing the same template (Freeman *et al.*, 1999). Thus, using cPCR, one can optimize the protocol so that most tubes will fall within the exponential phase but it is impossible to be sure that all the tubes are in the exponential phase. Additionally, because of the nature of rPCR, a signal is obtained from each tube individually and therefore, each tube is quantified individually. This is not the case in cPCR where a series of PCR reactions is required to obtain a single quantification. This characteristic also makes quantification using rPCR more accurate. Another drawback of using cPCR is that it is extremely time consuming to carry out the actual experiments and complete the data analysis. This makes screening numerous samples rather impractical. Additionally, because there are many steps in this process, and

amplification of a large amount of message, there is often opportunity for contamination of the workspace with attendant PCR problems. This is avoided in rPCR by removing the agarose gel step altogether. Therefore, in rPCR, the PCR tubes are not opened after amplification, and the risk of contamination is low. Additionally, dUTP is used in the rPCR reactions and uracil-DNA glycosylase is added to the PCR reactions prior to amplification to degrade any nucleic acids containing uracil; elimating the risk of amplification of prior PCR products.

Both methods rely on equal amounts of total RNA being used in the reverse transcription. A few different techniques for measuring RNA, or correcting for the amount of RNA added to the reverse transcription were utilized in this thesis. Initially, RNA concentrations were measured using the absorbance at 260 nm. This method of measuring nucleic acids is very prone to inaccuracies due to the fluorescence of the aromatic amino acids present in proteins at around 280 nm. Thus, a small amount of protein contamination in the RNA drastically overestimates the actual amount of RNA in the sample. This was corrected for in our experimental design by: first, only using RNA samples that had A_{260}/A_{280} ratios of greater than 1.8 and that were almost identical in our cPCR reactions and later on by including glyceraldehyde phosphate dehydrogenase (GAPDH) external control reactions in early rPCR experiments. GAPDH is a metabolic enzyme that is expressed at a constant level within the cell. In the intial rPCR studies, most investigators were using GAPDH as an external control for their studies (Zamorano et al., 1996). Thus, we also normalized our GIP receptor expression to GAPDH expression, and this helped correct for any small differences in the amount of RNA added to our reverse transcriptions. In our intial studies that compared GIP receptor levels in

fatty and lean Zucker rats (Chapter 4), there was good agreement between the cPCR method using total RNA and the rPCR method using total RNA and GAPDH as an external control.

However, it has been consistently demonstrated that changes in metabolic state of the cell, as well as other manipulations can alter GAPDH expression levels (Zamorano *et al.*, 1996). These studies prompted us to investigate the possibility of using alternate means to determine the amount of total RNA added to our reverse transcription reactions. We thus switched to the Ribogreen fluorescent method (Molecular Probes) for RNA determination because it relies only on the binding of a fluorescent probe to RNA, and completely excludes protein from the measurements. The fluorescent dye utilized in this method does not efficiently bind to either nucleotide triphosphates or to small single stranded molecules; thus, there is little chance of including degraded RNA in the determinations. This method of quantification along with the development of the RNA standard allowed efficient and accurate quantification of GIP receptor RNA levels. Since the development of these techniques, we have repeated some of our initial experiments and observed very good agreement with our previous results. Thus, in our hands both cPCR and rPCR methodologies demonstrated that the GIP receptor was downregulated in the fatty Zucker rat, and both techniques demonstrated that GIP receptor expression was downregulated by approximately 70 % (figures 2 & 16).

Chapter 4 – GIP and the Vancouver Diabetic Fatty Zucker VDF Rat Model of Type 2 Diabetes

4.1 Background

A large proportion of postprandial insulin secretion is stimulated by hormones secreted from the small intestine. Glucose-dependent insulinotropic polypeptide, the proglucagon gene derived glucagon-like peptide-1-(7-37) (GLP-1) and the carboxy-terminal truncated form: GLP-1-(7-36)-amide are the major incretins that act via this endocrine system to potentiate glucose induced insulin secretion (reviewed in D'Alessio, 1997). GIP and GLP-1 both signal via serpentine, seven transmembrane, G-protein coupled receptors of the secretin/VIP superfamily. Binding of the incretins to their respective receptors on the β-cell surface activates adenylyl cyclase, increases cAMP and stimulates insulin secretion (Gremlich *et al.*, 1995; Moens *et al.*, 1996; Wheeler *et al.*, 1995). Recent studies have demonstrated that the GIP receptor displays similar characteristics to other G-protein coupled receptors in terms of ligand binding, desensitization and subsequent internalization (Gelling *et al.*, 1997; Wheeler *et al.*, 1999).

A wide range of experimental techniques have been utilized to demonstrate the physiological importance of GIP and GLP-1. *In vivo* administration of exendin-(9-39) and GIP-(7-30), GLP-1 and GIP receptor antagonists, resulted in decreased insulin responses to oral glucose (Schirra et al., 1998; Tseng et al., 1996b; 1999). Furthermore, both GIP and GLP-1 receptor knockout mice display compromised insulin release and, therefore, altered glucose tolerance to an oral load (Miyawaki *et al.*, 1999; Pederson *et al.*, 1998a; Scrocchi *et al.*, 1996). From these studies, it has been concluded that

secretion of the incretins could account for up to 70 % of the postprandial insulin response to glucose (Nauck *et al.*, 1993a). GIP and GLP-1 both require elevated levels of ambient glucose to stimulate pancreatic β-cell insulin secretion; hence, there is considerable interest in using incretin analogs of these peptides in the treatment of T2D (Brown *et al.*, 1978; Jia *et al.*, 1995; Nauck, 1998; Pederson & Brown, 1976; Rachman & Turner, 1995).

One shortfall of using GIP in therapy is the controversy over its effectiveness as an incretin in T2D (Nauck *et al.*, 1986; 1993b). Human studies have shown that there is a decreased incretin effect in T2D and this has been attributed mainly to an attenuation of GIP-stimulated insulin secretion either via a change in GIP receptor expression or a change in circulating GIP levels, although, altered signal transduction pathways could play a role (Holst *et al.*, 1997). Presently, there is no consensus regarding possible abnormalities in circulating levels of GIP in type 2 diabetics; studies have demonstrated increased, decreased and unchanged GIP levels (Ahren *et al.*, 1997; Fukase *et al.*, 1993; Jones *et al.*, 1989a; Vaag *et al.*, 1996). Thus, it cannot be concluded that chronic, homologous desensitization of the GIP receptor in T2D causes an ineffective incretin response (Hinke *et al.*, 2000a; Tseng *et al.*, 1996a). In addition, studies have shown point mutations in the GIP receptor gene in human populations that affect GIP signaling in cell models; however, it has not been possible to associate these mutations with T2D (Almind *et al.*, 1998; Kubota *et al.*, 1996).

In the current study we set out to test the hypothesis that the attenuated GIP-stimulated insulin responses observed in T2D can result from long-term downregulation

of GIP receptor expression in the β-cell plasma membrane. To test this hypothesis we utilized the Vancouver diabetic fatty Zucker (VDF) rat as a model of T2D.

4.2 Effect of GIP on glucose tolerance in the VDF rat.

In order to quantify the glucose lowering potency of GIP in VDF obese rats, the glucose lowering actions of GIP were first assayed in lean controls for comparative purposes. In initial experiments, the optimal GIP dose was determined by carrying out a bioassay with varying GIP concentrations ranging from 2 pmol/min/kg to 20 pmol/min/kg and monitoring the glucose lowering potency (figure 5). The optimum dose determined from this study (4 pmol/min/kg) produced a submaximal glucose lowering response in the lean animals but was still within the physiological range of doses. Thus, this dose was utilized for the remainder of the experiments. Figure 6 shows the blood glucose response to an IP glucose tolerance test in the presence or absence of infused GIP in lean, control animals. This figure clearly shows that GIP was able to significantly improve glucose tolerance in the lean animals as early as 30 minutes following IP glucose injection. Furthermore, this improvement in glucose tolerance was maintained as long as the GIP infusion was continued. Figure 6 (inset) shows that the integrated glucose response (over 65 minutes) for the lean animals receiving GIP was significantly smaller than those receiving saline.

The fat animals displayed basal hyperglycemia, with an average of 8.3 ± 0.4 mM, compared to 5.5 ± 0.2 mM for the lean animals (*cf* figures 6 and 7). The fat animals also
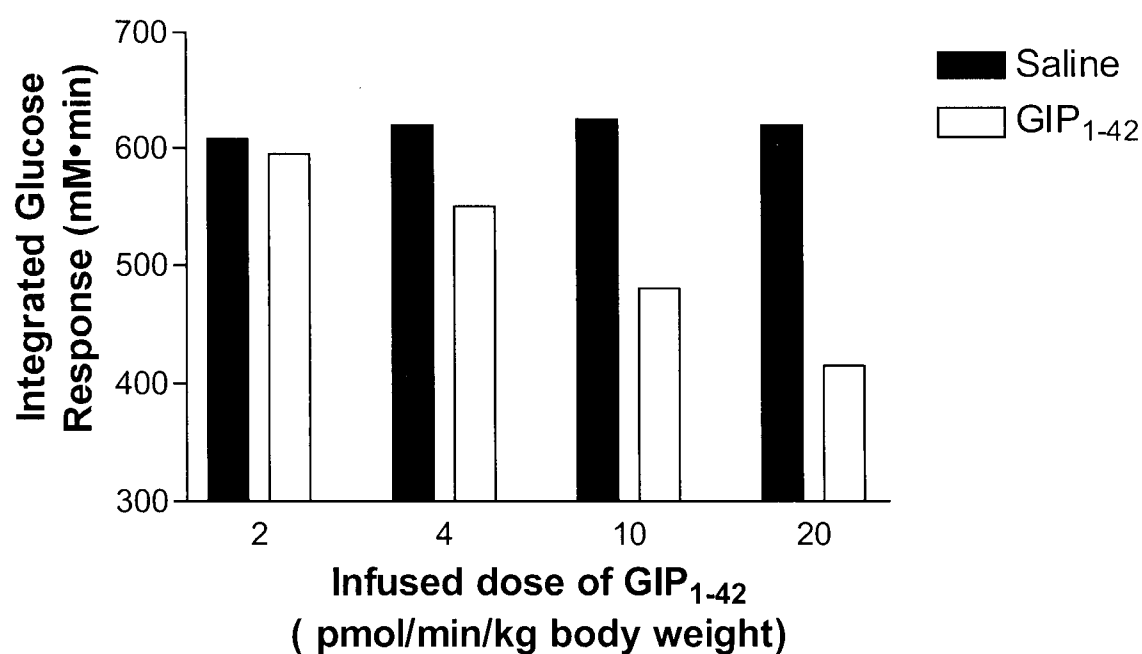
Figure 5: Dose related effects of GIP on plasma glucose during an IPGTT. Integrated glucose responses were determined from lean Zucker rats over 65 minutes of either GIP_{1-42} or saline infusion concomitant with a 1 g/kg intraperitoneal glucose tolerance test as outlined in the methods (n=2).
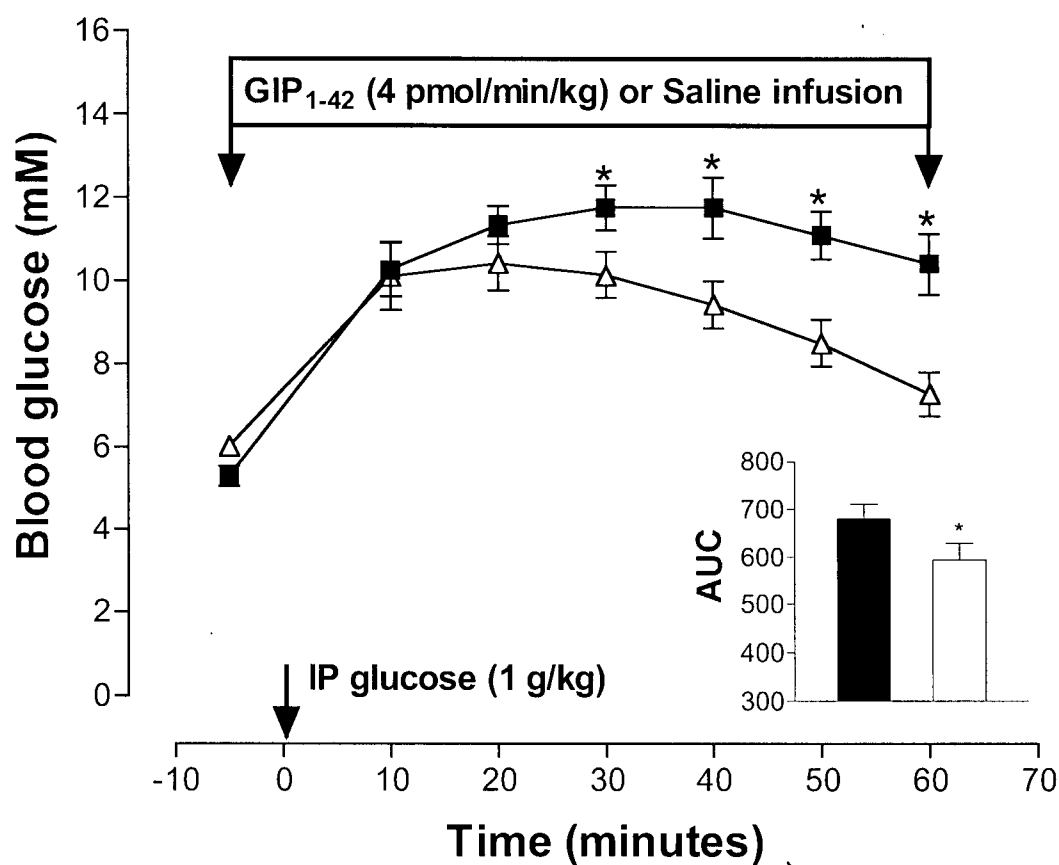
Figure 6: Glucose response to infused GIP (△) and saline (■) in control, *Fa/?* rats. Basal blood glucose samples were taken and then a 4 pmol/min/kg dose of GIP was infused into the jugular vein. Following five minutes of GIP infusion, glucose (1g/kg) was administered via an intraperitoneal injection. Blood glucose measurements were made on a handheld glucose analyser at 10 minute intervals. The inset indicates integrated area under the two curves (AUC). Asterisks indicate statistical significance (n=12, P ≤ 0.05), values are expressed as mean ± S.E.M.
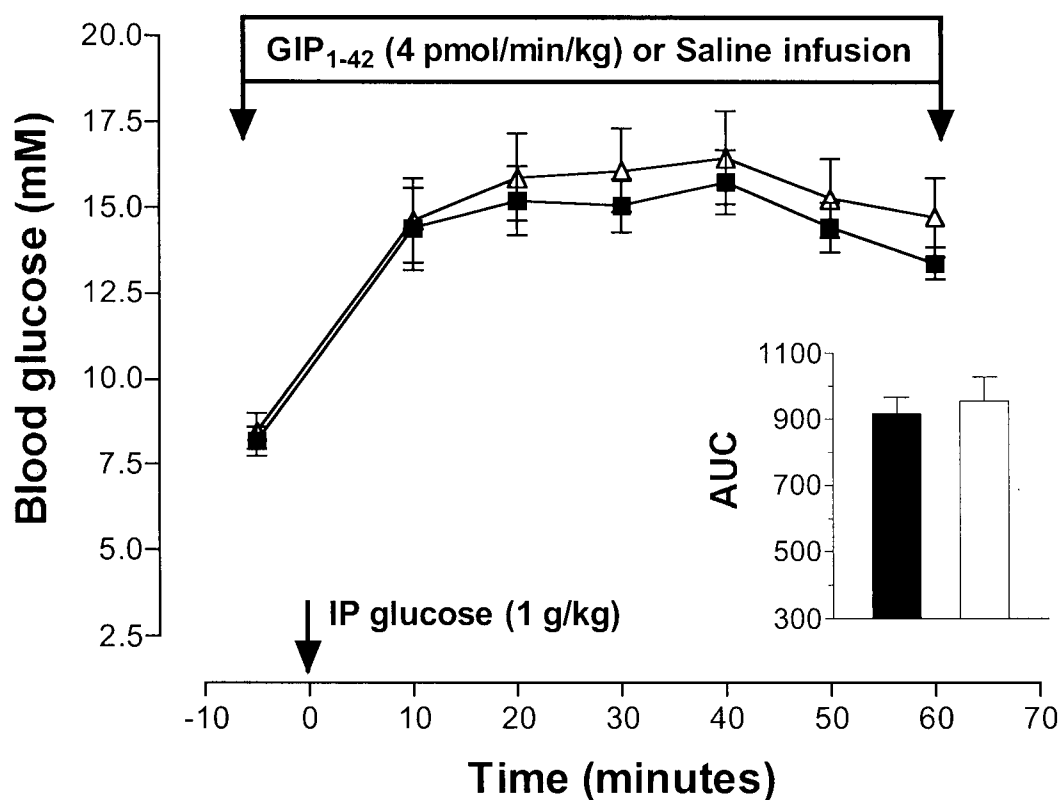
Figure 7: Glucose response to infused GIP (△) and saline (■) in VDF (fa/fa) rats. Basal blood glucose samples were taken and then a 4 pmol/min/kg dose of GIP was infused into the jugular vein. Following five minutes of infusion, glucose (1g/kg) was administered via an intraperitoneal injection. Blood glucose was measured with a handheld glucose analyser at 10 minute intervals. The inset indicates integrated area under the two curves (AUC). Values are expressed as mean ± S.E.M (n=12, P ≤ 0.05).

had significantly higher peak glucose levels in response to the glucose challenge, with the control (saline) values peaking at 15 mM (compared to 11 mM in lean animals); thus the fatty animals were glucose intolerant and hyperglycemic. The GIP infusion did not result in a decrease in circulating glucose levels in the fat animals, as no difference ($P>0.05$) was observed between GIP and saline infusions at any time following IP glucose (figure 7). Furthermore, the integrated glucose response for the fatty animals that received GIP was not different from the integrated response of saline infused animals (inset figure 7). Thus, GIP at an effective glucose-lowering dose in lean rats, yielded no improvement in glucose tolerance in the diabetic fatty animals.

4.3 Effect of GIP on insulin secretion in the Zucker rat.

In the same IPGTT experiments, plasma was collected at –5, 10, 20, 30 and 60 minutes following IP glucose and assayed for insulin and IR-GIP content by RIA. There was no difference observed in basal circulating IR-GIP levels between the fat (16.7 ± 1.8 pM) and lean (15.5 ± 1.6 pM) animals. Additionally, there was no difference in the integrated GIP response of the saline infused animals during the IPGTT, indicating both that the circulating levels of GIP are not different in the two phenotypes and that the IPGTT was not stimulating endogenous GIP release (figure 8).

GIP yielded a significant increase in circulating insulin levels in the lean animals with a peak of 400 pM at 20 minutes, which was prior to the glucose peak, observed in the same study (*cf* figures 6 & 9). Additionally, the integrated insulin response was significantly greater in the lean animals that received GIP (inset figure 9). Due to the insulin resistant state of the fatty animals, insulin levels were much higher at all times during the infusion protocol in this group (figure 10). Furthermore, there was neither a
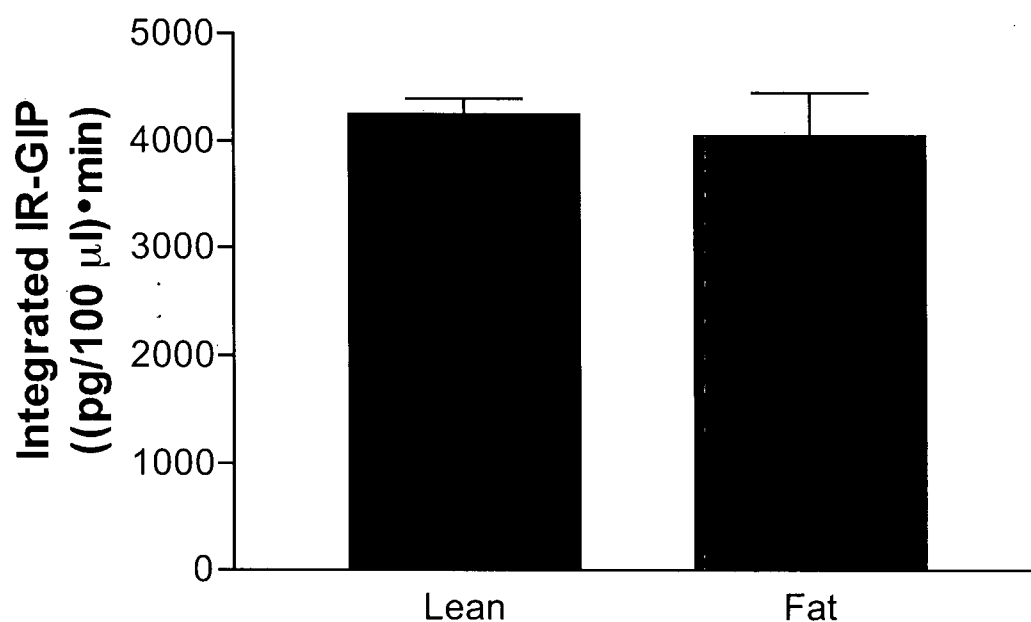
Figure 8: The integrated GIP response of saline infused control (*Fa/?*,lean) and VDF (*fa/fa*,Fat) rats during the IPGTT. Basal blood glucose samples were taken and then saline was infused into the jugular vein at a rate of 30 μl/min. Following five minutes of infusion, glucose (1g/kg) was administered via an intraperitoneal injection. Blood samples (500 μl) were collected from the tip of the nicked tail and plasma GIP was assayed using radioimmunoassay. Values are expressed as mean ± S.E.M (n = 9, P ≤ 0.05).
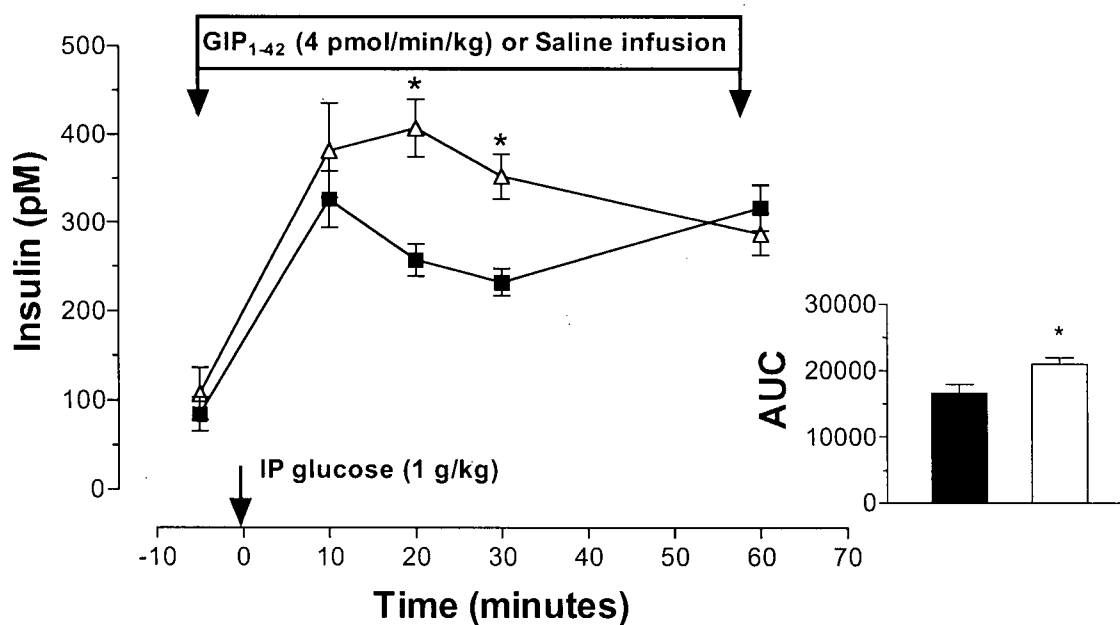
Figure 9: Insulin responses to infused GIP ($\triangle$) and saline (■) in control, *Fa/?* rats. Basal blood glucose samples were taken and then a 4 pmol/min/kg dose of GIP was infused into the jugular vein. Following five minutes of infusion, glucose (1g/kg) was administered via an intraperitoneal injection. Blood samples (500 μl) were collected from the tip of the nicked tail and plasma insulin was assayed using radioimmunoassay. The inset was obtained by taking the area under the curves (AUC) from the timecourse study. Asterisks indicated statistical significance ($n = 4$, $P \leq 0.05$). Values are expressed as mean ± S.E.M.
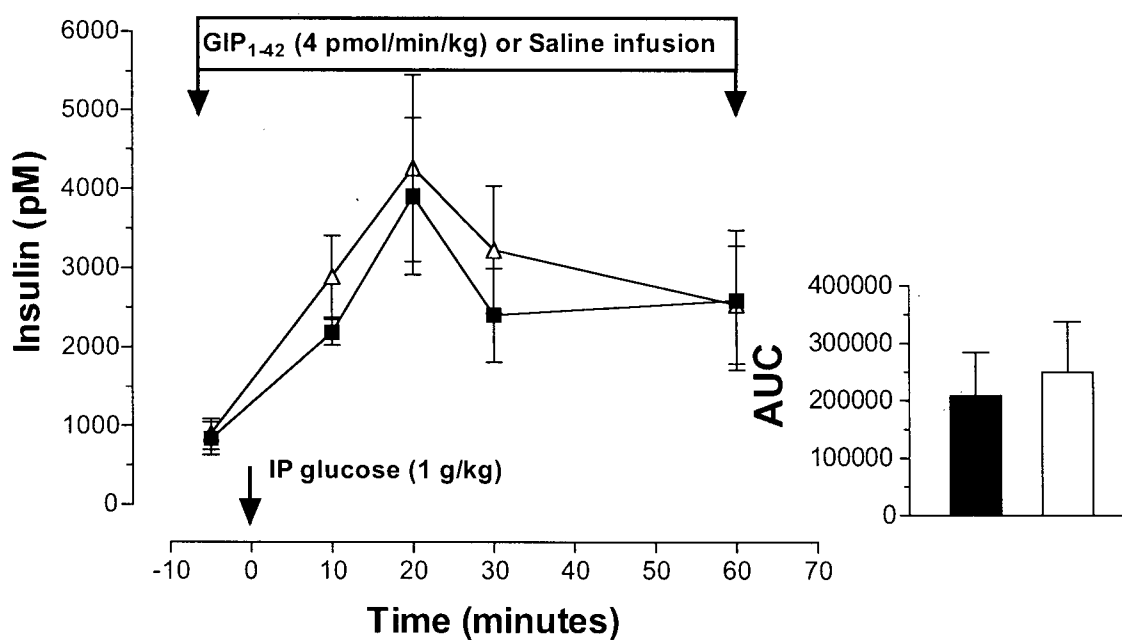
Figure 10: Insulin responses to infused GIP ($\triangle$) and saline ($\blacksquare$) in VDF (*fa/fa*) rats. Basal blood glucose samples were taken and then a 4 pmol/min/kg dose of GIP was infused into the jugular vein. Following five minutes of infusion glucose (1g/kg) was administered via an intraperitoneal injection. Blood samples (500 µl) were collected from the tip of the nicked tail and plasma insulin was assayed using radioimmunoassay. The inset was obtained by taking the area under the curves (AUC) from the timecourse study. Values are expressed as mean ± S.E.M (n = 4, P ≤ 0.05).

significant increase in insulin secretion elicited by GIP infusion (figure 10) nor an increase in the integrated insulin response in these animals (inset figure 10).

4.4 Effect of GIP on insulin release from the pancreas of the Zucker rat.

Pancreatic perfusions were carried out to determine if the defect in the secretory response to GIP in obese animals was confined to the pancreas and was not a result of extrapancreatic effects of this peptide. As indicated in figure 11, 10 pM GIP and 50 pM GLP-1 in the presence of 8.8 mM glucose evoked a significant 1.5 fold increase (~ 6-fold increase in area under the curve between 20 and 40 minutes) in insulin secretion from the lean perfused pancreas. However, this augmentation in insulin secretion was not observed in the VDF Zucker pancreas (figure 12) where insulin secretion decreased from approximately 8480 pM to around 3650 pM during the high glucose and GIP infusion period. Although GLP-1 did produce an approximate 5-fold increase (~ 10-fold increase in the area under the curve) in insulin secretion from the perfused VDF pancreas.

As seen in figure 13, 10 nM GIP in the presence of 16 mM glucose was able to stimulate insulin secretion from the lean Zucker islet, with a peak level 10 times the basal level of 62 pM. High glucose (16 mM) alone only produced a 4-fold increase in insulin release from the islets. Figure 14 illustrates the effects of GIP on the islets from the fatty Zucker rat. As illustrated, there was little effect of 10 nM GIP on insulin release from these islets in the presence of 16 mM glucose. The peak GIP-stimulated insulin release from the fat islets in the perifusion system was 4.3 ± 1.5 -fold basal, whereas glucose alone produced about a 3.2 ± 1.5 -fold increase from a basal level of 95 pM. This
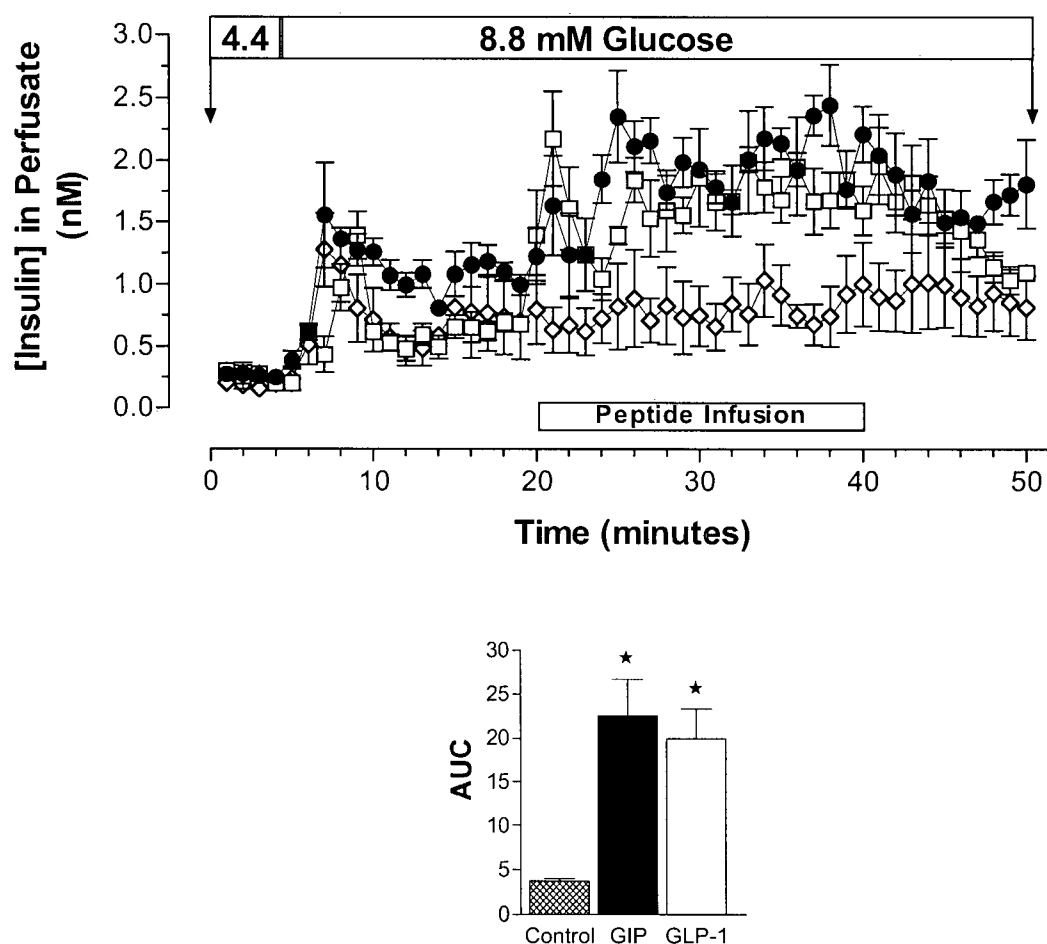
Figure 11: Insulin responses from the perfused pancreata of control rats.
Pancreata were perfused at a rate of 4 ml/min with Krebs buffer. Four minutes (t=4)
following equilibration, the preparations were subjected to 8.8 mM glucose (open
diamonds, glucose alone). GIP (●, 10 pM) or GLP-1 (□, 50 pM) was then added via an
infusion pump and a side arm at twenty minutes. Samples were collected every minute
and assayed for insulin content using radioimmunoassay. The area under the curves
(inset, AUC) was determined using Graphpad software (Prism). Values are expressed as
mean ± S.E.M. (n=3-6). Asterisks indicate statistical significance P<0.05.
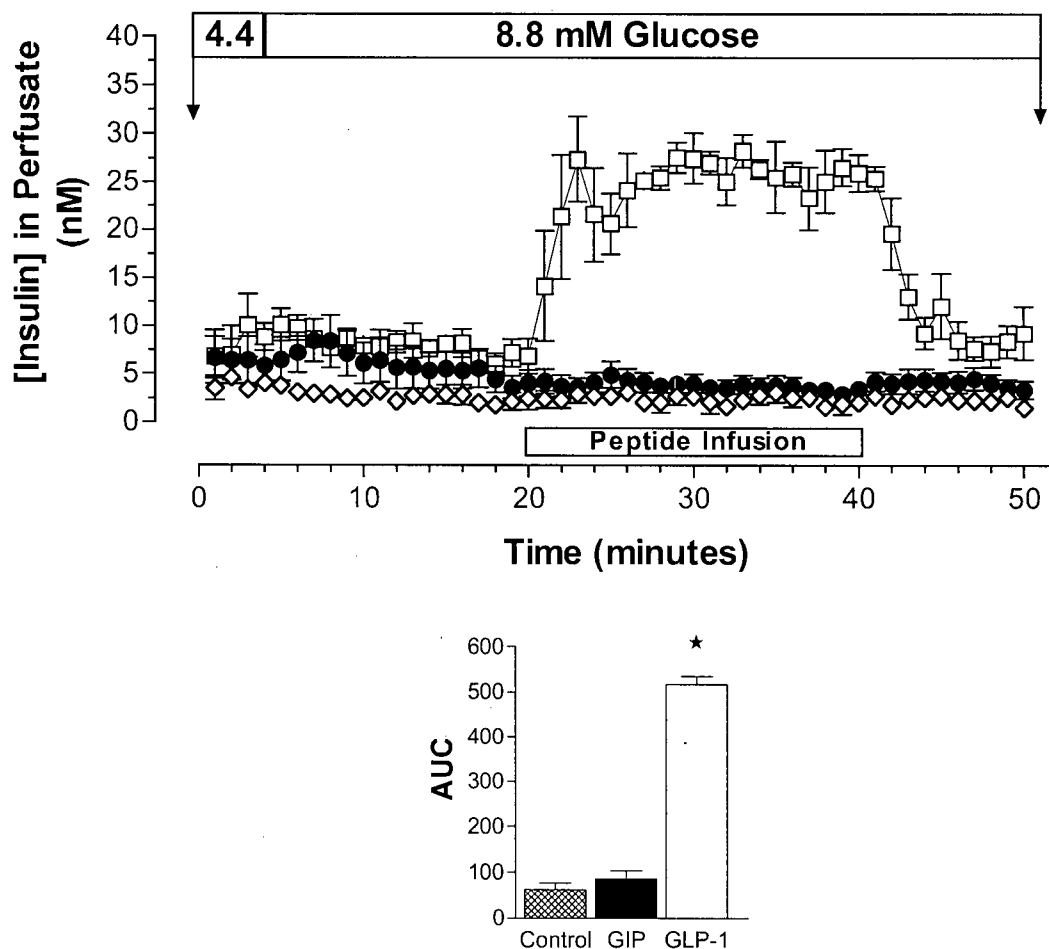
Figure 12: Insulin responses from the perfused pancreas of VDF *(fa/fa)* rats.
Pancreata were perfused at a rate of 4 ml/min with Krebs buffer. Four minutes (t=4)
following equilibration, the preparations were subjected to 8.8 mM glucose (open
diamonds, glucose alone). GIP (●, 10 pM) or GLP-1 (□, 50 pM) was then added via an
infusion pump and a side arm at twenty minutes. Samples were collected every minute
and assayed for insulin content using radioimmunoassay. The area under the curves
(inset, AUC) was determined using Graphpad software (Prism). Values are expressed as
mean ± S.E.M. (n=3-6). Asterisks indicated statistical significance P< 0.05.
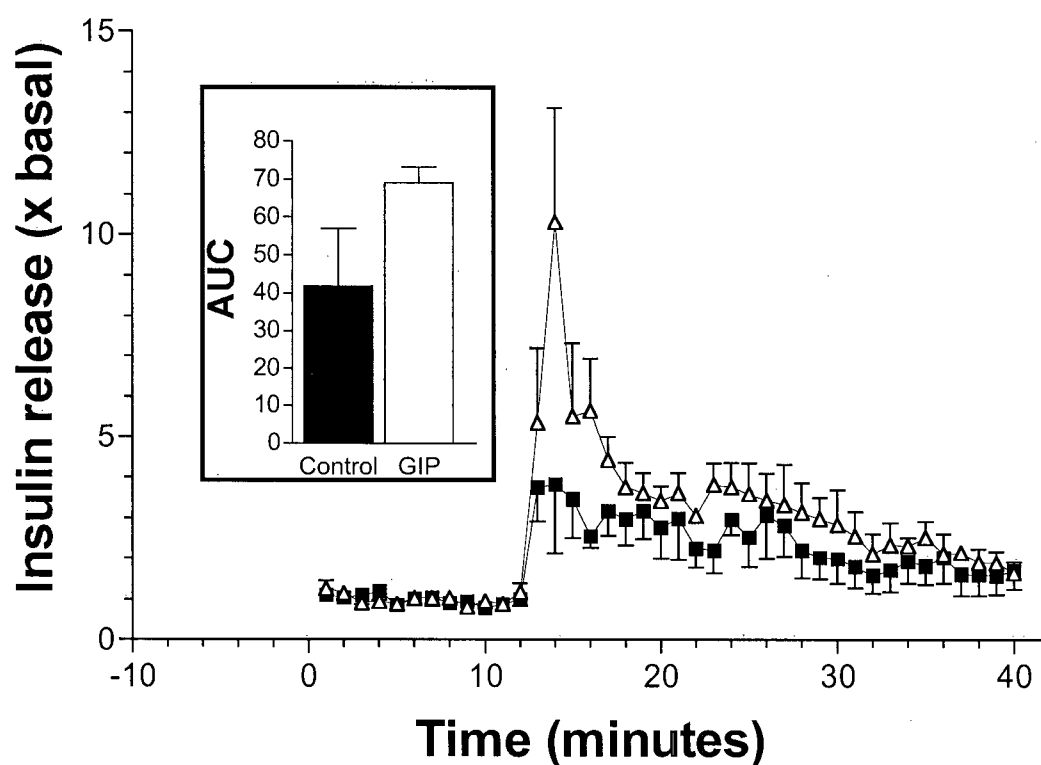
Figure 13: Insulin release from perifused islets isolated from control (*Fa/?*) rats.
Islets were isolated, cultured and perifused as described in Chapter 2. Following a 70
minute equilibration at 2.8 mM glucose, 10 nM GIP in 16.6 mM glucose ($\triangle$) or 16.6 mM
alone (■) was applied and continued for the remainder of the experiment. The inset
depicts area under the curves (AUC) that were determined using the trapezoid rule
(Graphpad, Prism). Fractions were collected every 2 minutes and perifusate was assayed
for insulin using radioimmunoassay. Data is expressed as mean ± S.E.M. (n=3).
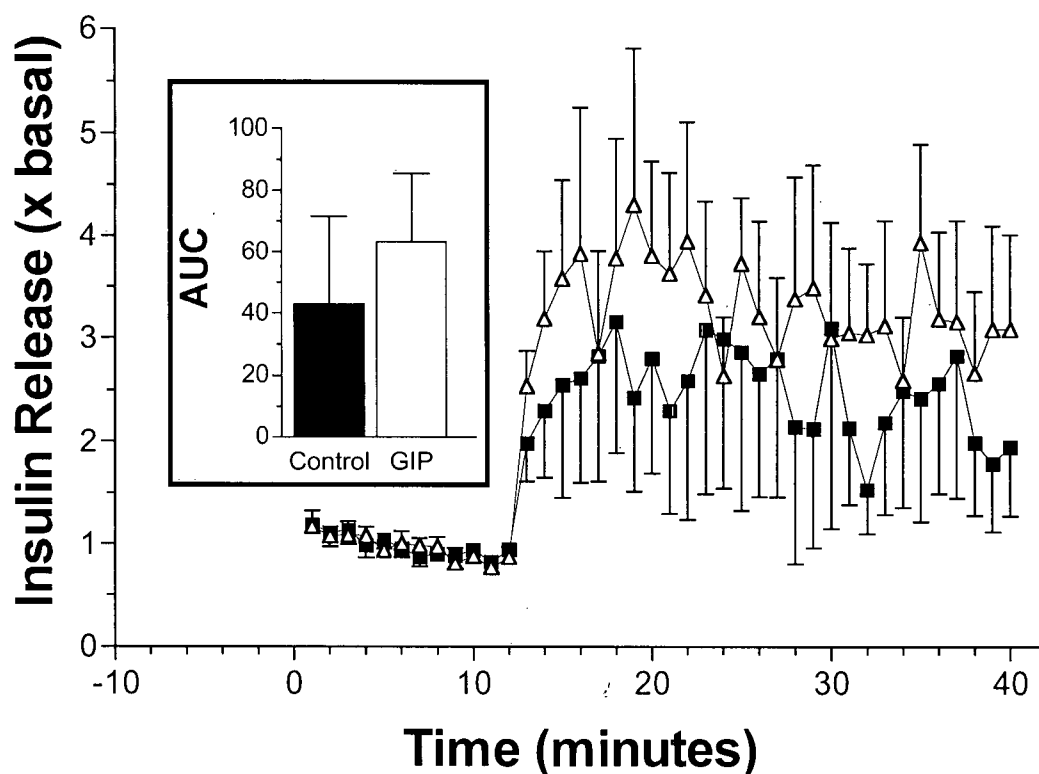
Figure 14: Insulin release from perifused islets isolated from VDF (*fa/fa*) rats.
Islets were isolated, cultured and perifused as described in Chapter 2. Following a 70
minute equilibration at 2.8 mM glucose, 10 nM GIP in 16.6 mM glucose (△) or 16.6 mM
alone (■) was applied and continued for the remainder of the experiment. The inset
depicts area under the curves (AUC) that were determined using the trapezoid rule
(Graphpad, Prism). Fractions were collected every 2 minutes and perifusate was assayed
for insulin using radioimmunoassay. Data is expressed as mean ± S.E.M. (n=3).

response was similar to the response seen in the lean rat islets to glucose alone (*cf* figures 13 & 14).

4.4 Effect of GIP on cAMP production in Zucker rat islets.

Islet cAMP studies were carried out to locate the defect in the GIP signaling pathway in the fatty animals Figure 15 illustrates the effect of GIP on cAMP production from fat and lean rat islets. GIP (10 nM) produced a marked response in the lean, control islets; however, there was no observable cAMP response to GIP in the islets from obese animals. The basal values of cAMP production did not differ significantly between the two phenotypes. Forskolin was included in these experiments to control for islet size and viability. As seen in figure 15, forskolin-stimulated cAMP production did not differ significantly between islets that were isolated from the two phenotypes.

4.5 GIP receptor mRNA expression in the Zucker rat islets.

The cAMP data suggested that a decrease of GIP receptor expression or a defect proximal to adenylyl cyclase could be responsible for the decreased effectiveness of GIP signaling in the fatty Zucker rat. This hypothesis was tested by carrying out reverse-transcription, real time PCR on RNA isolated from islets of lean and fat animals. We observed a significant (75 ± 5 %) decrease in GIP receptor mRNA in the islets from the fatty Zucker rats, as seen in figure 16 A. Additionally, the reduction of GIP receptor mRNA was obtained when measured with RT-competitive PCR: an alternate means of measuring RNA expression (figure 16 B).
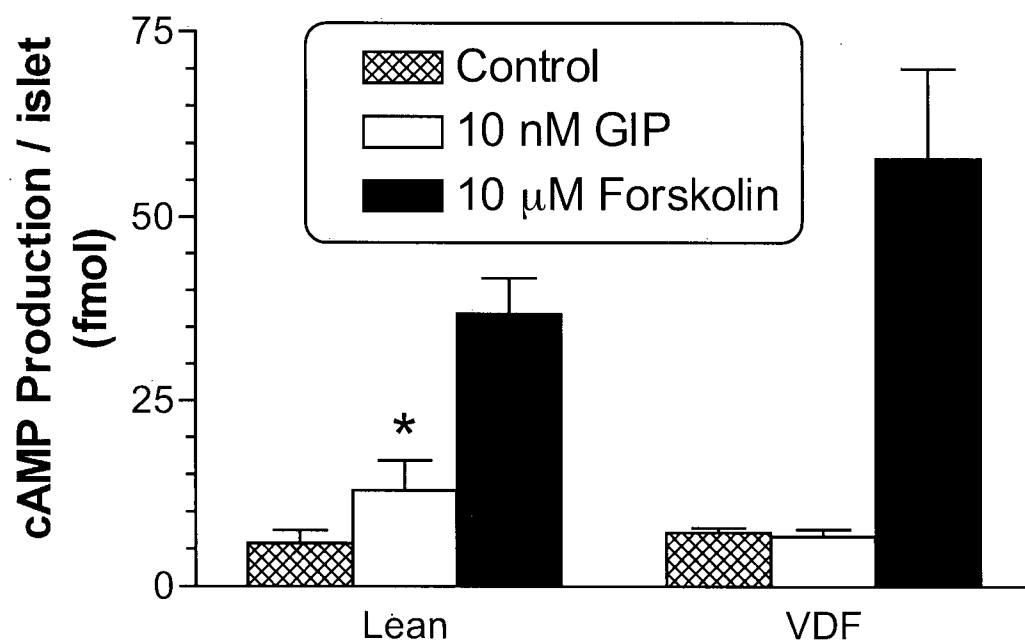
Figure 15: Islet cAMP responses to GIP and forskolin from control and VDF rat islets. Islets were isolated and cultured as described in Chapter 2. Forty islets were allowed to equilibrate in 8.8 mM glucose KRBH at 37 °C for 30 minutes prior to GIP stimulation (10 nM). GIP, or forskolin (10 μM) or glucose alone was then added to the islets in KRBH supplemented with 0.5 mM IBMX. Islets were stimulated for 30 minutes prior to cAMP extraction and measurement using radioimmunoassay. Data are expressed as mean ± S.E.M. (n=4) with asterisks indicating statistical significance between control and GIP stimulated conditions (P ≤ 0.05).
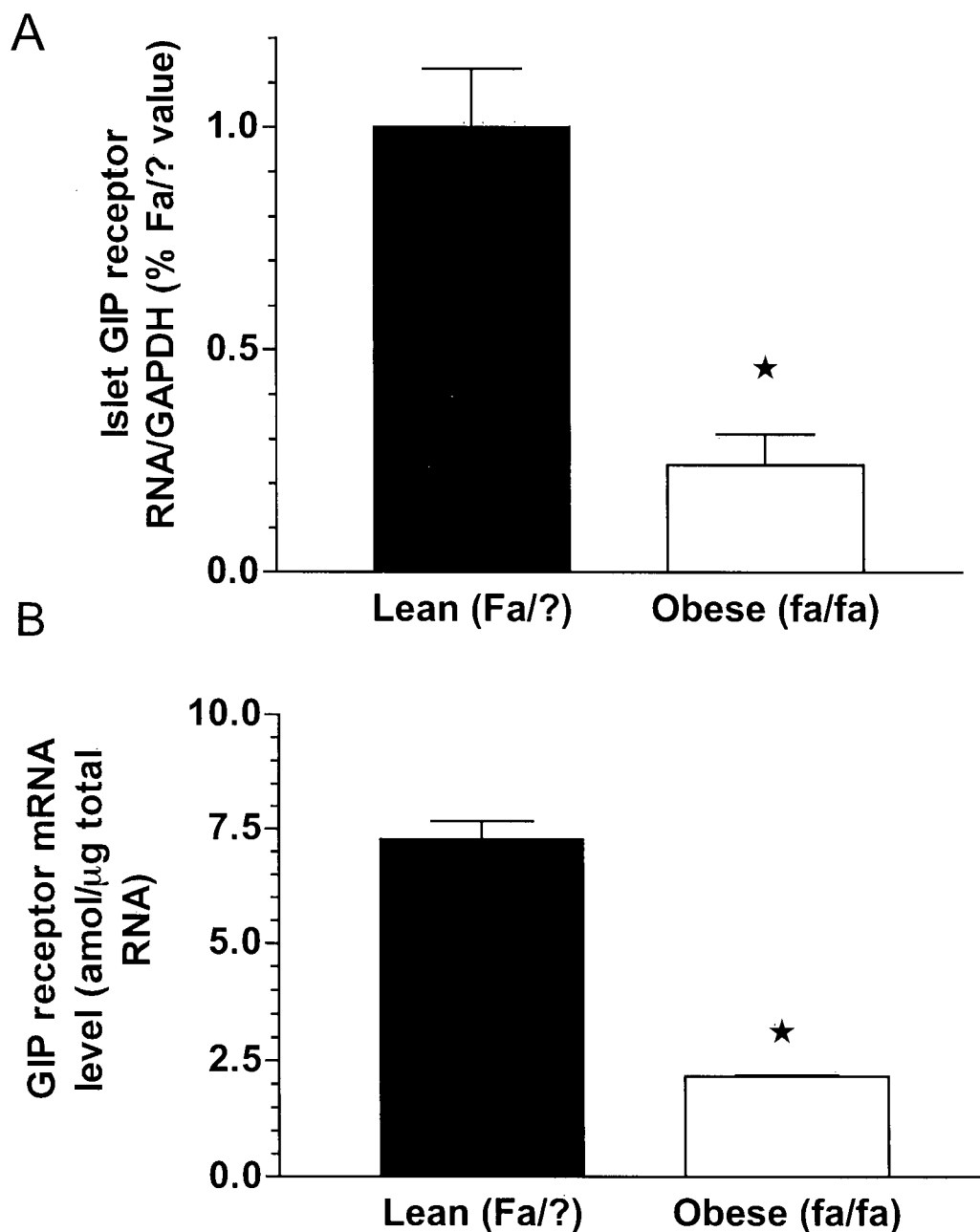
Figure 16: GIP receptor mRNA levels in the islets of control (*Fa/?*) and VDF (*fa/fa*) rats measured by (**A**) real time RT-PCR or (**B**) competitive RT-PCR. Islets were isolated as described in Chapter 2. Following isolation, RNA was extracted from the islets and subjected to reverse-transcription PCR. GIP receptor mRNA was normalized to glyceraldehyde-phosphate dehydrogenase mRNA content (**A**) and expressed as a fraction of control islet content. Data are expressed as mean ± S.E.M. (n=4) with asterisks indicating statistical significance (P ≤ 0.05).

4.6 GIP receptor protein expression in the Zucker rat islets.

The Western blot shown in figure 17 was typical of those observed when comparing fat and lean islet GIP receptor protein content. The post-translationally modified GIP receptor appears to run at around 65 kDa which is in agreement with previous work (Amiranoff et al., 1986). Figure 17 illustrates a marked decrease in GIP receptor protein level in islets from the VDF rats. This decrease is in accordance with that seen with mRNA levels as well as insulin release and cAMP stimulation of islets with GIP.

4.7 Glucose Tolerance, Insulin Secretion, and GIP Receptor Expression in Prediabetic VDF Rats

Experiments were carried out using 4 week old prediabetic animals to determine if GIP receptor downregulation was a result of impaired glucose tolerance or whether it was a genetic defect inherent to this animal model and present at birth. The prediabetic animals displayed significantly elevated blood glucose levels in a fasted state (time 0) as well as 10 minutes following glucose administration during an oral glucose tolerance test (OGTT). However, the glucose levels in young VDF animals and control animals were superimposed for the remainder of the OGTT (figure 18). These data indicate that at four weeks of age, these animals were only mildly hyperglycemic and thus, in a prediabetic state.

The small size of these animals made it impossible to collect sufficient blood to carry out IPGTT experiments similar to those performed on older animals. Thus, islets were isolated and the insulin secretory response to GIP was assessed. As seen in figure 19, GIP stimulated a significant amount of insulin secretion from the islets of both the

Figure 17: GIP receptor protein expression in the islets of control (*Fa/?*) and VDF (*fa/fa*) rats. Islets were isolated as described in Chapter 2. Following isolation islets were lysed in ice-cold RIPA buffer. 50 µg of total cellular protein was run on a 13 % polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and blotted with GIP receptor antibody, followed by HRP-conjugated goat anti-rabbit secondary antibody. The immunoreactive bands were visualized using ECL, and GIP receptor molecular weight (65 kDa) was determined using R_f analysis. This figure is a representative of an n=3.
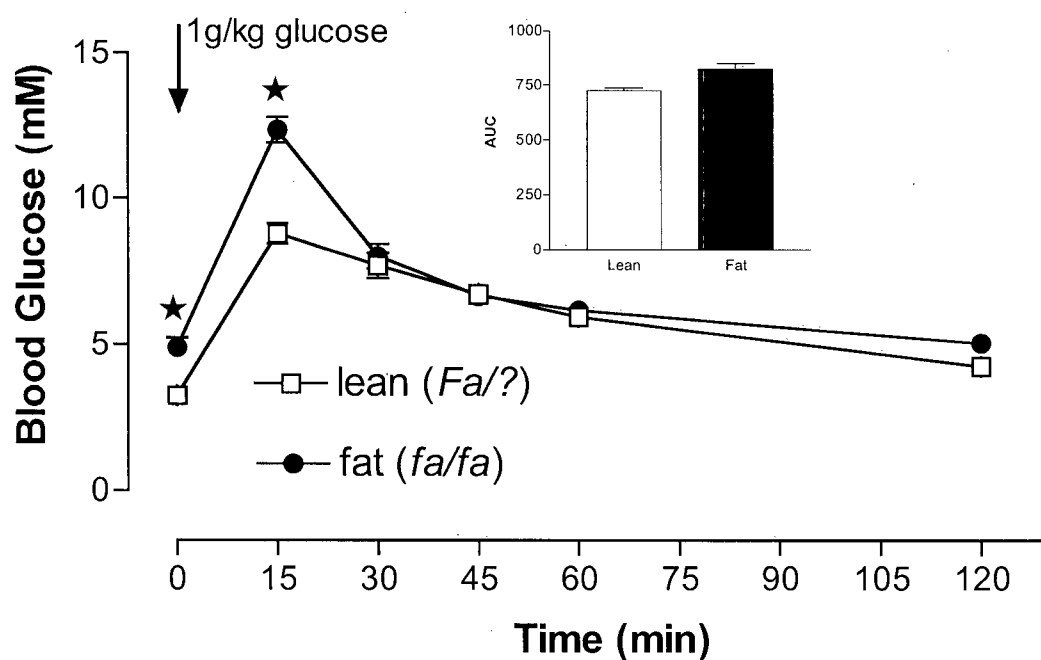
Figure 18: Oral glucose tolerance test from 4 week old control (*Fa/?*) and VDF (*fa/fa*)
rats. Rats were fasted overnight before an oral glucose tolerance test was carried out.
Glucose (1g/kg) was administered after a basal blood glucose reading was taken from the
tail vein using a Surestep handheld blood glucose analyzer. Further blood glucose
readings were taken as indicated. The inset shows the integrated area under the curve for
the control (white) and VDF (black) animals (n=10).

Figure 19: Insulin release from isolated islets from 4 week old control (lean, *Fa/?*) and VDF (fat, *fa/fa*) rats. Islets were isolated and cultured as described in Chapter 2. Forty islets were allowed to equilibrate in 2.0 mM glucose KRBH at 37 °C for 60 minutes prior to GIP stimulation (10 nM) at 16.7 mM glucose. Islets were stimulated for 30 minutes prior to collection of the media, extraction of total insulin and measurement of both using radioimmunoassay. Data are expressed as mean ± S.E.M. (n=4) with asterisks indicating statistical significance between control and GIP stimulated conditions (P ≤ 0.05).

young VDF and young control animals. Thus, it appears as if GIP retains insulinotropic potency in islets of young VDF rats.

To determine whether the insulin secretory profile in response to GIP was similar in the 4 week old animals, the pancreata from control and prediabetic VDF animals were perfused with a 0-50 pM gradient (figure 20). GIP perfusion stimulated insulin secretion from both the control and the prediabetic pancreata when compared to saline infusions. However, it appears that the 4 week old prediabetic VDF animals are hypersensitive to GIP: as stimulation with a low GIP concentration caused a profound insulin secretory response compared to the GIP response obtained from the 4 week old control animals (figure 20). In addition, the prediabetic VDF animals were not able to increase their insulin response to higher concentrations of GIP to the same degree as the control animals. This could be a result of a rapid desensitization of the islets of the prediabetic VDF animals to GIP or because the β-cells of these animals are already maximally secreting insulin in response to low GIP concentrations.

Finally, when the GIP receptor expression levels were analyzed in the islets of young control and VDF animals (figure 21), it was determined that there was a statistically significant 32 % decrease in the expression level of the GIPR in the islets from young VDF animals. This decrease amounted to approximately one-half the level of downregulation observed in the older VDF rats.

4.8 DISCUSSION

Human type 2 diabetics have been characterized by a decreased incretin response (Ahren *et al.*, 1997; Nauck *et al.*, 1986). This has been attributed to dysfunction in the
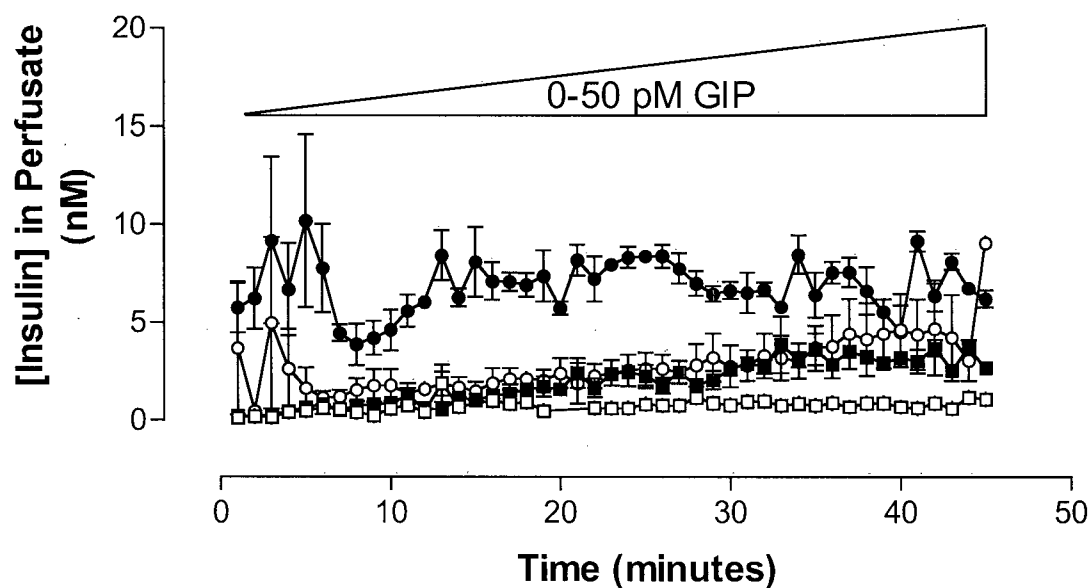
Figure 20: Perfusion of 4 week old control and VDF Zucker rat pancreata with saline or with a 0-50 pM gradient of GIP. Rats were anesthetized, pancreata isolated as described in materials and methods and perfused with a gradient of GIP in the presence of 8.8 mM glucose. Open circles denote saline infused VDF rats, filled circles denote GIP infused VDF rats. Open squares denote control saline infused animals and filled squares denote control animals infused with GIP. Perfusate was collected at one minute intervals and analyzed for insulin content using radioimmunoassay (n=4).
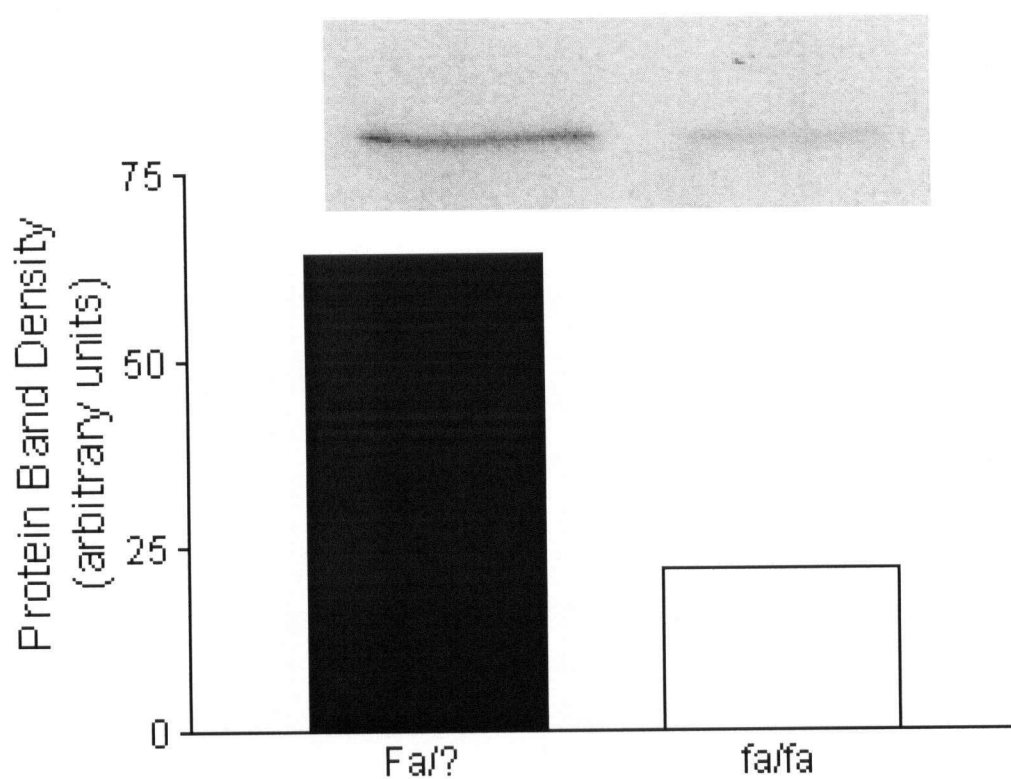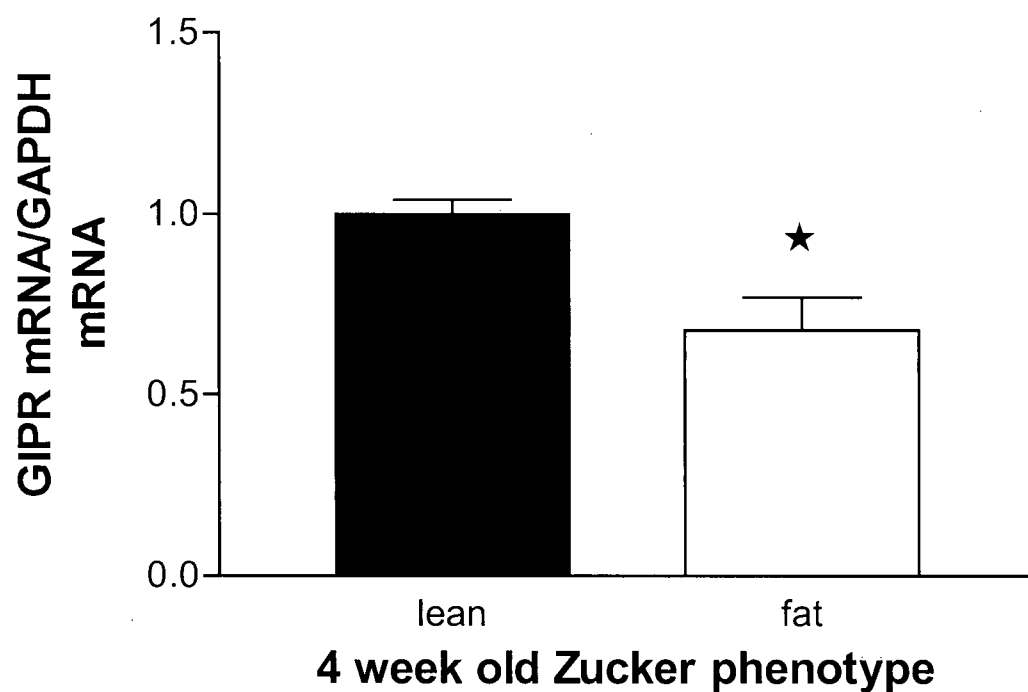
Figure 21: GIP receptor mRNA levels in the islets of control (lean, *Fa/?*) and VDF (fat, *fa/fa*) rats. Islets were isolated as described in Chapter 2. Following isolation, RNA was extracted from the islets and subjected to real time reverse-transcription PCR. GIP receptor mRNA was normalized to glyceraldehyde-phosphate dehydrogenase mRNA content and expressed as a fraction of control islet content. Data is expressed as mean ± S.E.M. (n=4) with asterisks indicating statistical significance (P ≤ 0.05).

GIP portion of the enteroinsular axis since GLP-1 continues to exert relatively normal insulinotropic and blood glucose lowering actions in these individuals (Ahren *et al.*, 1997; Lewis *et al.*, 2000; Nauck *et al.*, 1993b). The studies described in this chapter examined the expression and function of the GIP receptor on the pancreatic β-cell of the VDF rat - an animal model of T2D. It was shown for the first time that there is a decreased level of GIP receptor mRNA in the pancreatic islets of animals exhibiting characteristics of T2D: hyperglycemia, and insulin resistance. Additionally, this decrease in GIP receptor mRNA, decreased the ability of islets in these animals to respond to physiological GIP doses.

The 14-16 week old VDF rats used in these experiments were both glucose intolerant (figure 7) and hyperinsulinemic (figures 9 and 10). The pancreas perfusions (figures 11 and 12) demonstrated that there is a blunted first phase insulin secretion in the fat animals: 125 % increase compared to a 600 % increase in lean animals in response to the introduction of 8.8 mM glucose. Furthermore, in the normal, lean animals, 10 pM GIP stimulated a characteristic, biphasic insulin response; whereas, this hormone had no effect on second phase insulin secretion in the fat animals (*cf* figures 11 and 12). The perifusion data demonstrate that GIP is able to significantly increase first phase insulin secretion in islets from the lean animals, but continued exposure to 10 nM GIP probably leads to desensitization of β-cell surface GIP receptors and no further significant effect of GIP on insulin secretion is observed (Hinke *et al.*, 2000a).

Recently, there has been considerable interest in using dipeptidylpeptidase IV (DP IV) inhibitors in T2D therapy (Demuth et al., 2002). Circulating DP IV inactivates both GLP-1 and GIP by cleaving the amino terminal dipeptide from the parent incretin

polypeptides rendering them biologically inactive; thereby, decreasing the circulating half-life of the 'active' incretins. Presently, there is controversy as to whether GIP levels are elevated, normal, or lowered in T2D and animal models of the disease (Ahren *et al.*, 1997; Fukase *et al.*, 1993; Jones *et al.*, 1989a; Kieffer *et al.*, 1995b; Vaag *et al.*, 1996). One possible explanation for the ineffectiveness of GIP in the VDF rat is an increase in DP IV in these animals, which could inactivate GIP prior to its actions on the β-cell. Since it was impossible to measure differences in intact versus amino terminally truncated GIP with our radioimmunoassay, it was not possible to determine the role of DP IV in our findings, nor was it possible to determine the concentration of bioactive GIP in these animals. However, it has been demonstrated that the levels of circulating DP IV in these fatty and lean Zucker rats are similar; therefore, this explanation for GIP ineffectiveness can be ruled out (Pederson *et al.*, 1998b; Pospisilik *et al.*, 2002).

Interestingly, exposure of the rat islet to GIP leads to desensitization of the islet to GIP (Hinke *et al.*, 2000a). Presently, it is not clear whether this is due to a reversible phosphorylation of the GIP receptor, a decrease in GIP receptor mRNA expression or both. However, it was demonstrated that there was a rapid, homologous desensitization of the GIP stimulated insulin secretion in mouse β-cells (βTC3) that occurred both at the receptor level, as well as further downstream in the signaling cascade (Hinke *et al.*, 2000a). In contrast, experiments reported here demonstrate a decreased ability of GIP to increase cAMP levels in the islets of diabetic VDF animals; suggesting there is a defect in the GIP receptor – adenylyl cyclase portion of the GIP receptor intracellular signaling pathway. Furthermore, since we were unable to measure elevated ambient GIP levels in the VDF rat (figure 8), there is no reason to believe that hyperGIPemia caused a loss of

functional cell surface receptors, either by desensitization or downregulation, in these animals. The expression of other G-protein coupled receptors (such as the glucagon receptor) in the superfamily is regulated by glucose as well as the adenylyl cyclase activator forskolin (Abrahamsen & Nishimura, 1995). Thus, it is possible that GIP receptor downregulation and dysfunction occurs in response to inappropriate stimulation of the β–cell by abnormal levels of glucose or cAMP elevating agents in the VDF rat.

The pancreases of the 4 week old Zucker rats were much smaller than those of the mature animals. This made it difficult to carry out experiments on these animals in the same manner as those done on the older animals. Thus, the data obtained from the 4 week old animals are not directly comparable to the data obtained from the older diabetic animals, and for that reason any direct comparisons and conclusions that are made must be considered as purely speculative. In addition, further experiments need to be conducted to further characterize the timeline for development of defective GIP receptor expression in these animals.

These prior considerations aside, the 4 week old (but not the mature) VDF animals retained an insulin response to GIP (figures 12, 19 and 20). This is of interest because these prediabetic VDF animals were only mildly hyperglycemic and were able to clear the glucose during an OGTT as efficiently as the young control animals (figure 18). Furthermore, this normal disposal of oral glucose is the result of a near normal level of GIP receptor expression in these animals (figures 18 and 21). However, the prediabetic VDF animals do display basal hyperglycemia and do not seem to respond as quickly to an oral glucose load as the control animals (figure 18). Lewis *et al* (2000) administered a GIPR antibody to rats and reduced the first phase insulin response by 35 %,

demonstrating that GIP may be important for early phase insulin response. It is tempting to speculate that the 30 % decrease in GIP receptor expression that we observed in the islets from prediabetic VDF animals may be sufficient to significantly impair first phase insulin secretion *in vivo* without affected the overall glucose tolerance of the animals. Therefore, a decrease in GIP-stimulated first phase insulin secretion could explain the elevated 10 minute blood glucose values in the prediabetic VDF animals during the OGTT.

The level of GIP receptor expression in the prediabetic VDF animals was decreased 30 %; however, there is no major change in overall oral glucose tolerance in these rats *i.e.* they are able to adequately clear the glucose over the course of the OGTT. To speculate further, if there were a genetic mutation in the promoter or GIPR gene in these animals that directly altered GIP receptor expression, it would be likely that GIP receptor expression would be downregulated throughout their lives. Additionally, the decrease in GIP receptor expression seems to be inversely correlated with the degree of overnutrition in these animals. Thus, GIP receptor expression is probably controlled by a metabolite that is abnormally regulated in T2D, such as glucose or fat. This observation provides a basis for future experiments designed to determine exactly how GIP receptor levels are modulated by nutrient status. Furthermore, this observation lends support to the concept that a genetic defect in GIP receptor expression is not a primary cause for T2D, but rather that downregulation of GIP receptor levels during the development of T2D may exacerbate the disease.

These experiments demonstrate, for the first time, that GIP receptor mRNA expression is downregulated in the pancreatic β-cell of the diabetic Zucker rat (figure 16).

This observation suggests that there is a decrease in receptor expression on the pancreatic β-cell, and that this decrease in cell surface expression leads to the decreased potency of GIP as an insulinotropic agent. Figure 17 demonstrates that there is a decrease in total GIP receptor protein within the islets of the VDF rats. However, we have been unable to develop a method to assess cell surface GIP receptor protein expression on the islets of rats, as we do not have an ample supply of reliable antibody directed against the GIP receptor nor have we been able to develop a reproducible saturation radioligand binding protocol for use in islets. However, we have been able to demonstrate in clonal beta cells that changes in mRNA expression also produce similar changes in cell surface GIP receptor expression using radioligand saturation binding curves (Chapter 5). Therefore, we believe that there is a decrease in cell surface GIP receptor concomitant with the decrease in intracellular GIP receptor mRNA in these islets.

Béguin *et al.* (1999), demonstrated that stimulation of the β-cell by GIP caused phosphorylation of the Kir6.2 (K_{ATP}) channel via protein kinase A on serine 372. Phosphorylation of this serine residue led to an increased open probability of the channel. This recent paper was the first demonstration that GIP stimulation of the β-cell leads to protein phosphorylation. However, the physiological basis for this phosphorylation event is still unclear since Béguin and colleagues believe that Kir6.2 is maximally phosphorylated in the basal state. It is tempting to speculate that decreased levels of GIP receptor on the β-cell surface, would decrease the phosphorylation state of the K_{ATP} channel and decrease the open probability. This in turn would lead to a membrane depolarization and insulin secretion. If this receptor deficit was great enough there could

be uncoupling of glucose stimulated insulin secretion and β-cell decompensation, as observed in the fatty rats.

In conclusion, glucose tolerance and insulin responses were studied following GIP infusion in the diabetic VDF rat. In these animals GIP did not potentiate glucose induced insulin secretion, either *in vivo* or from the perfused rat pancreas and isolated perifused rat islets. Moreover, GIP failed to stimulate cAMP production in isolated *fa/fa* islet static incubations. Finally, GIP receptor mRNA and protein levels were shown to be downregulated in the islets of these animals, and this was hypothesized to be the basis for their insensitivity to GIP. In addition, the pancreata of young, prediabetic VDF and control animals are responsive to GIP and GIP receptor mRNA downregulation is not as severe as in the prediabetic animals. Thus, it appears that GIP receptor expression is decreased, possibly by hyperglycemia, during the development of T2D. As a consequence, GIP stimulated insulin secretion is greatly compromised during the development of T2D, and this may contribute to the etiology of this disease.

Chapter 5: The Regulation of GIP Receptor Expression in Rat Clonal β-Cell Lines

5.1 Background

Postprandial insulin secretion is controlled in part by the gut derived incretin hormones GIP and GLP-1. These incretins stimulate pancreatic β-cell insulin secretion by binding to a serpentine, seven transmembrane, G-protein coupled receptor and subsequently activating adnenylyl cyclase, phospholipase A_2 (PLA_2), and extracellular regulated kinases (ERK, MAP) as well as changing cellular ion fluxes (Beguin *et al.*, 1999; Ding & Gromada, 1997; Ehses *et al.*, 2001; McIntosh *et al.*, 1996; Trumper *et al.*, 2001; Wheeler *et al.*, 1995).

Knockout mouse studies have demonstrated that both the GIP and GLP-1 receptors are integral to the release of insulin from the pancreas following a meal. Both GIP and GLP-1 receptor null mice displayed compromised insulin secretion and therefore exhibited poor glucose tolerance to an oral glucose load (Miyawaki *et al.*, 1999; Scrocchi *et al.*, 1996). Furthermore, *in vivo* administration of exendin (9-39) and GIP (7-30) antagonists at the GLP-1 and GIP receptors respectively decreased glucose tolerance to an oral glucose load in rats (Schirra et al., 1998; 1996b; Tseng et al., 1999). From these studies it has been estimated that together GIP and GLP-1 could account for over 50 % of the insulin secretory response to a meal.

The major stimuli for GIP secretion from the gastrointestinal tract are carbohydrates and fatty acids (Pederson *et al.*, 1975). Thus it follows that GIP may play a role in fat metabolism in the adipocyte as well as other cell types expressing its

receptor. Our laboratory has demonstrated that GIP is lipolytic in differentiated 3T3-L1 cells in a cAMP dependent manner (McIntosh *et al.*, 1999). Furthermore, McIntosh *et al.* suggested that this lipolytic activity of GIP could prime the β-cell for the ensuing meal by causing an increase in free-fatty acids in the circulation. However, other groups have shown GIP to be lipogenic in rat adipose tissue (Beck & Max, 1983; Oben *et al.*, 1991). The role of GIP in fat metabolism in other cell types is at present poorly defined.

The peroxisome-proliferator activated receptors (PPARs) are a family of nuclear TF that are activated *in vivo* by fatty acids; binding of an activator of PPARα stimulates heterodimerization with the retinoid X receptor followed by translocation to the nucleus where transcriptional regulation can occur (Desvergne & Wahli, 1999). PPARα is expressed in the β-cell and is activated by free fatty acids such as palmitate and oleate as well as synthetic fibrate drugs such as clofibrate and WY 14643 (Roduit *et al.*, 2000; Wang *et al.*, 1999). Furthermore, PPARα has been demonstrated to tightly control expression of genes involved in fatty acid oxidation in the pancreatic β-cell including upregulation of acyl-CoA-synthetase and carnitine palmitoyl transferase-1 (Zhou *et al.*, 1998). Additionally, it is believed that activation of PPARα is the main pathway by which leptin stimulates lipolysis in the pancreatic β-cell; thereby, protecting the β-cell from lipotoxicity (Unger *et al.*, 1999).

Recently, it has been demonstrated that GIP may be ineffective at stimulating insulin secretion in T2D and the VDF animal model of T2D, probably because there is a decrease in the expression of the GIP receptor on the β-cell in the disease (Holst *et al.*, 1997; Lynn *et al.*, 2001; Nauck *et al.*, 1986). However, GLP-1 stimulated insulin secretion remains normal or even augmented in T2D as well as in the hyperglycemic,

hyperlipidemic VDF rat animal model (Lynn *et al.*, 2001; Nauck *et al.*, 1993b). The

mechanisms governing GIP receptor downregulation in T2D are unclear; although, in

Chapter 4 it was hypothesized that GIPR downregulation may be elicited by

hyperglycemia or hyperlipidemia. Since it is difficult to manipulate glycemia and

lipidemia within the whole animal without inducing widespread metabolic changes, β-

cell lines and islets maintained in cultured conditions were utilized to examine the effects

of glucose and fat on receptor expression.

5.2 Characterization of GIP Binding, GIP-stimulated cAMP Production and GIP-stimulated Insulin secretion in the INS(832/13) Clonal β-Cell Line

GIP binds to INS(832/13) cells in a specific manner with an IC_{50} of 30 nM and a

maximum specific binding of approximately 500 cpm after incubating with 50 000 cpm

of label with a specific activity of 350 mCi/mg (figure 22A). This level of GIP receptor

expression and GIP affinity is similar to that observed in other β-cell lines; however, the

affinity is slightly right-shifted from cells tranfected with the wild-type GIP receptor

(chapter 7). cAMP production in INS(832/13) cells was stimulated by GIP with an EC_{50}

of 6.6 nM (figure 22B). The maximal cAMP production was approximately 1.5 times

basal. This degree of GIP stimulated cAMP production as well as the EC_{50} are also in

line with other β-cell models (Hinke et al., 2000a).

5.3 The Effect of GIP on Insulin Secretion from INS(832/13) Cells.

GIP stimulated insulin secretion from INS(832/13) cells in both a concentration

(data not shown) and glucose dependent manner (figure 23). Figure 23 illustrates that in

the presence of 0 mM glucose, 50 nM GIP was unable to stimuate insulin secretion.
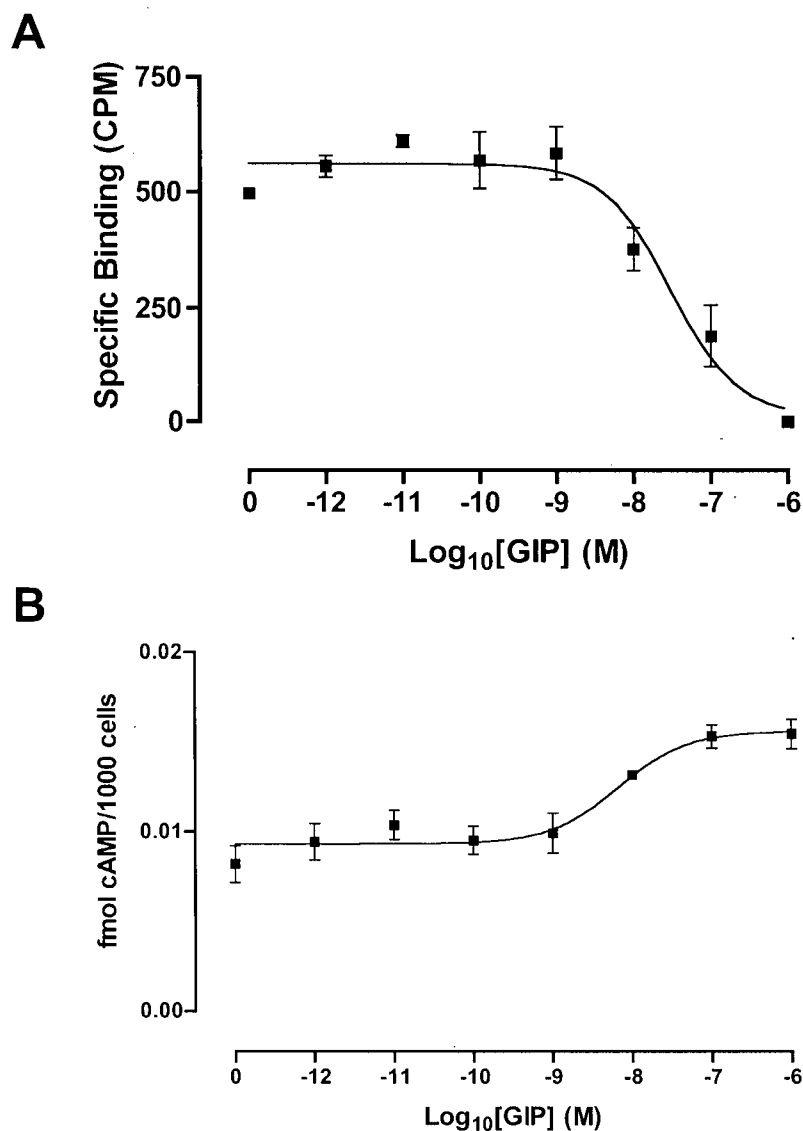
Figure 22: GIP receptor binding (A) and cAMP signaling (B) in INS(832/13) clonal β-cells. Cells were plated in 24 well plates at a density of 5×10^5 cells/well in 5.5 mM glucose and allowed to grow for 48 hours before experiments were conducted. For competitive binding analyses, various concentrations of unlabelled GIP were incubated for 4 hours at 4 °C in presence of ^{125}I-GIP. Cells were then washed and the amount of GIP bound was determined using a gamma counter. For cAMP studies (B), cells were preincubated in 5.5 mM glucose and then incubated for 30 minutes at 37 °C with 0.5 mM IBMX. cAMP was then extracted using 70 % ethanol and quantified using radioimmunoassay. Values are expressed as mean ± SEM of 4 independent determinations.
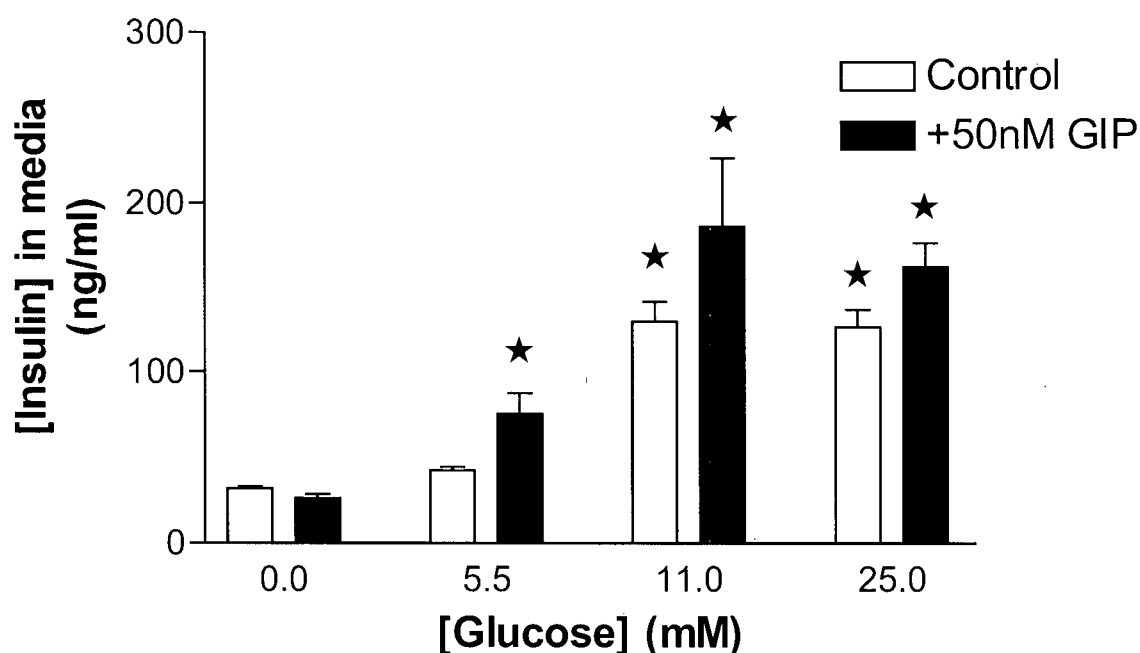
Figure 23: Insulin secretion from INS(832/13) cells in response to increasing glucose concentrations in the presence of GIP. Cells were plated at a density of 5 x 10^{5} cells/ml in 24 well plates and grown in 5.5 mM glucose for 48 hours. The experiment consisted of a 1 hour preincubation for 60 minutes in 1 mM glucose. Cells were then stimulated for 30 minutes at 37 °C in the presence of 50 nM GIP. Media was then collected and analyzed for insulin release using radioimmunoassay, as described in Chapter 2. Asterisks indicate statistical significance from 0 mM glucose conditions as determined by ANOVA and Dunnet's post hoc test (P< 0.05, n=5).

None of the GIP stimulated conditions were significantly different from control conditions (at that glucose concentration); however, there is a trend that indicates that GIP stimulated insulin secretion at all glucose levels, and it is probable that increasing the sample size would have resulted in statistical significance. The maximal glucose and GIP-stimulated insulin secretion occurred at 11 mM glucose, there was no further increase in the ability of GIP to stimulate insulin secretion at glucose concentrations greater than this.

5.4 GIP Stimulates Palmitate Oxidation in BRIN-D11 Clonal β-Cells.

GIP stimulated palmitate oxidation in the BRIN-D11 β-cell model. This stimulation of palmitate oxidation had an EC_{50} of 27 nM and a maximum of 1.6 times basal levels (figure 24). These values fit well with the IC_{50} values for GIP binding and insulin secretion in this cell model (data not shown)

5.5 The effects of glucose on GIP receptor mRNA expression in INS(832/13) cells.

Glucose strongly downregulated expression of GIP receptor mRNA in both a time and concentration dependent manner (figures 25 & 26). As illustrated in figure 26, there was a significant decrease in GIP receptor mRNA at glucose concentrations greater than 11 mM. Under the condition of 25 mM glucose, GIPR expression decreased to 30 % of that seen under zero glucose conditions. Furthermore, this decrease in receptor level occurred rapidly with a significant difference being observed at 6 hours following exposure to the 25 mM glucose (figure 25). No further decrease in receptor level was observed following the 18 hour time point (cf figures 25 & 26) at which time GIP receptor expression was reduced to 28 % of the basal level. Culture of cells longer than
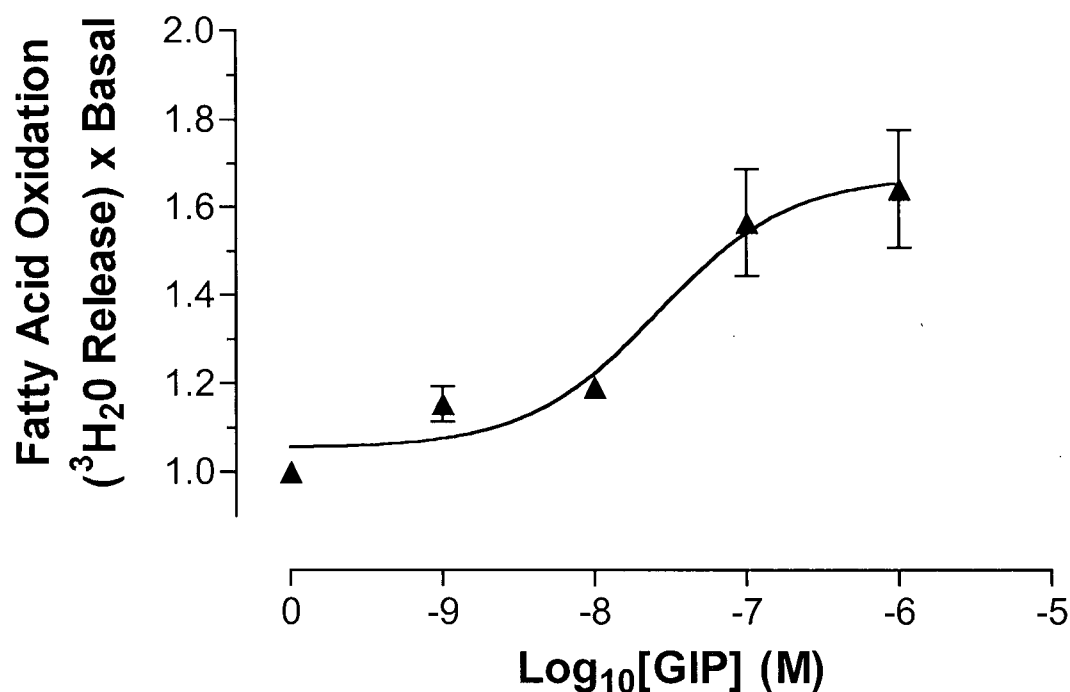
Figure 24: GIP-stimulated palmitate oxidation in BRIN-D11 clonal β-cells.
Cells were grown in the presence of ^{3}H-Palmitate for 48 hours, then washed and
stimulated with GIP for 4 hours. The medium was collected, and centrifuged following
stimulation with GIP. ^{3}H$_2$0 was used as a measure of fatty acid oxidation, ^{3}H$_2$0 was
allowed to equilibrate for 24 hours at 60 °C with non-labelled water. The radioactivity
was then measured in samples and normalized to 0 GIP conditions. Values are expressed
as mean ± SEM of 4 independent determinations.
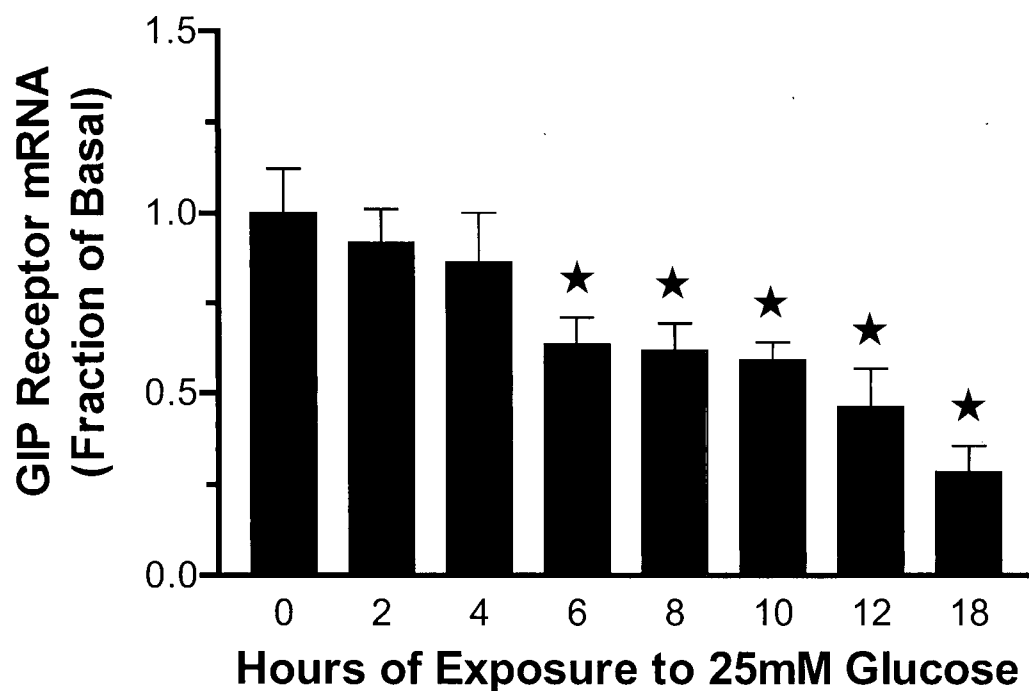
Figure 25: The effect of time of exposure of INS(832/13) cells to 25 mM glucose on GIP receptor mRNA expression. Cells were incubated in regular media supplemented with 25 mM glucose for times varying between 0 and 24 hours. Following incubation RNA was isolated and quantified using real-time RT-PCR as described in Chapter 2. Data were normalized to the basal conditions: *i.e.* for expression level at 0 hours. Asterisks indicate statistical significance compared to basal levels P<0.05 n=4
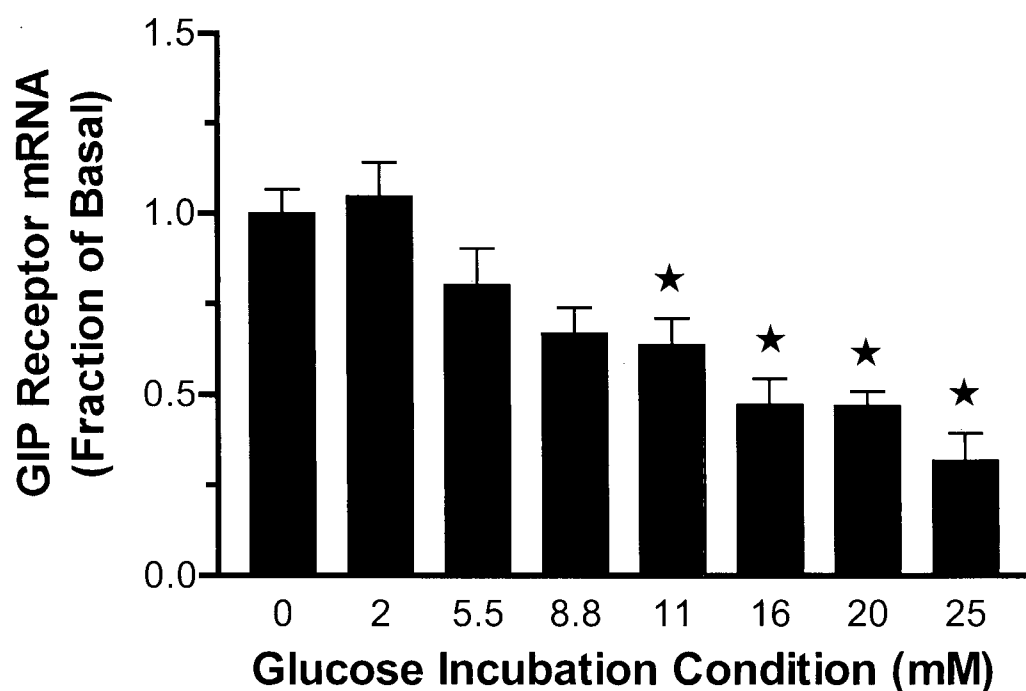
Figure 26: The effect of glucose on GIP receptor mRNA expression in INS(832/13) cells:
GIP receptor mRNA downregulation in response to graded glucose concentrations.
Cells were incubated for 24 hours in varying glucose concentrations between 0 and 25
mM. Following incubation, RNA was isolated and quantified using real-time RT-PCR
as described in Chapter 2. Data were normalized to the basal conditions: *i.e.* 0 mM
glucose. Asterisks indicate statistical significance compared to basal levels $P<0.05$ n=4

24 hours in 25 mM glucose does not allow the cells to desensitize to the high glucose conditions and the expression of GIPR mRNA remained at approximately 30 % of the basal level (data not shown). Saturation binding analyses (figures 27 & 28) showed a marked, statistically significant decrease in the amount of GIP receptor expressed on the cell surface of the INS(832/13) cells grown in high glucose conditions. The number of GIPR binding sites per cell grown at 5.5 mM glucose was 1930 ± 200, while the number of GIPR binding sites per cell grown at 25 mM was approximately 910 ± 130 (figure 28). In addition, the dissociation constant (Kd) for GIP was the same under both conditions, with Kd values of 400 ± 135 and 427 ± 145 pM in 5.5 mM and 25 mM glucose respectively (figure 27). This indicates that the kinetics of binding were identical under both high and low glucose conditions.

In an effort to determine how the downregulation of the GIPR mRNA was occurring we cultured INS(832/13) cells in the presence of various inhibitors of cell growth and proliferation (shown in figure 29). We did not see a reversal of the effects of 25 mM glucose in any of the conditions that we used. However, we observed that both wortmannin, a PI-3 kinase inhibitor and H89, a PKA inhibitor significantly increased GIPR mRNA levels above basal. Furthermore, we utilized insulin to ensure that high insulin levels were not contributing to the downregulation of the GIP receptor, as high insulin levels occur during incubation of these cells in high glucose. As seen in figure 29, insulin increased GIP receptor expression and therefore, was not contributing to the glucose-induced downregulation. Neither Bis (2 μM), a highly-specific, cell permeable PKC ($\alpha,\beta1,\beta2,\gamma,\delta,\varepsilon$ isoforms) inhibitor, nor PD 98059 (100 μM), a MEK inhibitor, had
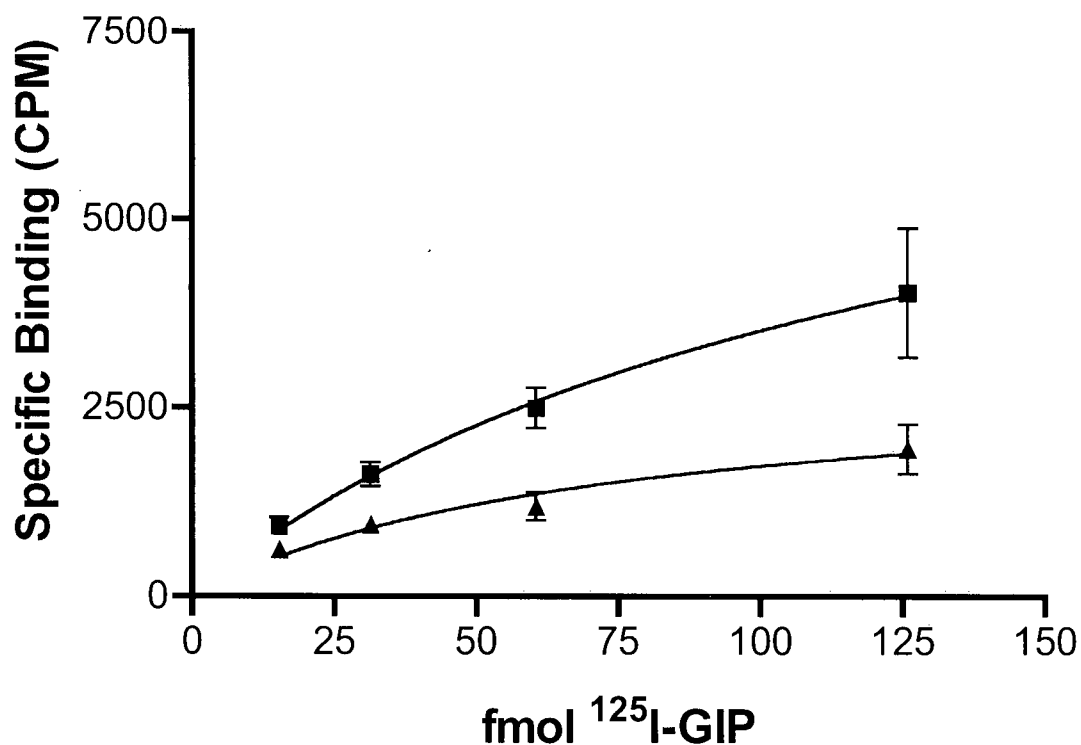
Figure 27: Saturation binding analysis of INS(832/13) cells treated with high glucose. Cells were incubated for 24 hours in either 5.5 mM (squares), or 25 mM (triangles) glucose. Following incubation varying amounts of ^{125}I-GIP was added to the cells and allowed to equilibrate over 4 hours at 4 °C. Cells were then washed, the amount of ^{125}I-GIP was counted and specific binding was calculated. The Y-asymptote at which the curve reaches a theoretical maximum denotes the number of specific GIP binding sites.
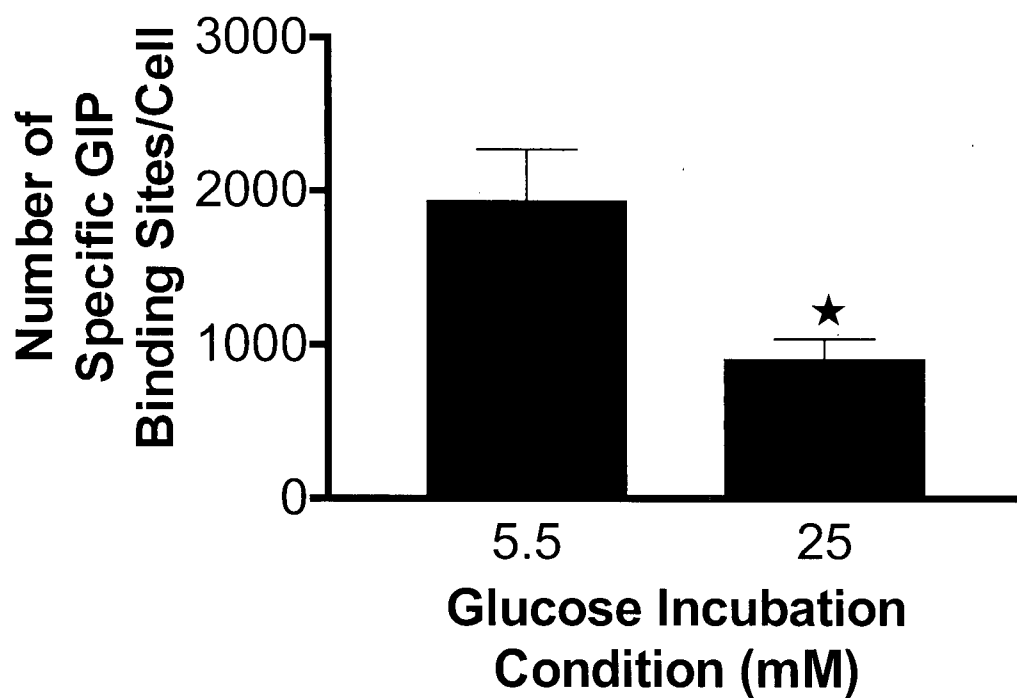
Figure: 28: Total cell surface GIP receptor numbers at 5.5 mM and 25 mM glucose. The theoretical cell surface receptor number was calculated from 4 independent saturation binding analyses carried out on INS(832/13) cells and plotted. The asterisks indicate statistical significance as determined by the two-tailed student's t-test (P<0.05).
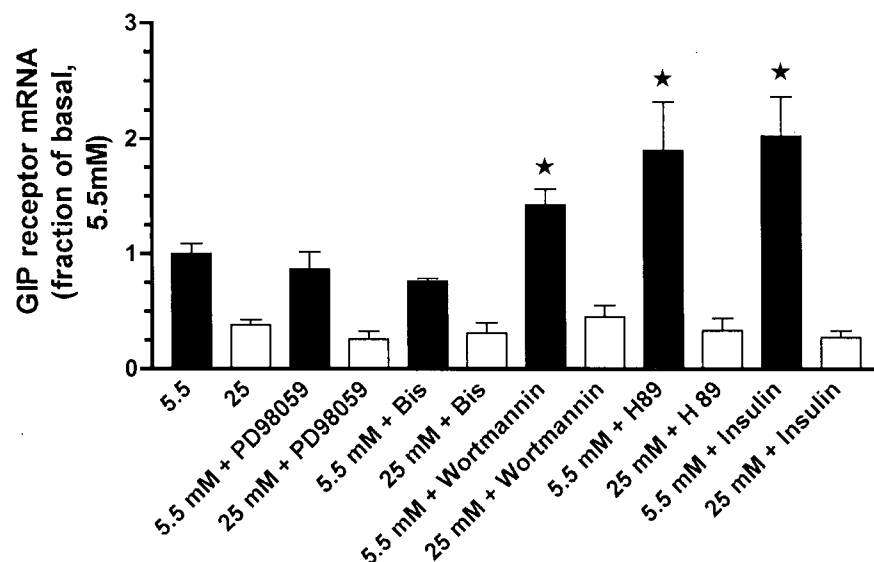
Figure 29: The effect of various inhibitors of cell growth and proliferation on glucose-induced GIP receptor mRNA downregulation in INS(832/13) cells. Cells were grown for 24 hours in either 5.5 or 25 mM glucose in the presence of drugs (5 μM H89, 100 μM PD 98059, 2 μM Bis, 100 nM wortmannin, 1 μM insulin) as described in Chapter 2. Following this incubation period, RNA was harvested and GIP receptor mRNA levels were measured using real-time RT-PCR. Data are expressed as a fraction basal (5.5 mM) conditions, asterisks indicate statistical significance from basal levels P<0.05, n=3.

any significant affect on GIP receptor expression at either glucose concentration (figure 29).

5.6 The Effect of Free Fatty Acids and PPARα Activation on GIPR Expression in Islets, BRIN-D11, and INS(832/13) Cells.

Prior to acquiring the recently developed INS(832/13) cell line, we carried out initial experiments in BRIN-D11 β-cells. As shown in figure 31, incubation of the BRIN-D11 cells in both 2mM palmitate and with the PPARα activator, WY 14643, produced significant increases in GIP receptor levels. Both stimuli produced an approximate 3-fold increase in GIP receptor expression under 5.5 mM glucose conditions. Additionally, incubation of these cells in a medium containing a high fatty acid concentration upregulated the GIP receptor expression at the cell surface as determined by saturation binding analyses. In fact WY 14643 and palmitate also significantly increased GIP receptor mRNA levels in islets isolated from lean Zucker rats as seen in figure 30. Palmitate was a stronger stimulant of receptor transcription in islets, producing an 11-fold increase in GIP receptor mRNA expression, while 100 μM WY 14643 caused a 7-fold increase in receptor expression.

Fatty acids were also capable of increasing GIP receptor expression in the INS(832/13) cells. Figure 32 demonstrates that there was significant induction of GIP receptor transcription after only 4 hours of stimulation with 2 mM palmitate. Furthermore, this upregulation of GIP receptor expression continues through 24 hours, reaching a maximum of approximately 5 times basal levels at 10 hours (figure 32). Figure 34 shows that in the presence of 5.5 mM glucose, 2 mM palmitate significantly increased receptor mRNA levels. Figure 36 illustrates that WY 14643 can increase
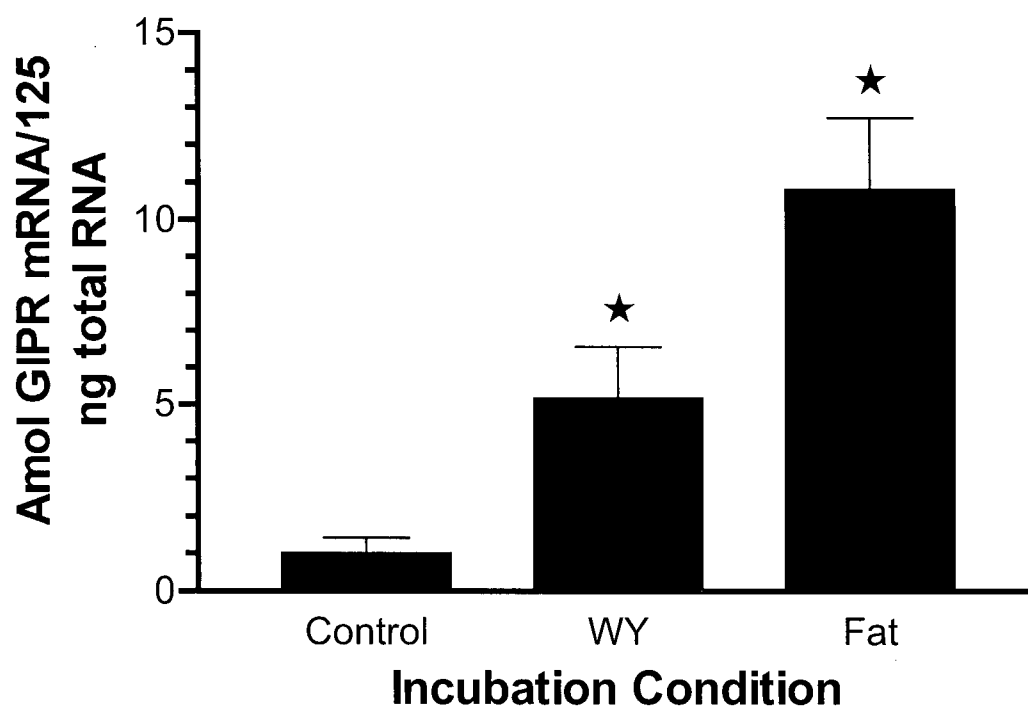
Figure 30: GIP receptor expression in islets following incubation with the PPARα activator WY 14643 (100 μM) or 2 mM palmitate. Islets were isolated from lean Zucker rats and then cultured overnight with 11 mM glucose. Following the recovery period, islets were incubated at 5.5 mM glucose with either WY 14643 or 2 mM palmitate for 8 hours before RNA was harvested. GIP receptor expression was determined by carrying out real-time PCR on total islet RNA. Asterisks indicate statistical significance from control conditions $P<0.05$, n=4.
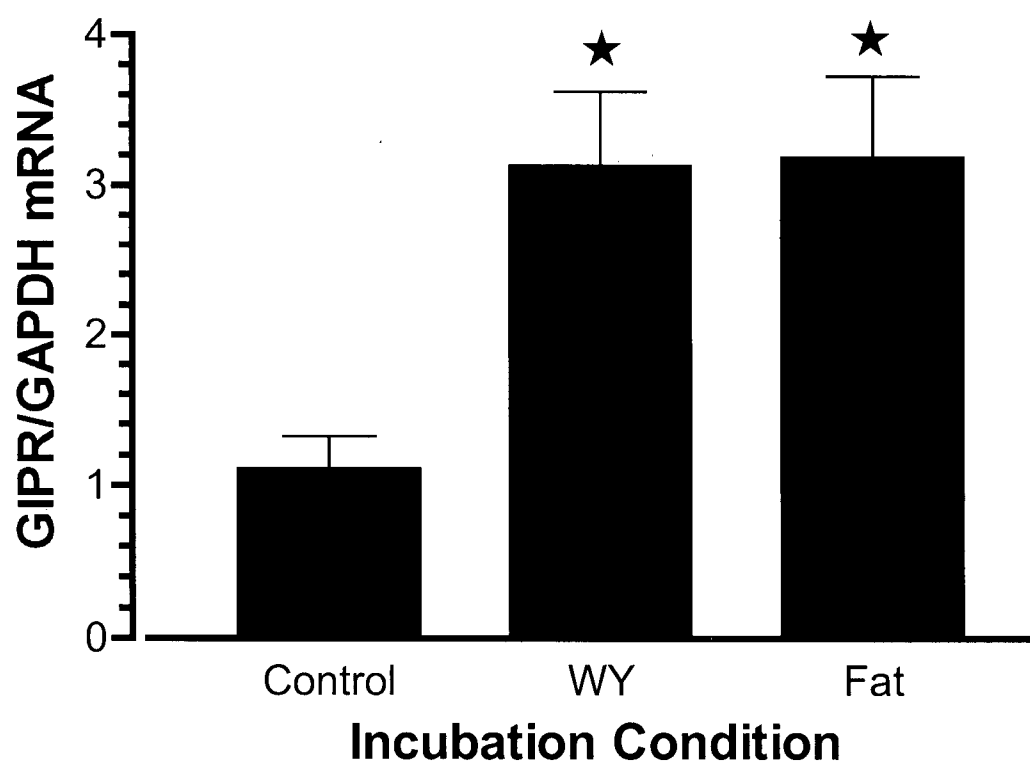
Figure 31: GIP receptor expression in BRIN-D11 cells following incubation with PPARα activator, 100 μM WY 14643 or 2 mM palmitate. BRIN-D11 cells were cultured for 24 hours in the presence of WY 14643 or 2 mM palmitate (Fat). RNA was then isolated and GIP receptor expression was quantified using real-time RT-PCR. GIP levels were normalized to GAPDH mRNA levels. Asterisks indicate statistical significance P<0.05, n=3.
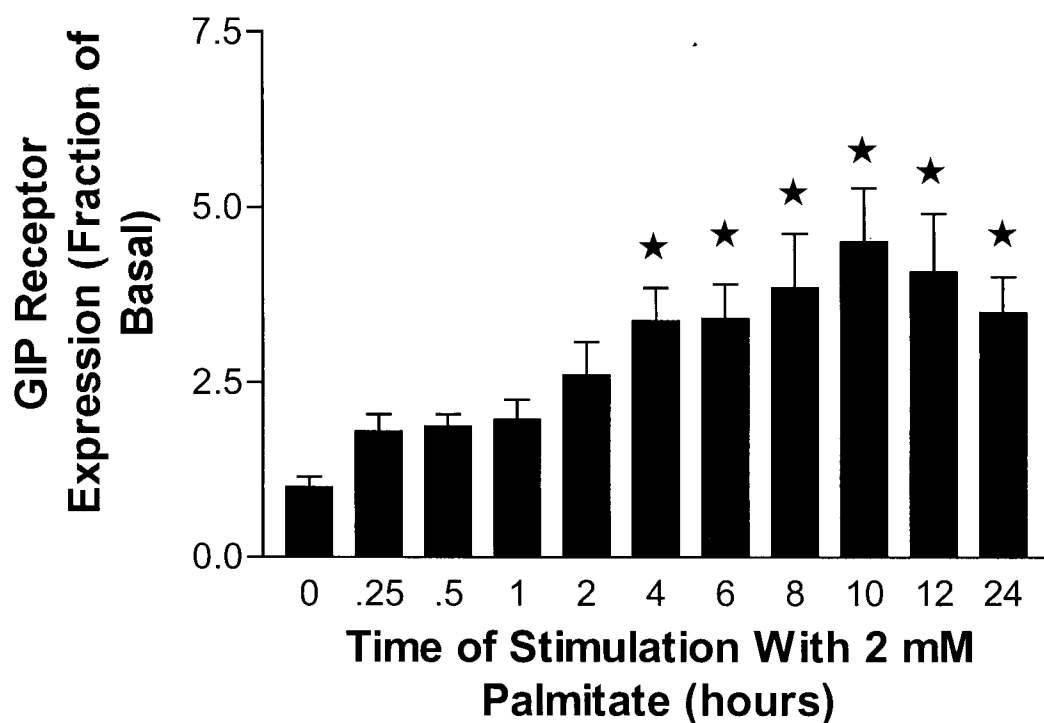
Figure 32: A time-course for palmitate-stimulated induction of GIP receptor expression in INS(832/13) clonal β-cells. Cells were grown in 12 well plates for 24 hours at 5.5 mM glucose before media was changed and cells grown for various times (0-24 hours) in 2 mM palmitate in the presence of 5.5 mM glucose. RNA was then isolated and GIP receptor expression was quantified using real-time RT-PCR. GIP levels were normalized to basal GIP receptor mRNA levels. Asterisks indicate statistical significance (P<0.05, n=4).
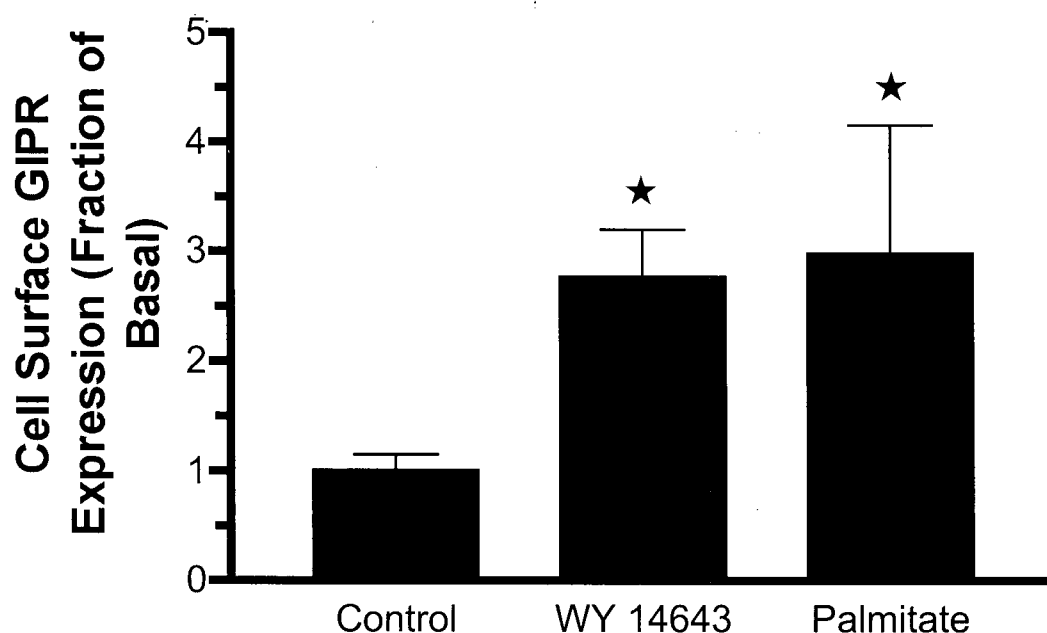
Figure 33: Saturation binding analysis of INS(832/13) cells treated with WY 14643 and 2 mM palmitate. Cells were incubated for 24 hours in 5.5 mM glucose with 100 µM WY 14643 or with 2mM palmitate. Following incubation, varying amounts of ^{125}I-GIP were added to the cells and allowed to come to equilibration over 4 hours at 4 °C. Cells were then washed, the amount of ^{125}I-GIP was counted and specific binding was calculated. The theoretical cell surface receptor number was calculated from 4 independent saturation binding analyses as described in Chapter 2 (Data Analysis) and plotted. The data are expressed as a fraction of basal (5.5 mM) cell surface receptors. The asterisks indicated statistical significance P<0.05.

receptor expression in INS(832/13) cells transfected with the mPPARα-G form of the the transcrition factor. mPPARα-G is a mutant (G282E) form of PPARα with low intrinsic transactivation properties but a higher affinity for WY 14643 and other fibrates than the wild-type form. Thus, both fatty acids and activation of PPARα were able to upregulate GIPR expression in the INS(832/13) cells. Additionally, stimulation of INS(832/13) cells with both WY 14643 and with 2 mM palmitate was able to increase cell surface GIP receptor expression approximately 3-fold (figure 33). Therefore, as in the case of glucose-stimulated downregulation of cell surface GIP receptor expression (figures 27 & 28), induction of GIP receptor mRNA expression is directly linked to an increase in cell surface expression.

5.7 The Interaction Between Fat and Glucose and the Effect on GIP Receptor Expression.

Recently Roduit *et al.* (2000) showed that glucose induced downregulation of PPARα in INS(832/13) cells. We hypothesized that, if GIP receptor expression was under the control of PPARα, then glucose may result in downregulation of the GIP receptor via a decrease in the ability of PPARα to stimulate or maintain the basal level of expression. To test this hypothesis we first incubated INS(832/13) cells in the presence of 2 mM palmitate in varying glucose concentrations. Figure 34 shows that at glucose concentrations higher than 8 mM, palmitate had no effect on GIP receptor expression. Furthermore, at high glucose levels (25 mM), fatty acids were unable to even maintain receptor levels at those seen basally and a significant decrease from basal level was observed. Furthermore, the PPARα antagonist, MK-886 (Kehrer *et al.*, 2001), caused a
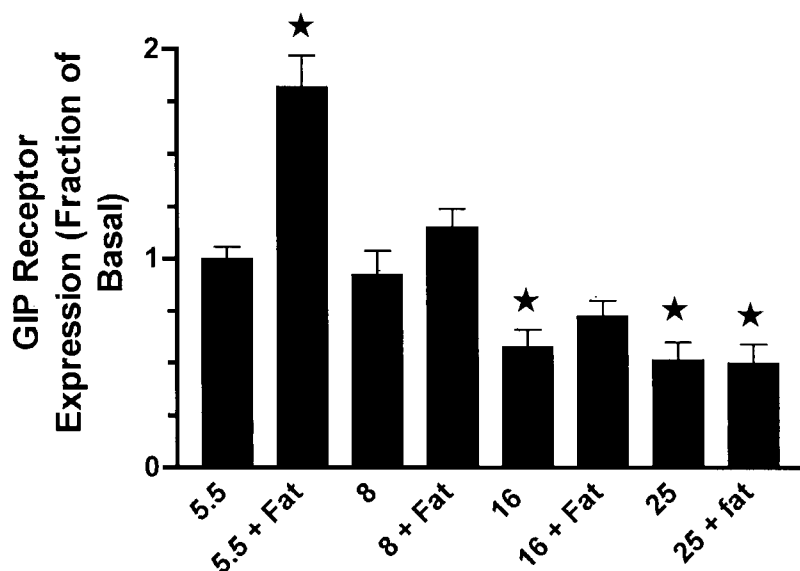
Figure 34: GIP receptor mRNA expression following culture of INS(832/13) cells for 24 hours in various glucose concentrations with 2 mM palmitate. Cells were incubated overnight in 5.5, 8, 16 or 25 mM glucose in the presence or absence or 2mM palmitate (Fat). Following this incubation, RNA was harvested and subjected to real-time PCR for quantification of GIP message. Asterisks indicate statistical significance compared to basal, 5.5 mM conditions $P<0.05$, n=4.

small decrease in GIP receptor expression at low glucose levels; however, it had no effect at levels higher than 8 mM glucose (figure 35).

Finally, transfection of INS(832/13) cells with a high affinity form of PPARα (mPPARα-G) increased GIPR mRNA levels to 1.7 x basal levels in the presence of WY 14643 (figure 36). Transfection of INS(832/13) cells with a dominant negative form of PPARα (Gervois *et al.*, 1999) caused a significant decrease in the expression of the GIP receptor to levels obtained with 5.5 mM glucose, while having no effect at 25 mM glucose. Taken together, these data strongly suggest that PPARα is able to maintain GIPR mRNA levels at low glucose but is ineffective at higher glucose levels.

5.8 Glucose, Palmitate , WY 14643 and Gene Transcription

GIPR mRNA half-life was analyzed because a decrease in the GIPR mRNA degradation rate would lead to an increase in the total amount of GIPR mRNA. This was carried out by incubating the cells with actinomycin D, an agent that intercalates into double stranded DNA and inhibits further nucleic acid synthesis. Figure 37 demonstrates that the high glucose induced downregulation of GIP receptor mRNA was not due to a reduced half-life of GIPR mRNA. The half-lives of the mRNA encoding the GIP receptor were not statistically different at 5.5 and 25 mM glucose (figure 37). Thus, it does not appear that high glucose affected the RNA degradation pathway, and it is likely that there was a decrease in GIPR mRNA synthesis resulting from high glucose levels.

As with the glucose studies, incubation of INS(832/13) cells in high fat or WY 14643 (figure 37) did not affect the degradation of GIPR mRNA. The half-lives of the GIPR mRNA in the cells that were grown in high fat (data not shown) and under control
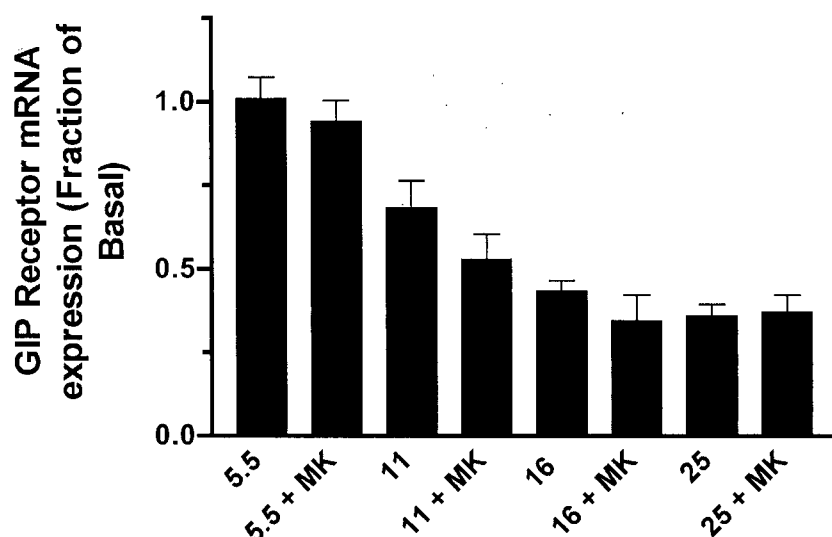
Figure 35: The effect of a specific PPARα antagonist on glucose induced GIP receptor downregulation. Cells were grown in 5.5, 11, 16 or 25 mM glucose in the presence or absence of MK-886, a PPARα antagonist, for 24 hours. RNA was then harvested and subjected to real-time RT-PCR for GIP receptor expression determination. Data are expressed as a fraction of the 5.5 mM condition; n=4.
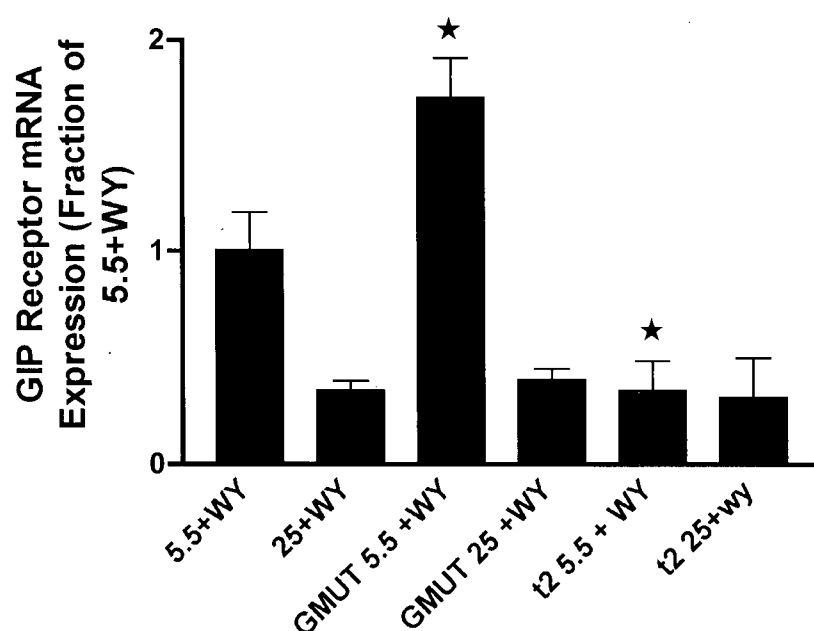
Figure 36: The effect of stimulating or blocking PPARα activity in INS(832/13) cells. Cells were transfected with two mutant PPARα isoforms: either the GMUT (mPPARα-G) form which has an increased affinity for WY 14643 or the hPPARα$_{tr}$ form which is a dominant negative protein as described in research design and methods (page 57). Cells were then grown for 24 hours in the presence of WY 14643 in either high (25 mM) or low (5.5 mM) glucose. RNA was then harvested and GIP receptor expression was quantified using real-time RT-PCR. Asterisks indicate statistical significance of 5.5 mM groups compared to 5.5 + WY 14643 (P<0.05; n=3).
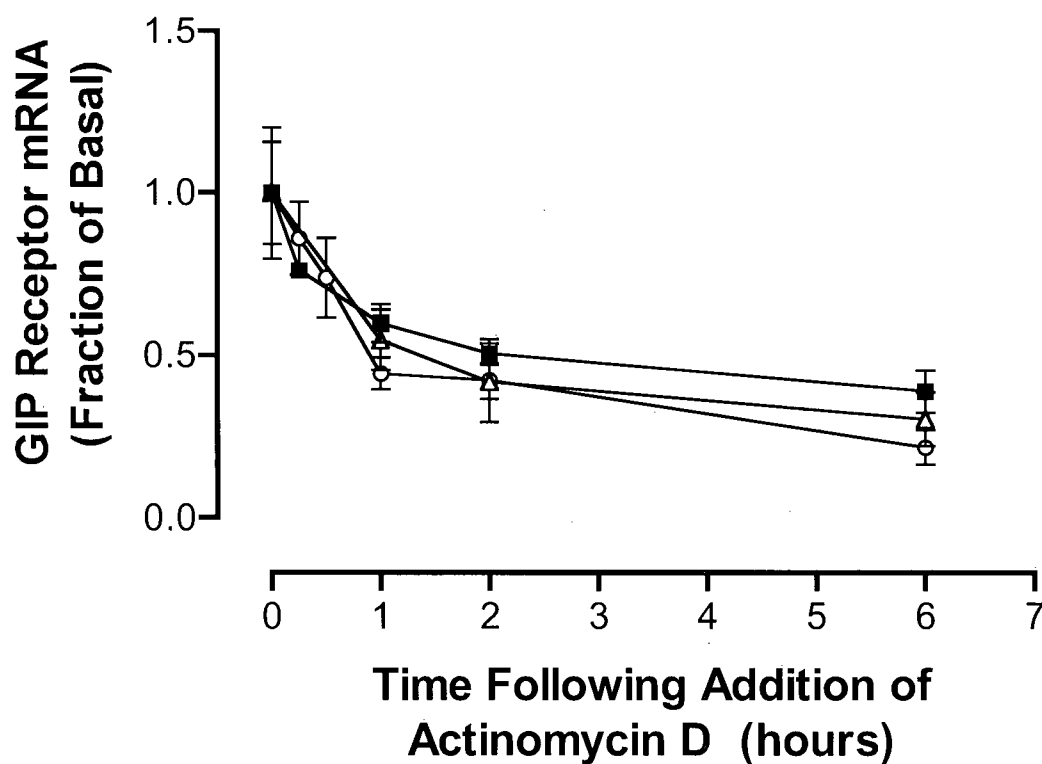
Figure 37: GIP receptor mRNA degradation curves in INS(832/13) cells. Cells were exposed to 5.5 mM glucose (■), 25 mM glucose (△) or WY 14643 (100 μM) (O) for 24 hours prior to the addition of 5 μg/ml actinomycin D. Cells were then allowed to incubate in actinomycin D for varying times between 0 and 6 hours before RNA was harvested and GIP receptor mRNA expression was assessed by real-time RT-PCR. Data are expressed as a fraction of that seen at basal conditions or before addition of actinomycin.

conditions were both approximately 30 min. Therefore, the increase in GIPR mRNA

levels was probably a result of an increase in the transcription of GIPR mRNA.

In addition both 100 μM WY 14643 and 2 mM palmitate stimulated increases in

GIP receptor promoter driven luciferase transcription of 1.4 and 1.7 times basal promoter

activity in INS(832/13) cells (figure 38). Furthermore, basal luciferase activity was

approximately 1.4 times greater than that seen with cells transfected with empty vector;

indicating that the proximal 2 kB of the GIP receptor promoter can actively control gene

transcription in this cell line (data not shown).

5.9 The Effect of Osmolarity on GIP Receptor Expression in INS(832/13) Cells.

When INS(832/13) cells were grown in high D-mannitol, hyperosmolar

conditions mimicking those of hyperglycemia, there was no effect on GIP receptor

expression (figure 39). In addition, it appears as if the INS(832/13) cell response to

hyperosmolarity is a slight, although non-significant, increase in GIP receptor expression

(figure 39). Thus, the increase in osmlarity of high glucose culture has no

downregulatory effect on GIP receptor expression.

5.10 The Effect of Activation of PPARγ on GIP Receptor Expression in INS(832/13) Cells.

There was no significant increase in GIP receptor expression at low glucose levels

when INS(832/13) cells were cultured in ciglitazone, a PPARγ activator (figure 40).

However, the glucose-stimulated downregulation of the GIP receptor was not as profound

as that observed in earlier experiments (c.f. figures 34 and 40). The reason for this is

Stimulation Condition
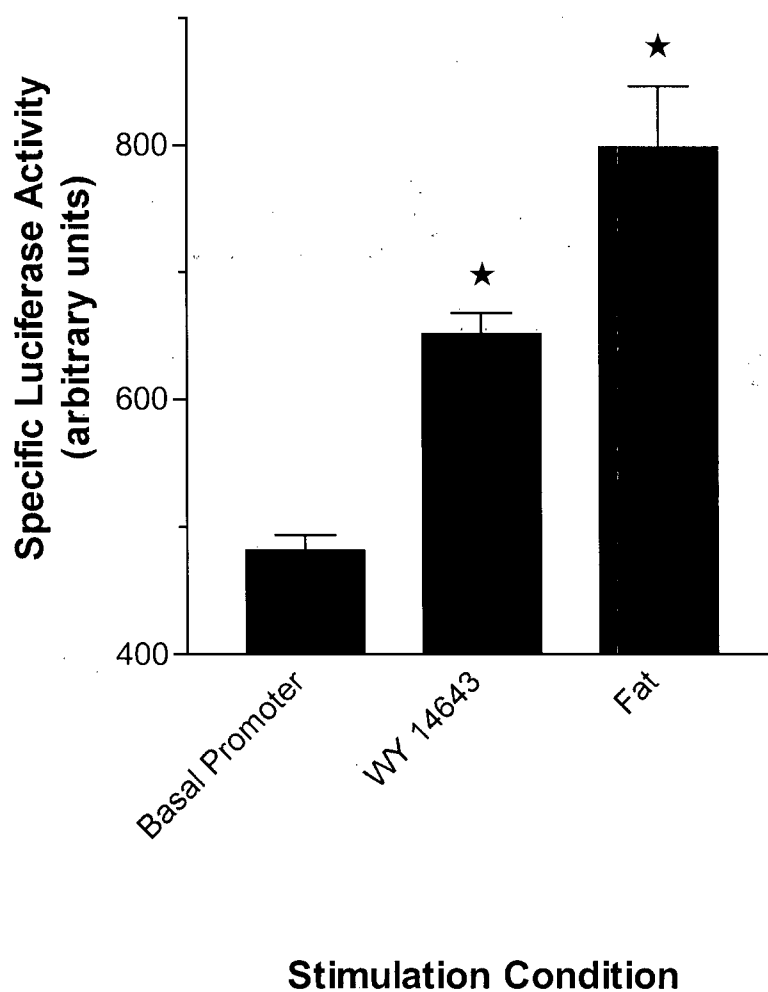
Figure 38: GIP receptor 5' promoter driven luciferase activity in response to WY 14643 and 2 mM Palmitate (Fat) in INS(832/13) cells. The proximal 1960 bp of the 5' GIP receptor promoter were cloned from Wistar Rat liver and inserted into the polylinker of the pGL3 luciferase promoter. INS(832/13) cells were transfected with the promoter construct as described in Chapter 2 and allowed to grow for 24 hours before media was changed and experimental stimuli were applied. Following a further 24 hours of growth medium was removed, fresh medium was added and luciferase activity was determined using the BrightGlo kit (Promega) and a 96 well, Turner Designs luminometer. Data is expressed as arbitrary light units, with asterisks indicating statistical significance from basal activity as determined by ANOVA and Dunnet's post-hoc tests ($p<0.05$, $n=6$).
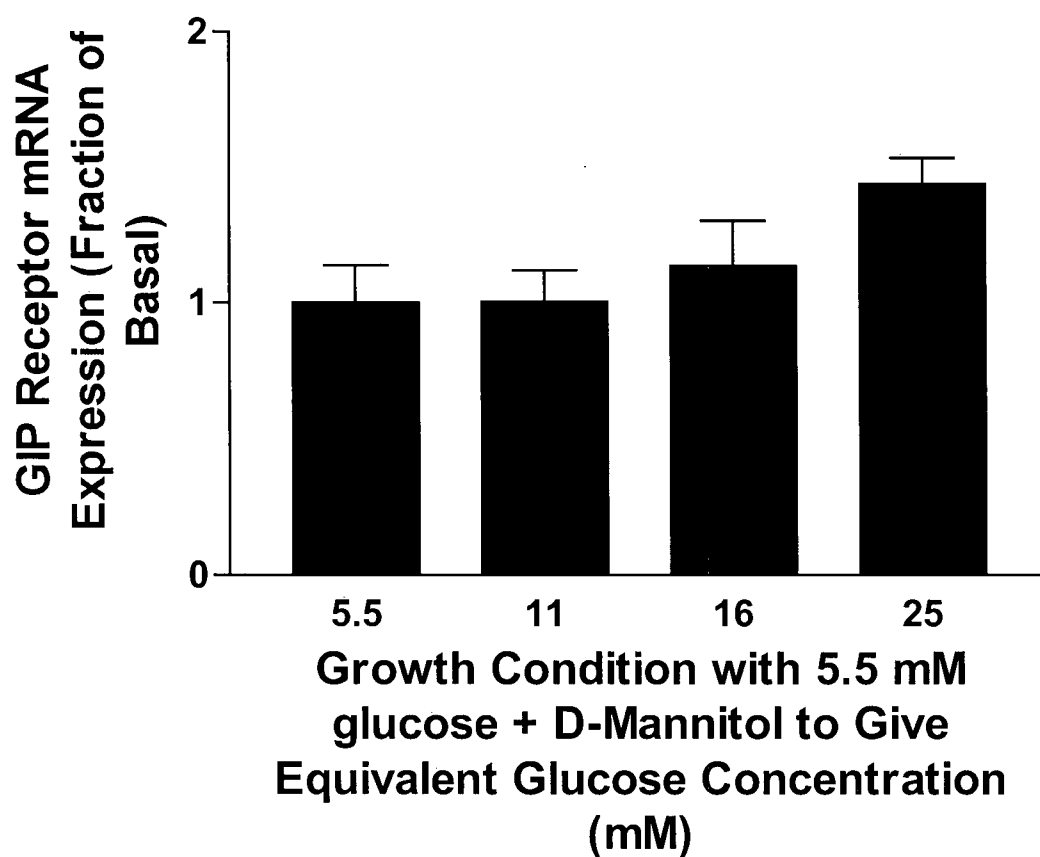
Figure 39: The effect of osmolarity on GIP receptor expression in INS(832/13) clonal β-cells. Cells were grown in 12 well plates for 24 hours in the presence of 5.5 mM glucose supplemented to the givien osmolarity with the non-metabolizable sugar D-mannitol to the given equivalent glucose concentration. RNA was then harvested and subjected to real-time RT-PCR for GIP receptor expression determination. Data are expressed as a fraction of the 5.5 mM condition; n=6.
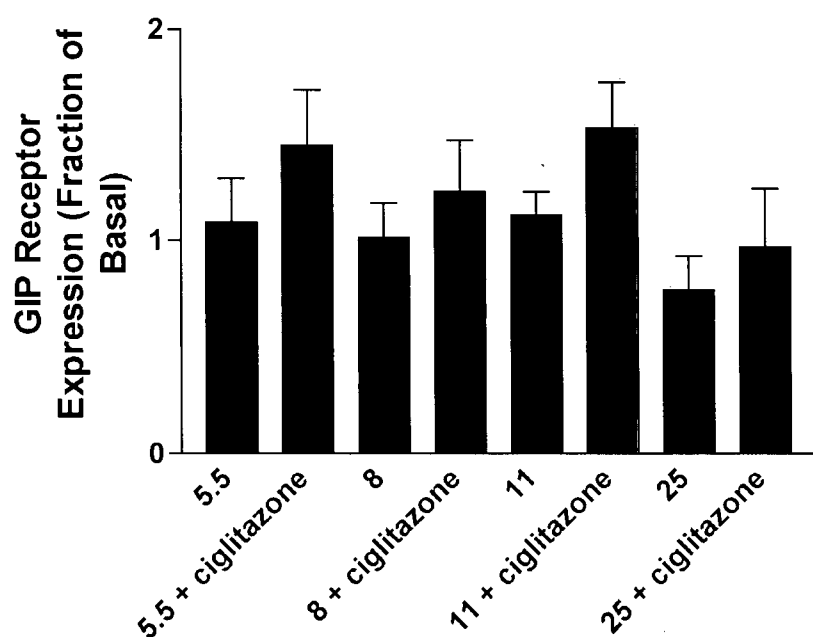
Figure 40: The effect of activation of PPARγ on GIP receptor expression at increasing glucose concentrations in INS(832/13) clonal β-cells. Cells were grown for 24 hours in varying glucose concentrations in the presence of 10 μM ciglitazone: a thiazolidinedione that activates PPARγ. RNA was then harvested and subjected to real-time RT-PCR for GIP receptor expression determination. Data are expressed as a fraction of the 5.5 mM condition; n=4.

unclear but it is possible that the higher passage number (passage 65) of the cells used in these experiments contributed to this effect.

5.11 Discussion

In T2D there is a marked reduction in the insulinotropic potency of GIP. Studies outlined in Chapter 4 demonstrate that the cause of this reduction in potency may be decreased GIP receptor expression on β-cells of the Vancouver diabetic fatty Zucker rat model of T2D. However, currently there are no data to suggest the mechanisms by which GIP receptor downregulation occurs in type 2 diabetic patients or in animal models of the disease. Here we demonstrate that elevated glucose levels are able to significantly reduce GIP receptor expression *in vitro* and *in vivo* and that this effect is not reversed by blocking any of the common cell growth and proliferation pathways. We also demonstrate a novel pathway for stimulation of GIP receptor expression at normal glucose levels through fat-stimulated PPARα activation, which is unable to reverse the GIP receptor downregulation associated with hyperglycemia.

Recently, Roduit *et al.* (2000) and Laybutt *et al.* (2001) demonstrated that high glucose caused downregulation of PPARα in both INS(832/13) cells and in pancreatectomized rats. The time-course for downregulation of PPARα in 20 mM glucose was almost identical to that seen in GIP receptor downregulation studies by high glucose reported here. Where we observed a significant reduction in GIP receptor expression after only 6 hours in high glucose (figure 25), Roduit *et al.* reported a significant and total ablation of PPARα expression at 6 hours (Roduit *et al.*, 2000). Their study also demonstrated that downregulation of PPARα led to a decreased expression of the mRNA for uncoupling protein 2 (UCP2), carnitine palmitoyltransferase 1 (CPT 1)

and acyl-CoA oxidase: genes that all have well defined PPAR response elements in their promoters. Therefore, downregulation of PPARα by glucose can cause a downregulation of genes normally controlled by this nuclear transcription factor. Finally, their paper demonstrated that 0.4 mM oleate had no effect on PPARα expression in INS(832/13) cells in the presence of either high or low glucose levels. Thus, our observation that FFA were unable to increase GIPR mRNA levels under high glucose conditions was likely not a result of downregulation of PPARα by fatty acids but rather because PPARα was downregulated by elevated glucose.

The upregulatory effect of palmitate on GIP receptor expression followed kinetics similar to those observed by Sato *et al.* (2002) in a recent paper in which they studied the time course for induction of various PPARα target genes in response to WY 14643. Here we demonstrated that 2 mM palmitate caused a significant increase in GIP receptor expression with approximately a 4 hour lag time following application of high palmitate containing media (figure 32). Similarily, it was demonstrated that AOX, L-FABP, long-chain acyl-CoA synthetase (LACS), and the peroxisomal bifunctional enzyme are induced in response to WY 14643 in rat hepatoma Fao cells with a 4-6 hour lag time (Sato *et al.*, 2002). Thus, the induction of the GIP receptor observed here fits with the kinetics observed for well-characterized targets of PPARα; therefore, one could speculate that PPARα stimulated transcription of GIP receptor expression occurs in a similar manner to other genes.

To determine if the downregulatory effects of glucose on GIP receptor expression could be attributed to the action of another common signal transduction pathway, various inhibitors of these pathways were applied to cells for 24 hours. As can be seen in figure

29, none of these inhibitors reversed the glucose-induced downregulation of GIP receptor expression. However, a number of interesting observations were made. First, we observed that wortmannin, a PI-3K inhibitor, H89, a PKA inhibitor and insulin all increased receptor expression. Insulin was included as a control because it was observed that under high glucose conditions the amount of insulin in the media was 2.5 times that of basal conditions (figure 23) and it was hypothesized that insulin could be causing the downregulation of the GIP receptor. However, insulin appeared to have the opposite effect. The apparent contradiction between the insulin and wortmannin data could be explained by a desensitization of the insulin signaling pathway in these β-cells by a prolonged, potent stimulation with insulin (Blake *et al.*, 1987; Kulkarni *et al.*, 1999). Thus, we expect that long-term stimulation with insulin probably had much the same functional effect as stimulation with wortmannin. Interestingly, the MAP kinase signaling module has been implicated in the activation of PPARα; and we did see a small but non-significant decrease in the expression of the GIP receptor at 5.5 mM glucose in the INS(832/13) cells that were incubated with the MEK inhibitor PD 98059. These data indicate that an actual decrease in PPARα expression is probably more important than the activation (phosphorylation) state of PPARα in the regulation of GIP receptor expression.

The control of GIP receptor expression by PPARα appears to be limited to low glucose conditions at which point it stimulates an increase in expression. The physiological significance of this is obscure since at low glucose levels GIP does not stimulate insulin secretion. However, in the presence of 0 mM glucose GIP is able to stimulate adenylyl cyclase, resulting in cAMP accumulation (Hinke *et al.*, 2000a) as well as activation of MAP kinase (Ehses *et al.*, 2002) and PLA$_2$ (Ehses *et al.*, 2001) .

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Therefore, it is possible that GIP has functional roles in the β-cell in addition to insulin secretion. Intraduodenal fat is probably the most potent stimulant of GIP secretion from the gut and therefore, it follows that fat should be able to regulate GIP receptor expression. In this manner, stimulation by free fatty acids or long chain acyl-CoA esters (LC-CoA) derived from either the adipocyte during the interdigestive period or early in the prandial process may ready the β-cell for the ensuing glucose stimulation. In addition recent data from our laboratory (figure 24) shows that GIP stimulates fatty acid oxidation within the pancreatic β-cell; thus, GIP may act to prime the β-cell with ATP, using intracellular fat stores. This would allow a more rapid glucose stimulation of insulin secretion.

When glucose levels are high, there is a dramatic and reproducible downregulation of the GIP receptor *in vivo* and *in vitro*; whereas, palmitate has the opposite effect (figures 25 & 26). However, fat is no longer able to induce GIP receptor expression at high glucose levels. This also makes physiological sense if GIP is acting to cause fat oxidation within the β-cell (figure 24) in order to prime the insulin secretory or metabolic pathways for the ensuing meal. It would thus be expected that when glucose levels are high, the β-cell would no longer have a need for GIP-stimulated oxidation of fatty acids. Therefore, expression of the GIP receptor is downregulated and GIP becomes ineffective at high glucose levels. The downregulation occurs quickly with a significant difference seen after only 6 hours in high glucose. This time-course would allow GIP to have an incretin effect on the β-cell, but would limit its actions in prolonged hyperglycemia. Additionally, our group has shown that GIP receptors are quickly internalized in response to GIP with a significant reduction in cell surface receptors

occurring after only 10 minutes of exposure to GIP (Hinke *et al.*, 2000a). Thus, within minutes of GIP receptor activation, bioactivity is probably governed by phosphorylation events but in the hours following, GIP receptor activity is probably controlled by the level of expression of the receptor at the cell surface. Accordingly, due to the chronic hyperglycemia in type 2 diabetic individuals, GIP receptor levels are decreased.

Interestingly, these data fit with the hypothesis put forward by Prentki *et al.* (1997) which suggest that glucose seems to positively regulate expression of genes involved in its metabolism and negatively regulate genes involved in metabolism of other fuels. In view of that, free or non-esterified fatty acids stimulate expression of genes involved in their metabolism such as CPT-1 (Assimacopoulos-Jeannet *et al.*, 1997). Thus, GIPR expression seems to be regulated in a manner that is consistent with other metabolic genes within the β-cell. In addition, one pathway by which GIP could stimulate β-cell function and cytoprotection could be by decreasing fatty acid levels within the cell, thereby preventing lipotoxicity.

It has been demonstrated that both palmitate and WY 14643 stimulate transcription of the GIP receptor gene as opposed to increasing the half-life of GIP receptor mRNA within the cell (figures 37 & 38). However, it is not known whether the action of PPARα on GIP receptor expression is a direct effect or if it occurs via activation of other TF. For example, Schinner *et al.* (2002) recently demonstrated that activation of PPARγ inhibits glucagon expression in the α-cell by inhibiting Pax6 transcriptional activity. The pancreatic β-cell also expresses Pax6 and it could conceivably interact with PPARα to induce receptor expression. Further studies using

the recently cloned GIP receptor promoter need to be carried out to determine the exact sequence elements that control the fatty acid stimulated increase in receptor expression.

In conclusion, the current studies have demonstrated a novel pathway by which glucose and fat can control GIP receptor expression in both clonal β-cells and under *in vivo* conditions. We found that free fatty acids were able to bind to and activate PPARα at low glucose conditions and stimulate GIP receptor transcription either directly or indirectly. However under high glucose conditions, PPARα itself is downregulated and is no longer able to maintain basal GIP receptor expression. These results may account for the downregulation of the GIP receptor that is observed in the hyperglycemic, hyperinsulinemic VDF model of T2D and may underlie the decreased responsiveness of type 2 diabetic patients to GIP.

Chapter 6: Glucose-Induced GIP Receptor Downregulation in the Lean VDF Rat

6.1 Background

The conclusions of the previous two chapters were that: 1) GIP receptor expression is decreased in the VDF model of T2D, and 2) Hyperglycemia may result in downregulation of the GIP receptor by decreasing expression of PPARα. Additional observations such as: the rapid time course of GIP receptor downregulation (6 hours), the degree to which GIP receptor expression was decreased (~ 75 %) and the mild hyperglycemic levels needed to stimulate a downregulation of the GIP receptor all led us to hypothesize that if we were able induce hyperglycemia in normal animals, we should be able to mimic the conditions that we observed in the VDF animals.

Hyperglycemic clamps were utilized to achieve this end. Anesthetized lean animals from the VDF colony were maintained at either a mild level of hyperglycemia (10 mM) or a severe level of hyperglycemia (25 mM) for 6 hours. Following this treatment, the animals were tested for either insulin secretory capacity in response to GIP using pancreas perfusions or the levels of GIP receptor mRNA were quantified using rPCR. The hypothesis to be tested was that both GIPR mRNA and GIP-stimulated insulin secretion would be attenuated in a dose-dependent manner by hyperglycemia in the clamped animals.

6.2 Glucose-Induced Downregulation of the GIP Receptor in Hyperglycemic Clamped Rats

Hyperglycemic clamps were performed on lean Zucker rats to determine if hyperglycemia was able to downregulate GIPR expression *in vivo*. Figure 41 demonstrates that islets of rats clamped at 25 mM glucose expressed only 33 ± 7 % the GIPR mRNA level seen in 5.5 mM clamped animals. Those animals that were glucose clamped at 10 mM also showed a significant reduction in GIP receptor expression of 60 ± 15 % of levels observed in 5.5 mM clamped animals.

Concomitant with reduced GIP receptor expression, there was a reduction in GIP stimulated insulin secretion from the perfused pancreata of animals clamped at 25 mM (figure 42). This is reflected as a 71 % reduction in the area under the curve in the treated animals as seen in the inset of figure 42. These data establish a causal link between GIPR levels and islet sensitivity to GIP.

6.3 Discussion

Data presented in the previous chapter demonstrated that GIPR expression is controlled reciprocally by the ambient glucose concentration in neoplastic clonal β-cells. However, the expression of genes in cell lines is often quite different from their expression *in vivo* and here we set out to determine if GIPR expression was controlled in the whole animal in a similar manner. As indicated in figure 41 we found that expression of the GIP receptor was decreased in animals that were subjected to hyperglycemic clamps. The GIPR downregulation was dose-dependent and resulted in a decrease in insulin secretion from the pancreata of these animals. Interestingly, the degree of downregulation in the pancreata from animals that were clamped to 25 mM glucose was
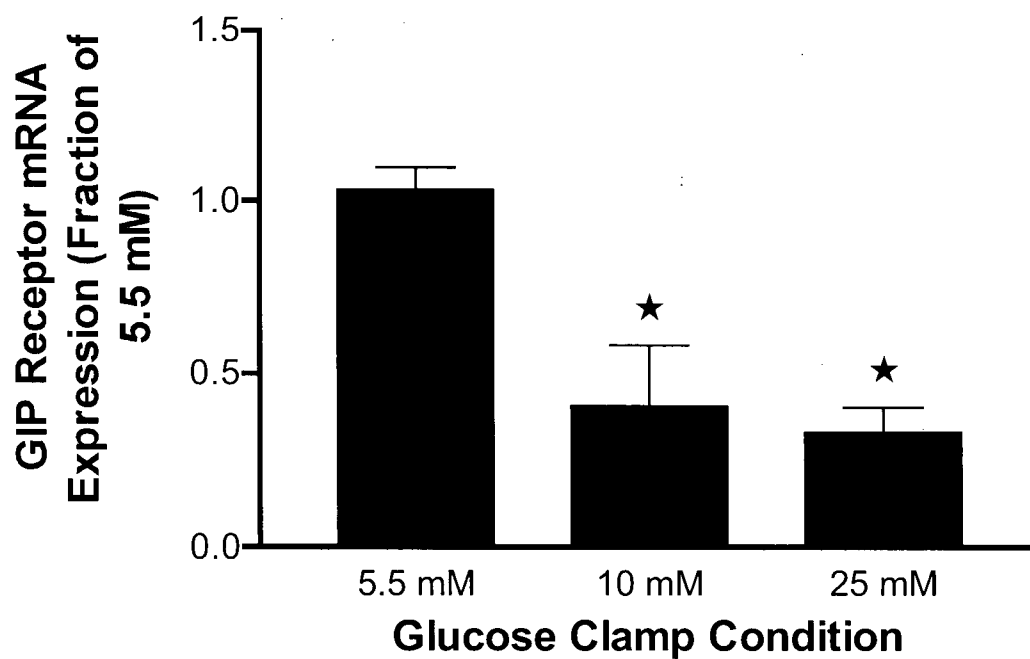
Figure 41: The effect of hyperglycemic clamping on GIP receptor expression in islets of lean Zucker rats. Plasma glucose levels of anesthetized lean Zucker rats were clamped at either 5.5, 10 or 25 mM glucose for 6 hours. Islets were then harvested at the clamped glucose concentrations and RNA was isolated for subsequent real-time RT-PCR. The inset depicts area under the curves (AUC) for the perfusion time interval in which GIP was included in the perfusate (10-30 min). Asterisks indicate statistical significance compared to basal conditions (P<0.05; n=3).
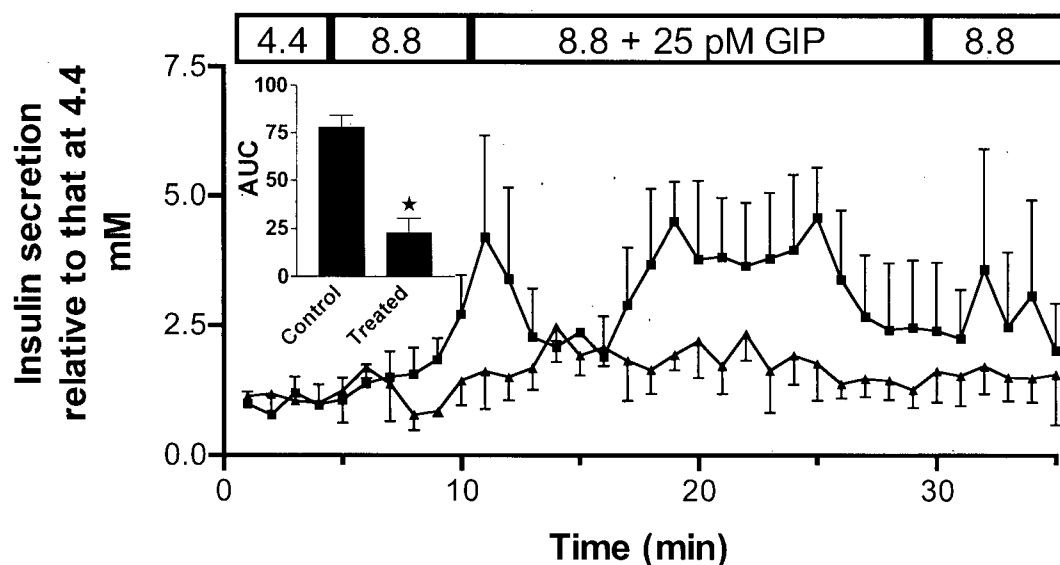
Figure 42: The effect of hyperglycemic clamp on GIP stimulated insulin release from the perfused lean Zucker rat pancreas. Lean animals were clamped at 5.5 (■) or 25 (Δ) mM glucose for 6 hours prior to pancreatic perfusion with the protocol outlined in the figure and in the research design and methods section. Insulin secretion is expressed as a fraction of that seen in the average of the first 5 minutes of the perfusion. Asterisks indicate statistical significance (P<0.05; n=3).

identical to the downregulation observed in the VDF animals (65 % decrease, figure 16). This is not surprising when the blood glucose concentrations in the VDF animals are examined. In the fasted state, a condition that would almost never occur in these obsese animals, the blood glucose levels are approximately 8 mM (Pederson *et al.*, 1998b; Pospisilik *et al.*, 2002). Furthermore, the genetic defect that these animals carry is a disruption in the leptin receptor. One of the results of a defect in the leptin signaling system is an inability to control nutrient intake. Thus, they are hyperphagic and the blood glucose levels in these animals rarely drop below approximately 10 mM and are often as high as 20 mM (Pederson *et al.*, 1998b; Pospisilik *et al.*, 2002). Therefore, a glucose clamp to 25 mM is within the range of plasma glucose expected in the VDF rat, and the degree of GIP receptor downregulation observed during these clamps should be similar to those observed in this animal model.

Additionally, figure 41 illustrates that the GIPR expression is significantly downregulated (40 % of control levels) with hyperglycemic clamps of only 10 mM: 5 mM above fasting levels in these lean animals. Furthermore, there is no statistical difference between the degree of downregulation observed with the 10 mM hyperglycemic clamp and the 25 mM hyperglycemic clamp. Therefore, this lends credence to the hypothesis that glucose could account solely for the downregulation observed in the VDF animals.

There was significant insulin secretion from the pancreata of control animals in response to GIP; however, the hyperglycemic clamped animals did not respond to GIP. This produced a significant difference (3 –fold) in the area under the curve for the two conditions for the perfustion interval between 10 and 30 minutes (figure 42). In

conclusion, both the GIP receptor level and the GIP-stimulated insulin secretory profile can be blunted by a 25 mM hyperglycemic clamp in lean Zucker animals. Furthermore, the levels of downregulation observed following glucose clamping are similar to those observed in the diabetic VDF rats; thus, hyperglycemia may be the primary factor resulting in GIP receptor downregulation in T2D.

Chapter 7: Glycosylation of the GIP Receptor, the Effect of Glycosylation on Cell Surface Expression and Insulin Secretion

7.1 Background

The initial biochemical characterization of the GIP receptor utilized ^{125}I-GIP crosslinking experiments to determine that the hamster β-cell GIP receptor was a protein with an apparent molecular weight of 59 kDa (Couvineau et al., 1984). Further studies demonstrated that dithiothreitol was able to reduce the electrophoretic mobility of the GIP receptor without effecting GIP binding indicating the presence of a disulfide bond (Amiranoff et al., 1986). These authors also demonstrated that the GIP receptor- ^{125}I-GIP complex could be adsorbed by wheat germ agglutinin and concanavalin A coupled to sepharose beads. This interaction could be specifically reversed indicating for the first time that the GIP receptor was a glycoprotein (Amiranoff et al., 1986).

More recently, cloning of the GIP receptor has shed more light on the degree of glycosylation that occurs on the GIP receptor. Studies in the mid-1990's indicated that the mature hamster GIP receptor protein contains 462 amino acids with a predicted molecular weight of approximately 52 kDa (Yasuda et al., 1994). Cloning studies in other animals indicate that the predicted molecular weight of the GIP receptor in all species is approximately 50 kDa (Gremlich et al., 1995; Wheeler et al., 1995). The difference between the predicted molecular weight and the actual electrophoretic size of the protein lends support for the indirect observation by Amiranoff et al. (1986) that the protein is a glycoprotein.

Wheeler *et al.* (1995) reported four asparagine (N) consensus sites for glycosylation within the sequence of the GIP receptor: N59, N69, N74 and N200. These four sites all fall on predicted extracellular regions of the receptor: three on the amino-terminal tail of the receptor and 1 on the first extracellular loop. However, the role of any of these sites in actual glycosylation of the GIP receptor has never been examined. Additionally, there has never been any direct evidence directed at proving that the receptor is glycosylated or what the effects of this glycosylation are on receptor structure, expression or function.

The glycosylation of the secretin, VIP, GLP-1, and gastrin-releasing peptide receptors has been demonstrated to affect the cell surface expression of the proteins (Benya et al., 2000; Couvineau et al., 1996; Göke et al., 1994; Pang et al., 1999). In addition, all of the receptors that have been examined to date have at least two glycosylation sites, which are located on the extracellular N-terminus or in the first extracellular loop. Removal of these sites by mutation decreases the ability of hormone to bind to the receptor because the receptor is not delivered to the plasma membrane or is incorrectly folded. For example, Couvineau *et al*, (1996) demonstrated that N58, N69 and N100 of the VIP receptor were all glycosylated; however, only mutation of all three sites affected delivery of VIP receptor to the cell surface and with this mutant receptor protein was retained in the perinuclear endoplasmic reticulum. In addition, they demonstrated that the 9 kDa carbohydrate residue at N58 was involved in the calnexin-dependent folding of the VIP receptor, but N69 was not. Others groups have demonstrated that receptor glycosylation does not seem to be necessary for delivery to the cell surface; however, it may increase the efficiency of the delivery of receptors to the

plasma membrane (Göke et al., 1994; Lanctot et al., 1999; Pang et al., 1999). Thus, glycosylation of GPCRs is important for optimal folding as well as delivery to the cell surface.

Because there is a dysregulation of glucose homeostasis within the β-cell of type 2 diabetics it is conceivable that there could be a defect in the glycosylation machinery within the cell. In fact, it has been demonstrated that glycation of insulin can occur in T2D, and that this leads to decreased biological activity of the hormone (Abdel-Wahab *et al.*, 1997). Furthermore, there has been a recent explosion in the amount of literature exploring the effects of advanced glycosylation end product (AGE) accumulation via non-enzymatic glycosylation in T2D: much of the research pointing to pathologic effects caused by the deleterious effect of adding sugars to the extracellular matrix (Brownlee, 1995). The following studies were designed to test the hypothesis that GIP receptor glycosylation is necessary for correct cell surface receptor expression and GIP binding. In addition, it is hoped that by understanding the role of glycosylation in GIP binding, signaling and cell surface expression, the potential role of inappropriate glycosylation in T2D could be predicted. It is hypothesized that glycosylation is necessary for the correct expression of the GIP receptor on the cell surface, as well as in binding and signaling.

To test this hypothesis four potential asparagine (N)-linked glycosylation sites (N 59, 69, 74, 200) in the extracellular amino-terminus and the first extracellular loop of the receptor were mutated to threonine residues using site-directed mutagenesis, generating 8 mutants. These mutants were fully sequenced, and expressed in HEK-293 cells, which have been shown to utilize complex glycosylation pathways. Furthermore, the effect of

glycosylation of the GIP receptor on insulin secretion from INS(832/13) cells was examined by treatment of the cells with tunicamycin (an inhibitor of glycosylation).

7.2 The ^{125}I-GIP Competitive Binding and Signaling Properties of the Glycosylation Site GIP Receptor Mutants

To determine whether the glycosylation sites were involved in expression of the protein at the cell surface, competitive binding and cAMP production analyses were carried out. When transfected into HEK cells, all of the mutant receptor proteins were able to bind GIP with an affinity in the near physiological range. The wild-type tagged GIP receptor (WTtag) had an IC_{50} of 3.56 ± 1.1 nM. The affinity of the WTtag receptor for GIP was found to be approximately 10-fold greater than the affinity observed in β-cell lines (Chapter 5) but similar to affinities of the non-tagged GIP receptor transfected into other cell lines (Wheeler *et al.*, 1995). There were minor differences in the affinities of the single glycosylation site GIP receptor mutants for GIP. The N59T mutant had an IC_{50} of 2.57 ± 1.4 nM, the N69T mutant had an IC_{50} of 6.16 ± 1.2 nM, the N74T mutant had an IC_{50} of 5.63 ± 2.4 nM and the N200T mutant had an IC_{50} of 5.51 ± 1.1 nM (figure 43A). All of these mutants had reduced maximal binding from the WTtag receptor, which by definition had a calculated maximal binding (B/Bo) of 100 ± 2 %. The N59T mutant had a maximal binding of 81 ± 3 %, the N69T of 71 ± 2 %, the N74T of 51 ± 4 % and the N200T of 81 ± 2 % (figure 43A). The maximal binding is related to the amount of receptor at the cell surface and since the transfection efficiency was equivalent in all of the transfections; thus, the WTtag mutant was most highly expressed followed by the N59T= N200T >N69T>N74T.

A
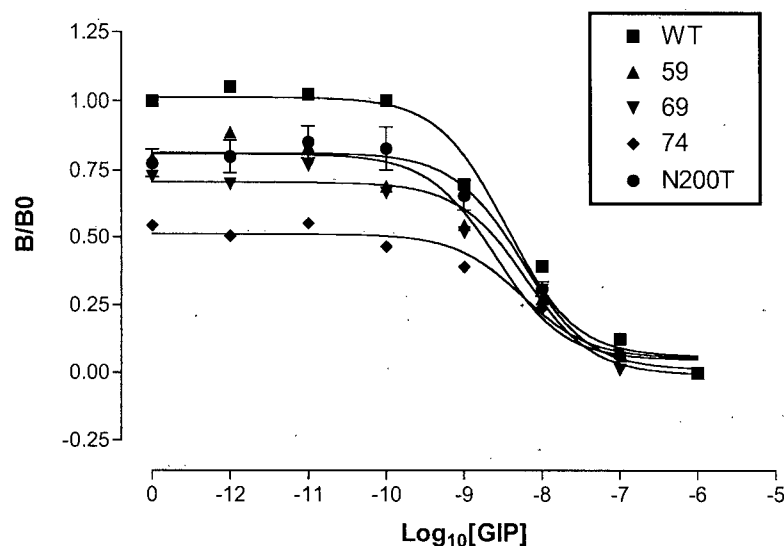
B
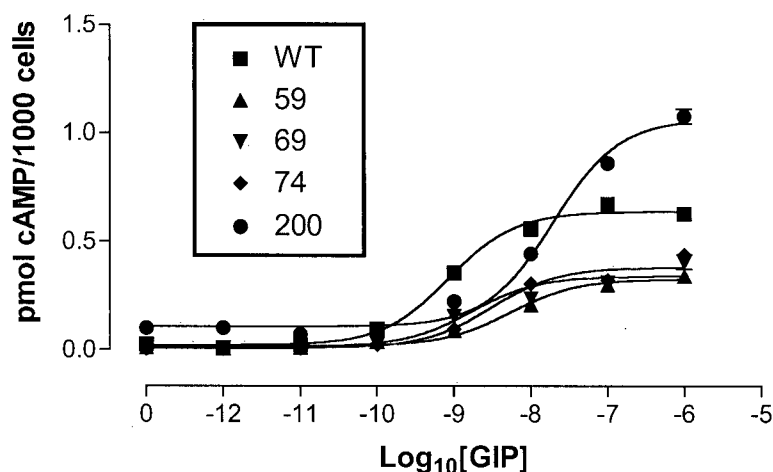
Figure 43: GIP binding (A) and cAMP production (B) by single site glycosylation mutants transfected into HEK cells. Single site mutants were constructed as described in Chapter 2.19. Mutant receptor DNA (2.5 μg) was then transfected into HEK 293 cells using Lipofectamine 2000, and cells were plated in 24 well plates. Following 48 hours of growth, cells were washed and incubated with (A) various concentrations of GIP at 4 °C for 4 hr in the presence of a constant amount of ^{125}I-GIP. Cells were then washed, and the amount of radiolabel was determined. Data are expressed as percent total binding of WTtag GIPR (mean ± S.E.M., n=4). (B) Here cells were incubated with various amounts of GIP for 30 min at 37 °C. Cells were then solubilized with 70 % ethanol and the cAMP production was determined using radioimmunoassay. Data are expressed as mean ± S.E.M (n=4).

The cAMP production by the single mutants was also determined (figure 43B). The half maximal concentration of GIP or EC_{50} values for cAMP production for all the mutant proteins were slightly right shifted from the WTtag receptor value of 0.86 ± 0.5 nM (figures 43B and 44B). Furthermore, all the mutants had significantly decreased maximal cAMP stimulating potency when compared to the WTtag receptor with the exception of the N200T mutant (figures 43B and 44B). The EC_{50} values for cAMP production by the single glycosylation site mutants were: N59T = 5.44 ± 1.0, N69T = 1.70 ± 1.5 nM, N74T = 3.08 ± 1.4 nM and N200T = 19.5 ± 1.2 nM. The maximal cAMP production from the WTtag receptor was 0.64 ± 0.01 pmol cAMP/1000 cells. The maximal cAMP production by the N59T mutant was 0.33 ± 0.01 pmol cAMP/1000 cells, 0.34 ± 0.02 pmol cAMP/1000 cells for the N69T mutant, 0.38 ± 0.02 pmol cAMP/1000 cells for the N74T mutant and 1.06 ± 0.04 pmol cAMP/1000 cells for the N200T mutant (figure 43B). These maximal cAMP levels were all lower than the WTtag receptor, with the exception of the N200T mutant. The multiple glycosylation site mutants were also tested for GIP binding and GIP stimulated cAMP production (figure 44). All of these mutants bound GIP with significantly decreased maximal binding than the WTtag receptor. The N59/69T double mutant displayed 22 ± 2 % WTtag binding, the N59/74T double mutant displayed 10 ± 1 % WTtag binding, the N69/74T double mutant displayed 28 ± 6% WTtag binding and the N59/69/74T (567) triple mutant displayed 25 ± 4 % WTtag maximal binding. However, the affinities of each of these mutant receptors for GIP did not differ dramatically from the WTtag receptor. These mutants had the following IC_{50} values for ^{125}I-GIP displacement by unlabelled GIP: N59/69T = 14.3 ± 3

nM, N59/74T = 2.28 ± 2.4 nM, N69/74T = 12.3 ± 4 nM and N59/69/74T = 76.7 ± 10 nM.

The maximal cAMP production values for the double mutants also differed from the WTtag receptor (0.64 ± 0.01 pmol cAMP/1000 cells). The N59/69T mutant had a maximal cAMP production/1000 cells of 24 ± 0.2 pmol, the N59/74T mutant had a maximal cAMP production/1000 cells of 22 ± 0.8 pmol, the N69/74T mutant had a maximal cAMP production/1000 cells of 14 ± 0.4 pmol and the triple mutant had a maximal cAMP production/1000 cells of 11 ± 0.1 pmol. The EC_{50} values for GIP stimulated cAMP production for the mutiple glycosylation site mutants were right-shifted from the WTtag mutant (0.86 ± 0.5 nM) with the N59/69T, N59/74T, N69/74T and the N59/69/74T mutants having values of 3.62 ± 0.5 nM, 3.76 ± 1.2 nM, 8.76 ± 1.4 and 86.2 ± 5 nM respectively.

7.3 THE EFFECTS OF MUTATION OF GIP RECEPTOR GLYCOSYLATION SITES ON RELATIVE ELECTROPHORETIC MOBILITY.

From the previous series of experiments it is clear that the glycosylation site mutants of GIP receptors were expressed on the cell surface to differing degrees. To determine if this was due to a difference in the glycosylation state of the receptors, electromobility shift assays (EMSA)s were carried out with samples of affinity purified GIP receptor that had been treated with PNGase F: an amidase that cleaves between the innermost N-Acetylglucosamine (GlcNAc) and the asparagine residues of high mannose, hybrid and complex oligosaccharides in N-linked glycoproteins. When the WTtag receptor was western blotted with the mouse anti-V5 tag antibody two bands appeared in the wild-type, non PNGase F treated extract. The major band ran with a molecular mass
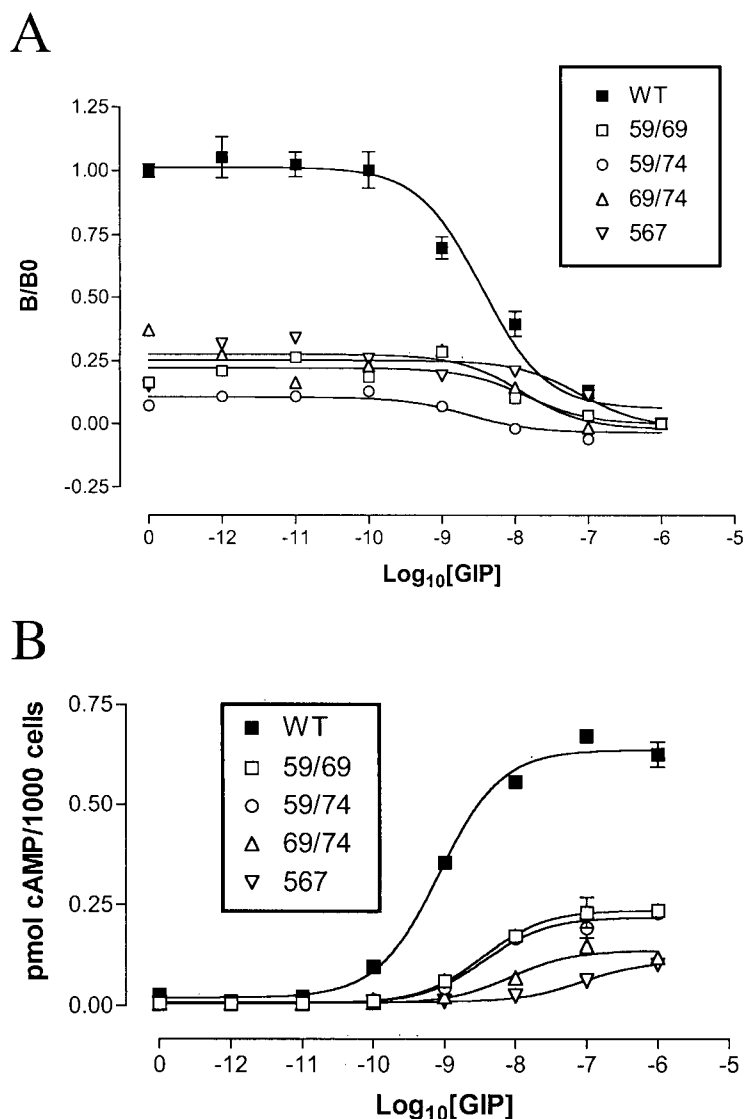
Figure 44: GIP binding (A) and cAMP production (B) by multiple site glycosylation site mutants transfected into HEK 293 cells. Multiple glycosylation site mutants were constructed as described in Chapter 2. Mutant receptor DNA (2.5 μg) was then transfected into HEK 293 cells using Lipofectamine 2000, and cells were plated in 24 well plates. Following 48 hours of growth, cells were washed and incubated with (A) various concentrations of GIP at 4 °C for 4 hr in the presence of a constant amount of ^{125}I-GIP. Cells were then washed, and the amount of radiolabel was determined. Data are expressed as percent total binding of WTtag GIPR (mean ± S.E.M., n=4). (B) Here cells were incubated with various amounts of GIP for 30 min at 37 °C. Cells were then solubilized with 70 % ethanol and the cAMP production was determined using radioimmunoassay. Data are expressed as mean ± S.E.M (n=4).

of 68 ± 3 kDa, while a minor band was detected at approximately 58 ± 2 kDa. The identities of the first band was presumed to be fully glycosylated GIP receptor and the second, less dense band was thought to be incompletely glycosylated or immature GIP receptor. It is hard to distinguish the difference between these because whole cell membrane and not plasma membrane preparations were utilized. When the WTtag GIP receptor was treated with PNGase F there was a shift in the mobility of the band to approximately 51 ± 2 kDa (figure 45). There was only one band observed with no smaller bands present. The major band from the N59T mutant ran with an apparent molecular weight of approximately 63 ± 3 kDa. However, there were two other bands observed in this lane; one that ran at 56 ± 2 kDa and another that ran with a similar mobility to the digested WTtag protein. When the N59T mutant affinity purified protein was treated with PNGase, the major band also appeared at 49 ± 1 kDa. The non-digested N69T mutant protein ran with an electrophoretic mobility similar to the N59T mutant with the major band running with an apparent molecular mass of 64 ± 3 kDa, and one minor band appearing at 56 ± 2 kDa. Furthermore, when this protein was digested with PNGase F, two bands also appeared: the major one at 49 ± 1 kDa and the second faint band around 51 kDa. The N74T His-tag purified proteins ran with an apparent molecular mass of 68 ± 3 kDa, with a minor band in the non-digested extract with an apparent mobility of 56 ± 2 kDa. When this extract was digested with PNGase F, the apparent molecular weight of the single band was the same in all the extracts and ran at 52 ± 3 kDa (figure 45). The N200T mutant was also analyzed for gel shift in response to PNGase F treatment. The apparent molecular weights of this mutant were similar to that of the
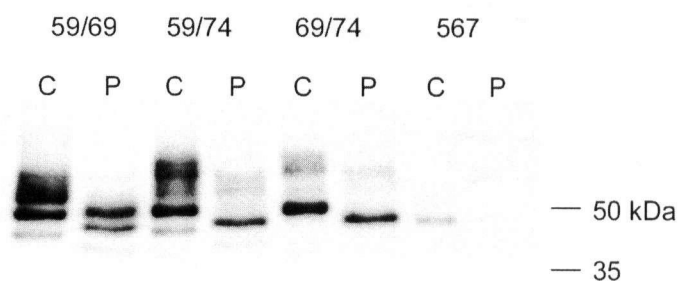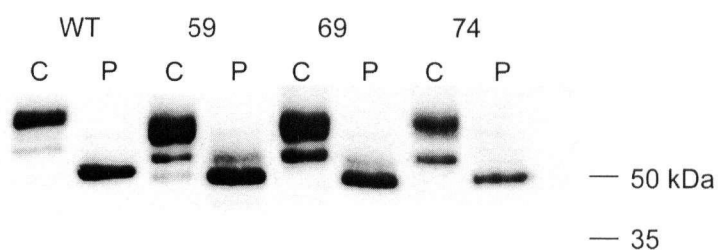
Figure 45: A representative electromobility shift assay using affinity purified GIP receptor extracted from transfected HEK 293 cells. Cells were transfected with GIP receptor constructs (5 µg) in 10 cm dishes and then protein was extracted as described in Chapter 2.20. The protein concentrations of the extracts were determined and 5 µg of purified protein was digested with 200 U of PNGase F for 1 hr at 37 °C. Control (C) and digested (P) proteins were run on a 12 % polyacrylamide gel and subjected to Western blot as described in section 2.20. Apparent molecular weights were calculated using R_f analysis.

N59T banding pattern except the major non-digested band ran at around 60 kDa (data not shown).

The same experiments were done on the double glycosylation site mutants to substantiate the single site mutant data. The major band of the N59/69T non-digested mutant protein extract ran with an apparent molecular mass of 59 ± 4 kDa, with minor bands running at 51 ± 2 kDa and 48 ± 2 kDa. When this extract was digested, two bands appeared at 51 kDa and 48 kDa in figure 45; however, other experiments only had the 48 ± 2 kDa band. The major band in the non-digested N59/74T protein extract had an apparent molecular mass of 67 ± 7 kDa, with a minor bands running at 53 ± 3 kDa. The digestion of this extract yielded a major band at 48 ± 2 kDa. The control N69/74T protein extract ran at 70 ± 9 kDa with a minor band in most extracts at 51 ± 4 kDa (however, the major band in figure 45 was at 47 kDa). When this extract was digested with PNGase F the major band ran at 49 ± 2 kDa. Finally, the triple mutant protein ran with an apparent molecular mass of 50 ± 3 kDa, when this protein extract was digested with PNGase F, the running distance did not change (figure 45). In addition, the amount of His-tagged protein purified from the triple mutant was never near the level of protein obtained from the other mutants indicating that this triple mutant is not post-translationally processed properly: perhaps one of the sites for glycosylation is needed for correct targeting for expression at the cell surface.

7.4 The Effect of Treatment of INS(832/13) Cells with Tunicamycin on Cell Surface GIP Receptor Expression

Figure 46 illustrates a representative saturation binding profile of INS(832/13) cells that have been treated with tunicamycin: an antibiotic which prevents the transfer of

GlcNAc-1-P from UDP-GlcNAc to dolichyl-P *i.e.* the first step in the glycosylation process. These experiments were carried out to determine the effect of glycosylation of proteins on GIPR expression and GIP-stimulated insulin secretion in β-cells. In all the experiments testing the effect of tunicamycin on INS(832/13) cells, a decrease in cell surface GIP binding was observed, indicating that GIP receptors were not being delivered as efficiently to the plasma membrane when glycosylation was blocked. Control cells expressed an average of 2443 ± 400 GIP receptors on the cell surface; whereas tunicamycin treated INS(832/13) cells expressed 760 ± 70 GIP receptors on the cell surface. Additionally, the dissociation constants (Kd) of GIP from the surface of these cells did not differ between the control (455 ± 50 pM) and tunicamycin (345 ± 100 pM) treated cells.

7.5 The Effect of Tunicamycin on GIP-Stimulated Insulin Secretion from INS(832/13) Cells

The GIP-potentiated secretion of insulin from cells treated with tunicamycin was also attenuated (figure 47). As illustrated in figure 47, GIP caused a small increase in insulin secretion at 5.5 mM glucose in control conditions. No difference in insulin secretion was also observed in cells treated with tunicamycin and then stimulated with GIP in 5.5 mM glucose conditions. In 11 mM glucose conditions, GIP caused a significant increase in insulin secretion from control cells; however, GIP was unable to potentiate 11 mM glucose-induced insulin secretion from cells that were treated with tunicamycin (figure 47). Additionally, growth in tunicamycin did not change the glucose stimulated insulin secretory response in these cells in response to either 5.5 mM or 11
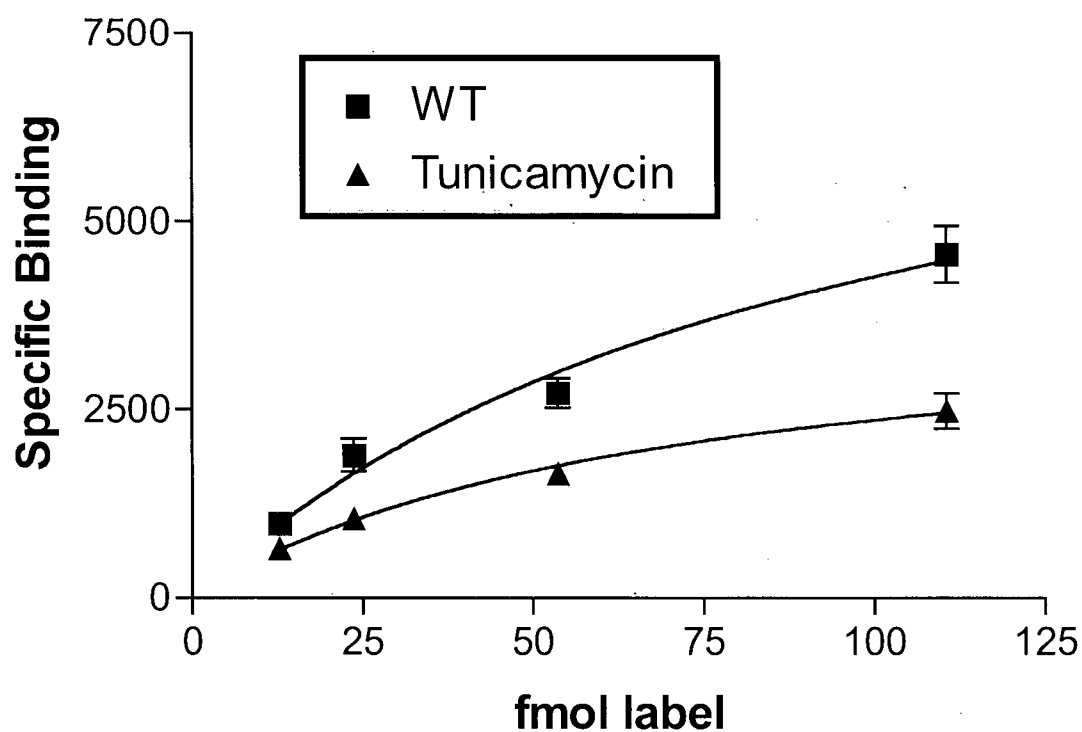
Figure 46: GIP saturation binding analysis from INS(832/13) cells treated with tunicamycin. Cells were plated into 24 well plates (5 x 10^5 cells/well) and grown for 24 hours. Cells were then treated with 1 µg/ml tunicamycin, an antibiotic that inhibits glycosylation, for 24 hours before the saturation binding analyses were carried out as described in section 2.14 and 2.26.
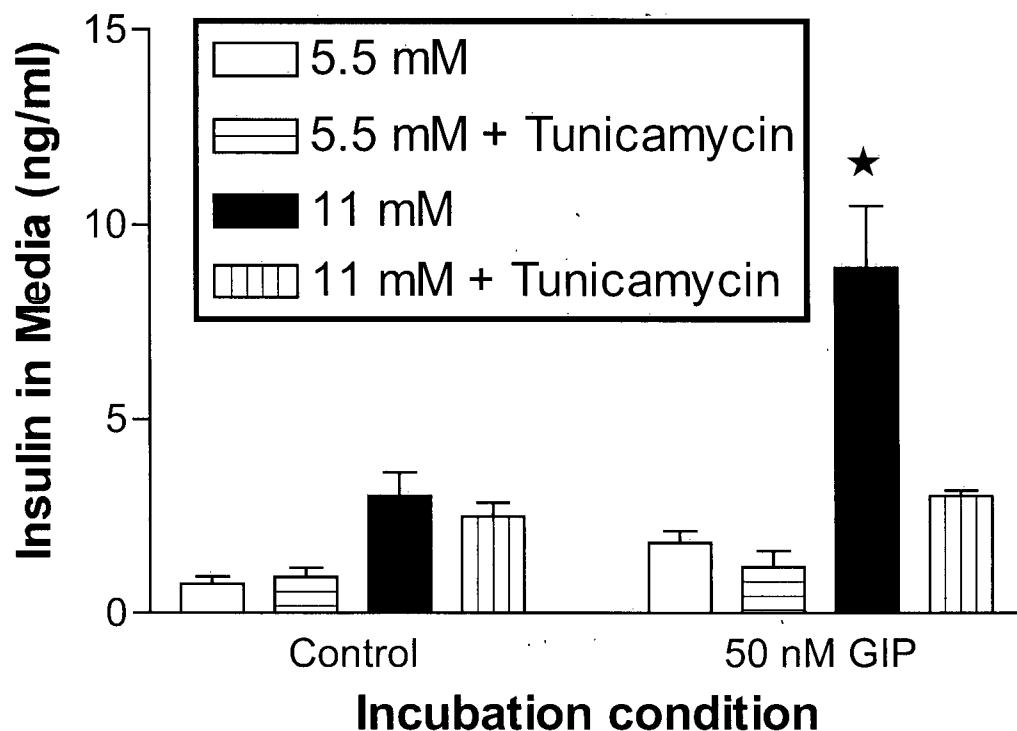
Figure 47: Tunicamycin decreases GIP-stimulated insulin secretion from INS(832/13) cells. Cells were plated into 24 well plates (5 x 10⁵ cells/well) and grown for 24 hours. Cells were then treated with 1 µg/ml tunicamycin, an antibiotic that inhibits glycosylation, for 24 hours before insulin release experiments were carried out (section 2.22). These were done by incubating cells in presence of either 5.5 mM or 11 mM glucose with or without 50 nM GIP for 30 minutes at 37 °C. The supernatant was then collected and assayed for insulin content using RIA. Data are expressed as mean ± S.E.M., n=4. Asterisks indicate statistical significance compared to basal conditions (P<0.05).

mM glucose; therefore, tunicamycin was probably not having adverse toxic effects on the cells.

7.6 Discussion

The decrease in GIP responsiveness in type 2 diabetics may be a result of factors other than hyperglycemia. For example, glycosylation of many GPCRs such as the GLP-1 receptor and the VIP receptor has been demonstrated to be important in the correct cell surface expression (Couvineau et al., 1996; Göke et al., 1994). Here we demonstrate that glycosylation of the GIP receptor affects cell surface expression and subsequent function of the receptor, and it is interesting to postulate that the GIP responsiveness in T2D may be affected in part by abnormal glycosylation within the β-cell.

Here we verify that the wild-type GIP receptor is a glycosylated protein with a molecular mass of approximately 59 kDa. Furthermore, when this receptor is treated with PNGase F, there is a band shift to an apparent molecular mass of 48 kDa. This indicates that N-linked carbohydrates account for a large portion of the GIP receptor structure (i.e. approximately 35 % by mass) (figure 45A). The literature indicates that the wild type GIP receptor is a 59 kDa glycoprotein (Amiranoff et al., 1986). Furthermore, molecular mass analysis of the cloned sequence of the GIP receptor predicts a mass of 50 kDa (Usdin et al., 1993; Wheeler et al., 1995). Here we overestimate the molecular mass of both the intact glycoprotein by approximately 10 kDa. This is probably a result of the reducing conditions that were utilized in our study; whereas, Amiranoff et al. (1986) used non-reducing conditions in their study. In fact when they used DTT to reduce disulfide bonds they saw a shift of the apparent molecular weight of the GIP/

receptor complex to around 73 kDa, which is in the molecular weight range determined in this study. This observation indicates that the GIP receptor is "normally" glycosylated in the HEK 293 cell line.

In any case, due to the structural contribution of the glycosylation to the GIP receptor, we set out to determine the exact effect of glycosylation on cell surface expression and function. The first series of experiments that we carried out was to look at the effect of tunicamycin on GIP receptor expression in the INS(832/13) rat β-cell model. When these cells were grown in the presence of tunicamycin, glycosylation was blocked and there was a decrease in ^{125}I-GIP binding to the surface of the cells on saturation: which corresponded to a 70 % decrease in cell surface GIP receptor number (figure 46). Concomitantly, the dissociation constant did not change in these experiments, indicating that the binding affinity of label for the wild-type receptor was not affected by glycosylation of the receptor. Furthermore, the majority of the glycosylation-site knockouts did not display dramatic differences in IC_{50} values from the WTtag receptor when competitive binding analyses were carried out; the exception was the triple mutant that was not highly expressed making an accurate binding isotherm hard to obtain (figure 44A). The amount ot total binding in a competitive binding analysis is a rough determination of total cell surface expression of the receptor. Figure 44A and 45 demonstated that there is much lower cell surface expression observed with the multiple glycosylation site mutants than with the single site mutations. Using these figures we can see that the WT receptor is most highly expressed followed by this sequence: N59T~N200T>N69T>N74T>N69/74T~N567T~N59/69T>N59/74T. These data indicate

that glycosylation affects GIP receptor expression, but does not change the affinity of the receptor for GIP; an observation that is in agreement with most of the studies done to date (Benya *et al.*, 2000; Pang *et al.*, 1999; Walsh *et al.*, 1998).

The GIP signaling properties of the mutants were also measured using cAMP as a marker of receptor activation (figures 44B & 45). These studies indicated that the mutants had right-shifted EC_{50} values for cAMP production. However, only the N200T and N567T mutants were significantly right-shifted with respect to the WTtag receptor. The maximal cAMP stimulation levels that were obtained from each of these mutant receptors were in line with their relative cell surface expression, with the single mutants producing much greater responses than the double mutants (*c.f.* figures 44B & 45). The only exception to this was the N200T mutant which produced a maximal cAMP response that was 1.6 times the WTtag receptor. This mutant was not more highly expressed at the cell surface; therefore, it is believed that removal of the carbohydrate, addition of a threonine residue, or both affects signaling. The affinity of the N200T receptor for GIP was found to be normal; however, EC_{50} for cAMP was right-shifted for this mutant meaning that coupling of this mutant receptor to G-proteins is disturbed.

Using gel shift analysis, the sites within the GIP receptor that were glycosylated were determined (figure 45). Figure 45 shows that both the N59T and N69T mutants have greater electrophoretic mobility than the wild-type tagged protein: each running approximately 5 kDa faster than the WTtag protein. Furthermore, the major band observed for the non-digested N59/69T mutant ran at approximately 59 kDa, corresponding to removal of the the 2 single glycosylation sites. The GIP receptor does not seem to be glycosylated at asparagine 74, since none of the mutants of this site had

gel shifts from the WTtag receptor. Finally, the N200T mutant was gel shifted in the non-digested form and ran with an apparent molecular mass of 63 kDa, indicating that this mutant was also glycosylated. Therefore, these data indicate that asparagines 59, 69 and 200 are all glycosylated in the GIP receptor.

Another interesting observation from the gel analysis was that most of the non-digested lanes included a band that ran with a greater mobility than the major glycosylated band. The identity of this band was not determined; however, it is absent in many of the PNGase digested lanes which indicates that it is in fact a glycosylated protein that runs with the same eletrophoretic mobility as the WTtag GIP receptor. This band was present in almost all the gels that were run, and it is believed to be GIP receptor protein that has not undergone full posttranslational processing. In these studies the GIP receptor was expressed at superphysiological levels under control of CMV promoter. In many cell lines this leads to the concentration of the overexpressed protein within inclusion bodies that generally surround the nuclear membrane and never fully mature. It is believed that the minor band represents GIP receptor that has been shuttled into this pathway; however, additional studies need to be carried out to identify this protein.

Another observation from the Western blots was that the triple mutant (N59/69/74T) was not glycosylated at all, and was generally very poorly expressed. None of the gels that were run showed a marked expression of this mutant, and it is believed that upon translation (if translated at all), the majority of the protein is incorrectly folded or inserted into the membrane leading to immediate degradation. This is supported by the competitive binding and cAMP studies which demonstrated very little cell surface expression of this mutant.

The final series of experiments in this study examined the role of GIP receptor glycosylation on the ability of GIP to stimulate insulin secretion. Figure 47 illustrates GIP was unable to potentiate insulin secretion in INS(832/13) cells that have been treated with tunicamycin. However, GIP was able to fully stimulate insulin secretion from INS(832/13) non-treated cells. There was no change in glucose-stimulated insulin secretion: indicating that insulin secretion was otherwise unaffected. An explanation for these findings is that non-glycosylated GIP receptor was not expressed on the cell surface and therefore GIP was not able to stimulate insulin secretion (figure 46).

In conclusion, the GIP receptor is glycosylated at asparagine residues 59, 69 and 200. This glycosylation is important for correct expression in the plasma membrane, although, it does not seem to be involved in modulating the binding of GIP. Furthermore, the removal of glycosylation at N200 seems to augment the ability of GIP to signal via cAMP, but does not change the affinity of GIP for the receptor. Finally, disruption of the glycosylation of proteins could affect cell surface GIP receptor expression. Although it seems unlikely that it could abrogate GIP binding completely, it is possible that overglycosylation could adversely affect GIP signaling. GIP seems to lose its insulin secretory ability in diseases such as T2D. One reason for this attenuation of GIP-stimulated insulin secretion could be the overglycosylation of proteins that is concomitant with high ambient glucose levels in T2D.

Chapter 8: Discussion and Future Directions

Previous reports have demonstrated that the insulin response to GIP is blunted in

T2D (Elahi *et al.*, 1994; Krarup *et al.*, 1987; Nauck *et al.*, 1993b). However, these

studies have not demonstrated a mechanism by which this occurs (Holst *et al.*, 1997).

The goal of this thesis was to elucidate the mechanisms by which GIP receptor

expression is controlled and to determine how this control is disturbed in T2D; with the

possible goal of altering disease therapy to allow GIP to have normal effects in the

disease. The data in this thesis describes a mechanism by which fat is able to upregulate

GIP receptor expression in the presence of low glucose; however, at high glucose GIP

receptor expression is downregulated in part by the downregulation of PPARα.

The degree of GIP receptor downregulation was found to be dependent on the

ambient level of glucose in young and diabetic VDF animals, as demonstrated in Chapter

4. Here it is shown that the mature animals display an approximate 70 % downregulation

of the GIP receptor mRNA and protein. On the other hand, the GIP receptor levels in the

4 week old prediabetic VDF animals are only reduced by 30 %. The ambient blood

glucose levels in these animals are positively correlated to their age: with old VDF

animals having a fasted blood glucose of 7.5 mM and young prediabetic animals having a

fasted blood glucose of 5 mM. Furthermore, older animals are glucose intolerant with

peak levels of approximately 19 mM after 60 minutes. In contrast, the prediabetic

animals have near normal glucose tolerance with a difference occurring only at the 15

min time point. After 60 minutes the blood glucose level in these animals has returned to

near basal levels. Based on the above discussion, one would predict that at these glucose

levels, there should not be any difference in GIP receptor expression in the prediabetic animals.

These *in vivo* data compare very well with the data presented in both Chapters 5 and 6. In these studies it was demonstrated that glucose is able to downregulate GIP receptor expression in both cell lines and in hyperglycemic clamped animals. Interestingly, glucose levels in the 10 mM range caused a significant decrease in GIP receptor expression in both cell lines (30 % decrease) and in lean animals (60 % decrease) that had undergone a hyperglycemic clamp for 6 hours. Blood glucose levels of 10 mM are easily obtained during the daily cycle of blood glucose in the same animals (Pospisilik *et al.*, 2002).

The cell and hyperglycemic clamp data also demonstrate that elevation in blood glucose is able to rapidly control GIP receptor expression. Thus, if there is a state of prolonged hyperglycemia, there could be concurrent reduction in the expression of the GIP receptor. The physiological basis for this downregulation is at present unclear, since most of the GIP within the circulation is degraded to an inactive form quickly following secretion and therefore is not biologically available to stimulate the β-cell (Kieffer *et al.*, 1995b). One possible explanation in an acute setting is that downregulation of the GIP receptor following a 6 hour period of hyperglycemia would protect the β-cell from further stress by limiting insulin secretion.

A more chronic situation could occur in Western society where overnutrition results in hyperglycemia throughout the day. In this case, a glucose induced downregulation of the GIP receptor would render the β-cell insensitive to physiological concentrations of GIP and therefore would remove the cytoprotective/antiapoptotic and

mitogenic effects of GIP that have recently been described in our laboratory. The cumulative effects of a decreased insulin secretion and a decreased ability of the β-cell to respond to GIP could lead to increased levels of blood glucose which hypothetically could stress the β-cell and lead to a decompensation in the insulin response to glucose and over time to T2D.

Another interesting observation from all of our studies was that in the β-cell there seems to be a basal amount of GIP receptor expression that cannot be regulated by hyperglycemia. In these studies this amounts to approximately 25 % total expression. For example, the maximal amount of downregulation observed in the VDF animal was around 75 % (Chapter 4), in the cell lines the maximal downregulation was also approximately 75% (Chapter 5), and in the hyperglycemic clamps a 75 % downregulation was also observed (Chapter 6). Therefore, a basal level of GIP receptor transcription is maintained that cannot be inhibited by glucose or by any of the other drugs that were tested in figure 29.

One pathway through which this apparent maximum amount of downregulation could be occurring is the PPARα pathway. Roduit *et al.* (2000) demonstrated that at 20 mM glucose, a 20 % basal level of PPARα is maintained. It is possible that this amount of PPARα expression is able to maintain receptor expression even in the face of hyperglycemia. However, this explanation does not fit exactly with our data since we demonstrate in figure 36 that transfection of β-cells with a mutant form of PPARα which is constitutively expressed does not elevate the GIP receptor expression level at hyperglycemia as would be expected. It is possible that the dimerization partner of PPARα, the RXR, is also downregulated by high glucose and therefore, our GIPR

expression in our tranfections may have been limited by the RXR levels as well. Expression of a coactivator of PPARs such as peroxisome proliferator gamma coactivator-1 (PGC-1) may also be regulated by glucose in the β-cell and may provide a mechanism by which tranfection of β-cells with PPARα does not lead to an increase in receptor transcription at hyperglycemia (Knutti & Kralli, 2001; Oberkofler *et al.*, 2002).

In these experiments we were unable to decrease the basal expression of GIPR promoter driven luciferase activity with hyperglycemia (data not shown). However, we did see an increase in luciferase activity following incubation of our promoter construct trasfected cells with both 2 mM palmitate and with the PPARα activator WY 14643 (figure 38). This is another observation that is hard to explain when we use the simplified explanation that glucose-induced PPARα downregulation leads to GIPR downregulation. It would be expected that since PPARα stimulates luciferase activity in the proximal 5' promoter, that a hyperglycemia induced decrease in PPARα would also decrease receptor expression in the same portion of the promoter. It is possible that there are multiple TF involved in binding and regulating GIP receptor transcription under hyperglycemia and that the a larger portion of the promoter is required for a full complement of these elements to bind and have effect at high glucose. Thus, there are probably factors (in addition to PPARα) involved in downregulation of the GIP receptor at high glucose levels. Further investigation of a more complete promoter is warranted in this regard.

The cloning of the GIPR promoter was carried out, however, time constraints prevented a complete characterization of the sequence elements within this promoter. These studies would provide important insight into the specific sequence elements that

are necessary for glucose and fat stimulated GIPR regulation. Gene therapy with a specific knockout of these putative sequences would allow the GIP receptor to be expressed at relatively normal levels on the β-cells of a model of T2D, and could further elucidate the role of GIP in T2D.

What are the possible therapeutic implications of a downregulation of the GIP receptor in the treatment of T2D? We have demonstrated that GIP receptors are downregulated significantly in this disease; however, it is probably still possible to use GIP or GIP analogues for therapy of T2D. Hinke *et al.* (2002) demonstrated that D-Ala^2-GIP stimulated insulin secretion from the pancreas of the VDF rat; however, pharmacological doses of 8 nmol/kg were required for an effect to be observed. One drawback of using high doses of modified GIP is that the long-term effects of administration of such a peptide are unknown and may result in further dowregulation of pancreatic GIP receptors. Another possible therapeutic strategy would be to use oral DP IV inhibitors such as those used by Pospisilik *et al* (2002) that were demonstrated to lower blood glucose levels in these animals. Drugs such as this in combination with GIP analogs in more physiological doses, and strict control of nutrional intake may be effective in the restoration of β-cell function without having any adverse affects. Another possible strategy would be to use GLP-1 analogs in conjuction with DP IV inhibitors because GLP-1 is much more effective at stimulating insulin secretion in T2D; presumably when the blood glucose levels had been lowered sufficiently GIP receptor expression would be restored and GIP would be able to stimulate insulin secretion and have other important effects. Future studies could examine the expression of GIP receptors in VDF rats that have had their hyperglycemia ameliorated by DP IV inhibition

or by an insulin sensitizing agent such as metformin. These studies would help to determine if improving glucose tolerance increases GIPR expression, and therefore, if the beneficial effects of GIP on β-cells could be fully restored in T2D.

Another potential mechanism of improving GIP responsiveness in diabetic individuals would be gene therapy possibly using virus vectors. One method of testing the efficacy of GIPR gene therapy would be to construct a β-cell specific inducible GIPR knock-in mouse model. This kind of system would allow a custom tailoring of GIPR expression to the organism's glucose intolerance by administering an oral drug such as tetracycline. A system like this would ideally lead to a restoration of GIP-stimulated insulin secretion in T2D without the need for peptide therapy.

Yaney *et al.* (2001) demonstrated GLP-1 stimulated lipolysis and FFA oxidation within the HIT-15 β-cell model and that this effect could be mimicked by forskolin and inhibited by orlistat, a lipase inhibitor. They proposed that GLP-1 activates hormone sensitive lipase, FFA are released from triglycerides and then LC-CoA is oxidized within the mitochondrion. Additionally, they demonstrated that this effect could be blocked by the addition of glucose, probably because glucose leads to an increase in malonyl-CoA and a subsequent inhibition of CPT-1.

Preliminary data are presented in this thesis, which indicate that GIP may also play a role in the oxidation of FFA within the β-cell. However, currently the pathway by which GIP stimulates FFA oxidation in the β-cell has not been determined, and future experiments could be directed towards this. One mechanism by which GIP could stimulate insulin secretion is by increasing the synthesis and oxidation of LC-CoA early following a meal presumably via cAMP and PKA. As in the case of GLP-1, this effect

would probably be inhibited by glucose. There is physiological evidence, which suggests that GIP is more important in early first phase insulin secretion (Lewis *et al.*, 2000). This could partly be explained by an effect of GIP on lipolysis within the β-cell, which is inhibited once the blood glucose levels elevated. Another series of studies could be designed to determine if lipolysis and/or fatty acid oxidation plays a role in the antiapoptotic action of GIP in β-cells.

In conclusion, GIP receptor expression is important for the normal stimulation of postprandial insulin secretion. In T2D GIP receptor expression may be decreased by hyperglycemia and the consequent downregulation of PPARα. The glycosylation of the GIP receptor may also be abnormal in T2D and may lead to a decrease in cell surface GIP receptor expression. Ultimately, for GIP receptor expression to be restored and for endogenous GIP to have full insulinotropic and mitogenic effects, blood glucose must be lowered to basal levels, using current therapeutic approaches or gene therapy must be developed to increase the expression of the receptor on the islets.

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