THE ROLE OF INCRETIN HORMONES IN GLUCOSE HOMEOSTASIS

by

Laurie L. Baggio

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Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

Abstract

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Incretins are hormones that are released from the gut in response to nutrient ingestion and act to enhance glucose-stimulated insulin secretion. The two major peptides which have been identified as incretin hormones are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Previous glucoregulatory studies using receptor knockout mice models suggest that the role of GIP is restricted to that of an incretin, whereas GLP-1 also exhibits non-incretin effects. However, studies with genetically-modified mice can be complicated by compensatory adaptive changes. To avoid this possibility, we assessed the incretin and nonincretin actions of GIP and GLP-1 in wild-type mice using the GLP-1 receptor antagonist exendin(9-39) and immunopurified anti-GIP receptor antisera to antagonize GLP-1 and GIP action, respectively. Our results indicate that GLP-1, but not GIP, plays an important role in regulating blood glucose levels in mice in a manner independent of oral nutrient ingestion.

In addition to its role as an incretin, GLP-1 contributes to blood glucose lowering through several different mechanisms and thus its therapeutic value as a treatment for diabetes is currently under investigation. However, GLP-1 is rapidly catabolized by dipeptidylpeptidase IV (DPP-IV) and therefore has a short plasma half-life, which may, in turn, limit its therapeutic potential. Inhibition of DPP-IV activity could thus provide a means to extend the half-life of GLP-1. However, DPP-IV may also act upon other substrates important for glucoregulation. To investigate this possibility, we examined the effects of

DPP-IV inhibition in GLP-1 receptor knockout mice. Our results suggest that in addition to GLP-1, other substrates are involved in the DPP-IV-mediated regulation of blood glucose control.

Exendin-4 is a potent GLP-1 receptor agonist that was originally purified from lizard venom and is currently being evaluated in clinical trials as a treatment for diabetes. Limited information is available regarding the long-term effects of exendin-4 treatment *in vivo*. To assess the physiological effects of chronic exendin-4 expression *in vivo*, we have generated transgenic mice in which exendin-4 expression is under the control of an inducible promoter. Our data indicate that sustained elevation of circulating exendin-4 has both predicted and unanticipated effects on GLP-1 receptor-dependent physiological end points.

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List of Abbreviations

° C	degree celsius
Ab	antibody
Ala	alanine
ANOVA	analysis of variance
ANTGIP	GIP (7-30)NH_2, a GIP receptor peptide antagonist
approx	approximately
Arg	arginine
Beta 2	Beta2/NeuroD basic helix-loop-helix factor
bp	base pairs
Brn 4	brain-4
C18	carbon-18
cAMP	cyclic 3', 5'-adenosine monophosphate
CAPs	CREB-associated proteins
CBS	CAP-binding sites
cDNA	complementary DNA
Cdx 2/3	caudal-related homeobox 2/3
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element-binding protein
C-terminal	carboxy-terminal
Cys	cysteine
DCS	distal cell-specific silencer
DNA	deoxyribonucleic acid
DPP-IV	dipeptidylpeptidase IV
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
ENTP	exendin N-terminal peptide
Ets	ubiquitious developmental transcription factors
Ex 4	exendin-4
FAP	fibroblast activation protein
G protein	GTP-binding protein
g	gram
GIP	glucose-dependent insulinotropic polypeptide
GIPR	glucose-dependent insulinotropic polypeptide receptor
GIPR-/-	GIP receptor knockout (homozygous)
glob	globulin
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLP-1R-/-	GLP-1 receptor knockout (homozygous)
GLP-2	glucagon-like peptide-2
Gln	glutamine
Glu	glutamic acid

Gluc	glucagon
Gly	glycine
GRF	growth hormone releasing factor
GRH	growth hormone-releasing hormone
GRP	gastrin-releasing peptide
GRPP	glicentin-related pancreatic polypeptide
GUE	glucagon upstream enhancer
h	hours
HbA_{1c}	hemoglobin A_{1c}
hGH	human growth hormone
His	histidine
HNF	hepatocyte nuclear factor
HPLC	high pressure liquid chromatography
i.p.	intraperitoneal
i.v.	intravenous
ICV	intracerebroventricular
IDDM	insulin-dependent diabetes mellitus
IDX-1	islet/duodenum homeobox-1
Ile	isoleucine
Inc.	incorporated
IP-1	intervening peptide-1
IP-2	intervening peptpide-2
IPGTT	intraperitoneal glucose tolerance test
IR	immuno reactivity
Isl-1	islet lim-homeodomain protein
Kb	kilobases
kDa	kilodalton
kg	kilogram
KIU	kallikrein inhibitory units
KLH	keyhole limpet hemocyanin
Leu	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LPI	lizard proglucagon I
LPII	lizard proglucagon II
MAPK	mitogen-activated protein kinase
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MPGF	major proglucagon fragment
mRNA	messenger RNA
MT-I	mouse metallothionein-I
MT-Ex	metallothionein-exendin-4
N	normal
n	number

NEP	neutral endopeptidase
ng	nanogram
NH_2	amide
nmol	nanomol
NPY	neuropeptide Y
nt	nucleotides
N-terminal	amino-terminal
OGTT	oral glucose tolerance test
p	observed significance level
^{32}P	phosphorous 32
PI	phosphatidyl inositol
PACAP	pituitary adenylate cyclase-activating polypeptide
Pax	paired homeobox
PBS	phosphate buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
PDX-1	pancreatic and duodenal homeobox gene-1
PGDP	proglucagon-derived peptide
PHI	peptide histidine isoleucine
PKA	protein kinase A
PKC	protein kinase C
Pro	proline
RIA	radioimmunoassay
RNA	ribonucleic acid
S	signal peptide
SD	standard deviation
sec	seconds
SEM	standard error of the mean
Ser	serine
STZ	streptozotocin
SURS	systematic uniform random sampling
TED	Trasylol/EDTA/Diprotin A
Thr	threonine
Trp	tryptophan
TSH	thyroid-stimulating hormone
Tyr	tyrosine
UTR	untranslated region
Val-Pyr	valine-pyrrolidide
VIP	vasoactive intestinal polypeptide
vol	volume
vs.	versus
wt	weight
X	any amino acid
Zn	zinc sulfate ($ZnSO_4$)

µg	microgram
µl	microliter

List of Appendices

Author Contributions and Dissemination of Research

Chapter 2. Differential Roles for GIP and GLP-1 in the Control of Glucose Homeostasis

A) Contributions:

All of the data in this chapter were generated by Laurie Baggio. The immunopurified GIP receptor antibody used in these studies were provided by Dr. T. J. Kieffer, University of Alberta, Edmonton, Canada.

B) Publications:

1) **Baggio, L.**, Kieffer, T.J. and Drucker, D.J. (2000). Glucagon-Like Peptide-1, But Not Glucose-Dependent Insulinotropic Peptide, Regulates Fasting Glycemia and Nonenteral Glucose Clearance in Mice. *Endocrinology* **141**. 3703-3709.

C) Abstracts and Presentations/Published:

1) **Baggio, L.**, Kieffer, T.J., and Drucker, D.J. (2000). Differential roles for GLP-1 and GIP in the control of glucose homeostasis in mice. Islets in Eilat 2000, 36th Annual Meeting, European Association for the Study of Diabetes Satellite Meeting. (Oral Presentation).

2) **Baggio, L.**, Kieffer, T.J., and Drucker, D.J. (2000). Incretin antagonists define contrasting roles for GLP-1 and GIP in the control of glucose homeostasis in vivo. The Endocrine Society 82nd Annual Meeting.

Chapter 3. Inhibition of DPP-IV Activity Improves Glucose Tolerance and Insulin Secretion in GLP-1R-/- Mice

A) Contributions:

All of the data in this chapter were generated by Laurie Baggio. The DPP-IV inhibitor, valine-pyrollidide, was provided by Dr. Nicolai Wagtmann, Novo Nordisk, Denmark.

The data generated in this chapter contributed, in part, to the manuscript by Marguet *et. al.* listed below. The data for figures 4 B,C and 6 A,B of the manuscript (corresponding to figures 14 A,B and 16 A,B of this thesis) were generated by Laurie Baggio.

B) Publications:

1) Marguet, D., **Baggio, L.**, Kobayashi, T., Bernard, A.M., Pierres, M., Nielsen, P.F., Ribel, U., Watanabe, T., Drucker, D.J., and Wagtmann, N. (2000). Enhanced Insulin Secretion and

Improved Glucose Tolerance in Mice Lacking CD26. *Proc. Nat. Acad. Sci. USA* **97**, 6874-6879.

Chapter 4. Sustained Expression of Exendin-4 does not Perturb Glucose Homeostasis, B-cell mass or Food Intake in Metallothionein-Exendin-4 Transgenic Mice

A) Contributions:

The data for figures 17, 18, 20, 21, 22, 23, 25 and 26 of this chapter were generated by Laurie Baggio. The data for figure 19 was generated by Feisal Adatia in Dr. Patricia Brubaker's laboratory in the Department of Physiology at the University of Toronto. The data for figure 24 of the thesis was generated by Troels Bock, Bartholin Institute, Denmark.

B) Publications:

1) **Baggio, L.**, Adatia, F., Bock, T., Brubaker, P.L., and Drucker, D.J. (2000). Sustained Expression of Exendin-4 does not Perturb Glucose Homeostasis, β-Cell Mass, or Food Intake in Metallothionein-Preproexendin Transgenic Mice. *The Journal of Biological Chemistry* **275**, 34471-34477.

C) Abstracts and Presentations/Published:

1) **Baggio, L.**, Adatia, F.A., Brubaker, P.L., and Drucker, D.J. (2000). Improved glucose tolerance but normal weight gain in mice expressing a MT-exendin transgene. The Endocrine Society 82nd Annual Meeting.

2) **Baggio, L.**, Adatia, F.A., Bock, T., Brubaker, P.L., and Drucker, D.J. (2000). Improved glucose tolerance but normal islet histology in mice expressing a MT-exendin transgene. Islets in Eilat 2000, 36th Annual Meeting, European Association for the Study of Diabetes Satellite Meeting.

Chapter 1. Introduction

The maintenance of normal glucose homeostasis is tightly regulated by the integrated processes of hepatic glucose production, glucose uptake and utilization by peripheral tissues and insulin secretion. Insulin, an important glucoregulatory hormone, is released in response to elevated blood glucose levels and, among other functions, promotes glucose uptake from the blood into adipocytes and muscle cells. Deficits in insulin secretion or action lead to impaired glucose tolerance and the development of diabetes mellitus.

The primary regulator of insulin synthesis and secretion is the plasma glucose concentration. In addition to glucose, insulin secretion is also modulated by the release of peptide hormones from the gastrointestinal tract. The concept that factors released from the gut in response to nutrient ingestion can stimulate secretion from the endocrine pancreas was first proposed in the early 1900's (1-4), and in 1929, the term "incretin" was introduced to describe these putative gut factors (5). This connection between the gastrointestinal tract and the endocrine pancreas was dubbed the "enteroinsular axis" (6). The subsequent observation by a number of investigators that oral glucose administration stimulates a greater increase in insulin secretion from pancreatic β-cells than an isoglycemic intravenous infusion (7-11) has been attributed to the action of incretins (3, 4). Thus, the term incretin refers to an agent that is released from the gut in response to nutrient ingestion and acts to potentiate glucose-stimulated insulin secretion (12).

The first incretin hormone to be identified was glucose-dependent insulinotropic polypeptide (GIP) (13-17). In agreement with its role as an incretin, GIP is synthesized in and released from intestinal K-cells, primarily in response to the ingestion of glucose or fat (18, 19), and enhances glucose-stimulated insulin secretion (13, 14). However, it was

concluded that GIP alone cannot account fully for the incretin effect *in vivo*, based on the following findings. (i) A portion of the incretin activity is preserved in rat gut extracts, despite immunoneutralization of endogenous GIP activity (20, 21). (ii) Anti-GIP antiserum depresses but does not eliminate insulin secretion in response to an oral glucose load in rats (22). (iii) Surgical patients who have most of their ileum resected exhibit a much smaller incretin effect relative to those who maintained a larger proportion of their ileum, despite both groups of patients having similar plasma GIP levels (23).

The cloning and sequencing of mammalian genes and cDNAs encoding proglucagon (24-26) led to the discovery of a second candidate incretin hormone, glucagon-like peptide 1 (GLP-1). GLP-1, a post-translational proteolytic product of the proglucagon gene, is released from intestinal L-cells into the circulation in response to the ingestion of glucose or a mixed meal and potentiates glucose-stimulated insulin secretion (27-30). In addition to its role as an incretin hormone, GLP-1 also lowers blood glucose by its ability to inhibit glucagon secretion (31-35), food intake (36-41) and gastric emptying (42-46). GLP-1 also confers glucose sensitivity to glucose resistant pancreatic β-cells (47-49) and may also promote glucose disposal in peripheral tissues (50-53). Recent studies have suggested that GLP-1 may also have a role in β-cell neogenesis and proliferation (54-57).

To date, only GIP and GLP-1 have been identified as incretin hormones. These two peptides augment glucose-stimulated insulin secretion in an additive manner and are thought to account fully for the incretin effect (58, 59). Due to their ability to augment glucose-stimulated insulin secretion, both GIP and GLP-1 represent potential therapeutic agents for the treatment of diabetes. However, subsequent analysis of the insulinotropic activity of these two peptides in type 2 (non insulin-dependent) diabetic patients revealed that the

incretin activity of GLP-1 was preserved in these patients but that of GIP was not (60). Thus, clinical studies have focused on the use of GLP-1 for the treatment of diabetes. However, the therapeutic potential of native GLP-1 may be limited as this peptide has a very short plasma half-life (approx 90 sec) (61) due, in part, to its rapid inactivation by the peptidase dipeptidylpeptidase IV (DPP-IV) (61-63).

The short half-life and potential therapeutic limitations of native GLP-1 prompted a search for more suitable alternatives. Exendin-4, a naturally occurring, long-acting GLP-1 receptor agonist, was originally purified from the venom of a *Heloderma suspectum* lizard (64) and subsequently found to elicit a variety of biological effects which mimic the actions of GLP-1, including its abilities to: (i) enhance glucose-stimulated insulin secretion (65, 66); (ii) regulate gastric emptying (67); (iii) act as a satiety factor (68-70), and (iv) promote pancreatic endocrine cell differentiation and expansion of β-cell mass (55-57). In contrast to GLP-1, exendin-4 is not a substrate for DPP-IV activity and thus has a much longer half-life than GLP-1 (71). In addition, preliminary *in vivo* studies suggest that exendin-4 may be a more potent insulinotropic agent than GLP-1 (68, 72). Currently exendin-4 is being evaluated in clinical trials as a potential therapeutic agent for the treatment of diabetes (Amylin Pharmaceuticals website; www.amylin.com).

The following sections summarize our current knowledge regarding the synthesis, secretion and biological activities of GLP-1, GIP and exendin-4, as well as the molecular and functional characteristics of DPP-IV and its role in glucose homeostasis.

1.1 Proglucagon and Proglucagon-Derived Peptides (PGDPs)

(a) Proglucagon Gene Expression

In mammals, the proglucagon gene consists of six exons and five introns and gives rise to a single, identical mRNA transcript in the α-cells of the endocrine pancreas, the L-cells of the intestine, and in hypothalamic nuclei and brainstem neuronal cell bodies in the central nervous system (CNS; Fig. 1) (3, 73). In general, pancreatic proglucagon gene expression is stimulated by fasting and hypoglycemia and inhibited by insulin (74, 75), whereas in the intestine, proglucagon gene expression is up regulated by nutrients (76) and possibly by peptides such as gastrin-releasing peptide (GRP) and GIP (77, 78). Agents which activate the protein kinase A (PKA) pathway also up regulate proglucagon gene expression in both the pancreas and intestine (79-81). Tissue specific expression of the proglucagon gene is regulated by the interaction of a variety of transcription factors with defined DNA regulatory sequences in the promoter and enhancer regions of the gene. Cell transfection and transgenic mice studies have identified transcription factors and DNA sequences that modulate proglucagon gene expression in the pancreas and intestine (Fig 2) (4, 30, 82).

Several *cis*-acting sequences within the rat proglucagon gene promoter have been identified as important control regions for gene expression. G1 is a promoter proximal element which binds to the homeobox transcription factors Brn4, Pax6, cdx2/3 and isl-1 and confers α-cell specific expression of the proglucagon gene (83-88). Recent studies have indicated that Pax6 and cdx2/3, in association with a co-activator protein, p300, interact synergistically to regulate proglucagon gene expression in islet cells (89). G2, and G3 represent proglucagon gene islet-cell specific enhancer-like elements (83). The G2 element

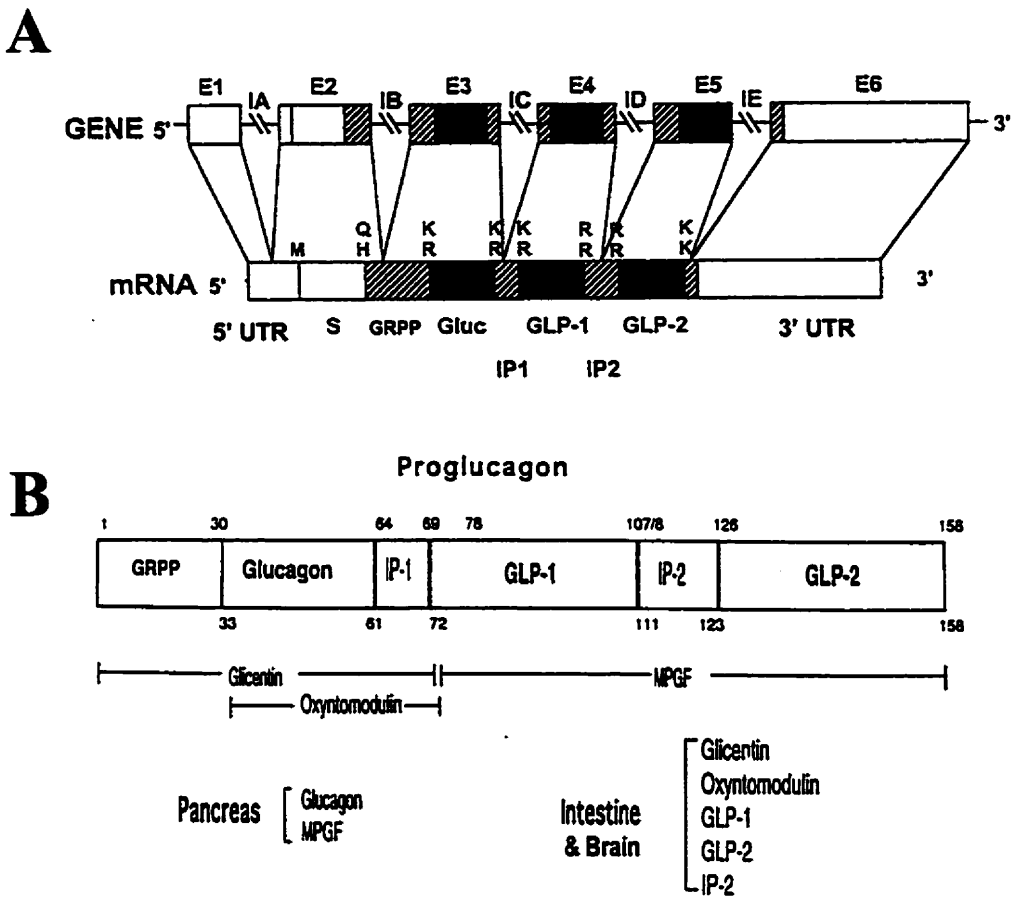


Fig. 1. Proglucagon gene, mRNA and precursor protein structures. A: The proglucagon gene is composed of six exons (E1-E6), which encode functional domains of the proglucagon precursor protein, and 5 introns (IA-IE) and gives rise to a single, identical mRNA transcript in the pancreas, brain and intestine. M, translation start site; S, signal peptide; UTR, untranslated region. The pairs of basic amino acids which serve as the sites for post-translational cleavage are indicated above the mRNA structure. Adapted from T.J. Kieffer and J.F. Habener, *Endocrine Reviews*, 20(6): 876-913, 1999. **B:** Tissue specific post-translational processing of proglucagon in the pancreas, intestine and brain. The numbers above and below the structure pertain to amino acid positions in proglucagon. For **(A)** and **(B)**: GRPP, glicentin-related pancreatic polypeptide; Gluc, glucagon; IP-1 and IP-2, intervening peptide 1 and 2; GLP-1 and GLP-2, glucagon-like peptide-1 and -2; MPGF, major proglucagon fragment. Adapted from D.J. Drucker, *Diabetes*, 47: 159-169, 1998.

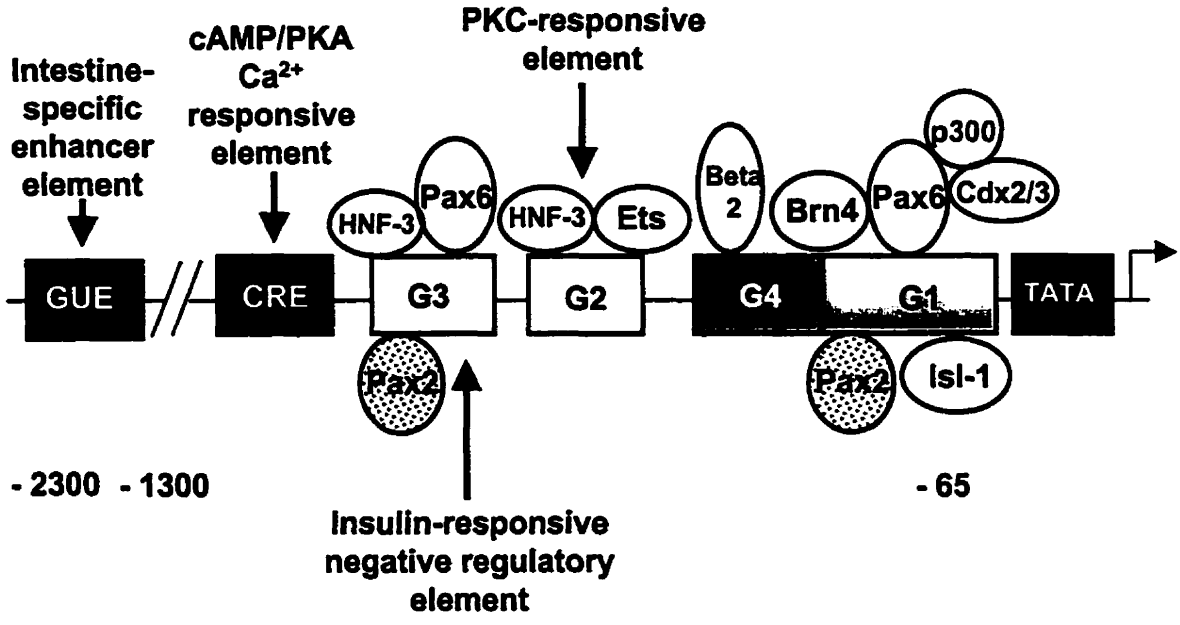


Fig. 2. DNA regulatory elements and their cognate transcription factors in the 5'-flanking region of the rat proglucagon gene. GUE, glucagon upstream enhancer; CRE, cAMP response element. G1 confers α-cell specific expression to the proglucagon gene. G2, G3, and G4 are islet-specific enhancer elements that confer pancreas-specific expression to the glucagon gene. TATA, TATA box. The right angled arrow indicates the transcription start site.

binds different members of the hepatocyte nuclear factor 3 (HNF-3) family of transcription factors to either enhance or repress proglucagon gene expression (90, 91). G2 also interacts with members of the Ets family of transcription factors (92), and mediates protein kinase C (PKC)-stimulated glucagon gene transcription in islets (93, 94). The G3 element interacts with members of the HNF family of transcription factors (95), as well as the transcription factor Pax6 (96). Recently, the transcription factor Pax2 has been detected in the endocrine pancreas and shown to bind to the G1 and G3 elements of the proglucagon gene (97). Thus, Pax2 may also be an important regulator of proglucagon gene expression. G3 also contains an insulin response element believed to modulate insulin-mediated negative regulation of proglucagon expression in α-cells (98). More recent studies have suggested that insulin responsiveness is conferred to the proglucagon gene via synergistic interactions between proximal promoter elements and more distal enhancer-like elements (99). G4 is a sub element of G1 that binds the transcription factor Beta2/NeuroD (4, 100). The proglucagon gene 5'-flanking sequences also contain a cAMP response element (CRE) which binds to a cAMP response element binding protein (CREB), conferring cAMP responsivity to proglucagon gene transcription in both the pancreas and intestine (79-81, 101). In addition to cAMP, the rat proglucagon CRE is activated by membrane depolarization or influx of calcium (102). CAP-binding sites (CBS) are located adjacent to the CRE, on either side, and are binding sites for CREB-associated proteins (CAPs), which inhibit CREB-mediated cAMP stimulation of proglucagon gene expression (103).

In contrast to proglucagon expression in the endocrine pancreas, very little is known about the factors that specify proglucagon gene expression in the intestine and brain. Studies in transgenic mice indicate that approximately 1.3 kb of the rat proglucagon gene 5'-

flanking sequences are sufficient to direct gene expression to the pancreatic α-cells and brain (104), whereas a larger (2.3 kb) proportion of the 5'-flanking sequences direct proglucagon gene expression to the pancreas, brain and intestine (105), suggesting that the sequences between –1300 and –2300 in the rat proglucagon promoter are essential for specifying intestinal proglucagon expression. The sequences upstream of –1300 in the rat proglucagon gene have been designated the proglucagon upstream enhancer element (GUE) (106). Transfection studies and electrophoretic mobility shift assays in enteroendocrine cell lines indicate that the proglucagon gene GUE is comprised of multiple positive and negative cis-acting DNA regulatory subdomains that contribute to the transcriptional control of proglucagon gene expression in both islet and intestinal cells (106). A recent study indicates that the transcription factor Pax6 is essential for proglucagon gene expression in the enteroendocrine cells of the intestine (107). In transgenic mice studies, the human proglucagon gene appears to utilize transcription factors and DNA sequences that are distinct from those used by the rat to specify tissue-specific gene transcription (108). In transgenic mice, approximately 1.6 kb of human proglucagon 5'-flanking sequences direct proglucagon gene transcription to the brain and intestine, but not the pancreas (108). Cell transfection studies using human proglucagon promoter reporter plasmids in rodent islet cell lines indicate that sequences within the first 6 kb of the human proglucagon gene 5'-flanking sequences are required for pancreatic expression (108).

(b) Proglucagon Post-Translational Processing and Biological Activities of the PGDPs

In all three tissues (islets, intestine and brain), the proglucagon mRNA is translated into a single 160 amino acid precursor protein which undergoes tissue-specific post-

translational processing (73, 109) to liberate the proglucagon-derived peptides (PGDPs; Fig. 1B).

In the pancreatic α-cells, the predominant post-translational processing products of proglucagon are glicentin-related pancreatic polypeptide (GRPP), glucagon, intervening peptide-1 (IP-1), and the major proglucagon fragment (MPGF; Fig. 1B) (3, 4). Glucagon elevates blood glucose levels, primarily by stimulating hepatic glycogenolysis and gluconeogenesis (110) and thus, is essential for maintaining glucose homeostasis in the fasting state. The functions, if any, of GRPP, IP-1 and MPGF are currently not known.

In the intestinal L-cells and brain, post-translational processing of proglucagon gives rise primarily to glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1), intervening peptide-2 (IP-2), and glucagon-like peptide-2 (GLP-2; Fig. 1) (3, 4). Glicentin appears to have trophic effects on the rat small-intestine (111) and oxyntomodulin has been shown to inhibit gastric acid secretion *in vivo* (112, 113). In addition to its role as an important incretin hormone, GLP-1 has a number of other biological functions which will be discussed in detail in the next section. A biological function for IP-2 has not been identified thus far. The major biological effect of GLP-2 appears to be that of an intestinal trophic factor (114-116). GLP-2 mediates its trophic effects by mechanisms which involve induction of intestinal crypt-cell proliferation and inhibition of apoptosis in both the intestinal crypt and villus compartments (115, 117). GLP-2 has also been shown to up regulate intestinal glucose transport (118, 119), inhibit gastric emptying (120) and gastric acid secretion (121), and improve intestinal barrier function by reducing intestinal epithelial permeability (82, 122). Very recent studies have identified GLP-2 as a specific neurotransmitter that inhibits food intake in rats (123).

The post-translational processing of proglucagon is carried out by the prohormone convertase (PC) enzymes which are endoproteases that cleave C-terminal to paired basic amino acid residues (124, 125). The PGDPs are flanked by pairs of basic amino acid residues, the recognition sites for PC cleavage. PC1/3 and PC2 have been detected in proglucagon-expressing cells (126, 127). PC1/3 has been localized to intestinal L-cells and shown to be both necessary and sufficient for the post-translational processing of proglucagon to its intestinal PGDPs (128-132). In contrast, PC2, but not PC1/3, has been localized to the pancreatic α-cells (133) and is believed to be responsible, at least in part, for the post-translational processing of proglucagon to glucagon in these cells (134-137).

1.2 Glucagon Like Peptide-1 (GLP-1)

(a) Biosynthesis and Secretion

GLP-1 secretion is controlled by a number of stimuli including nutrients and neural as well as endocrine factors (3, 4, 29). GLP-1 is produced in the enteroendocrine L-cells, the majority of which are found in the distal ileum and colon (138-141), and secretion from these cells is mediated by the activation of a number of intracellular signals, including PKA, PKC and calcium (78, 142-144). GLP-1 is released rapidly (within 10-15 min.) into the circulation following oral ingestion of nutrients in both rodents and humans (145-148). Oral administration of glucose in humans produces a bi-phasic increase in plasma GLP-1 levels, whereas intravenous infusion of glucose has no effect on GLP-1 secretion (148). Since the GLP-1-producing L-cells are located in the distal portion of the small intestine, it is likely that the rapid increase in plasma GLP-1 levels observed following nutrient ingestion are not due to a direct stimulatory effect of nutrients on the L-cell. Thus, the existence of a

proximal-distal loop that transmits nutrient-induced stimulatory signals, via neural or endocrine effectors, to the L-cells has been proposed (149). In support of this, *in vitro* and *in vivo* studies in rats have shown that the more proximally-located duodenal hormone GIP and the neurotransmitter acetylcholine can stimulate intestinal GLP-1 secretion (149-151). However, GIP does not stimulate GLP-1 secretion in humans (28, 60). The neuropeptide gastrin releasing peptide (GRP) stimulates GLP-1 secretion in both humans (152) and rodents (153). Recent studies in the GRP receptor knock out mouse have shown that these mice exhibit a reduction in the GLP-1 response to gastric glucose (154), indicating that a normal GLP-1 response to enteral glucose requires intact GRP receptor signaling. In addition, studies suggest that the stimulatory effects of GIP on GLP-1 secretion may be mediated indirectly, via GRP (153). Additional studies have indicated that the vagus nerve also has a role in mediating the rapid release of GLP-1 from the distal L-cells in response to ingested nutrients (155). More recently, a role for the neuropeptide calcitonin gene-related peptide (CGRP) in the regulation of GLP-1 release has been proposed (156). Thus, the biphasic nature of GLP-1 release appears to be mediated through a proximal-distal loop consisting of hormonal and neural factors which stimulate early (within 10-15 min) GLP-1 release, as well as direct nutrient contact with the intestinal L-cell to promote the second phase (30-60 min) of GLP-1 secretion (4).

Hormones such as insulin and somatostatin-28 (132, 157), as well as the neuropeptide galanin (158), have been shown to inhibit GLP-1 secretion from the intestinal L-cell in both *in vitro* and *in vivo* studies.

Multiple forms of GLP-1 are secreted from the L-cells, including GLP-1(1-37) and GLP-1(1-36)NH_2, which are biologically inactive (27, 31, 159), and GLP-1(7-37) and GLP-

1(7-36)NH_2, which are biologically active. GLP-1(7-37) and GLP-1(7-36)NH_2 are generated from their full-length precursors by the action of PC1/3 (130), and both peptides have equipotent insulin-stimulating activity (160). However, in humans, the majority of circulating GLP-1 is in the form GLP-1(7-36)NH_2 (161).

(b) Physiological Actions

The primary physiological role of GLP-1 is that of an incretin hormone. GLP-1, released from the gut in response to nutrient ingestion, is secreted into the circulation, and acts to augment glucose-stimulated insulin secretion (28, 30, 162). GLP-1 binds to its specific receptor on the pancreatic β-cell and stimulates both insulin secretion as well as insulin gene transcription and biosynthesis (163, 164), thereby contributing to the replenishment of β-cell insulin stores and preventing exhaustion of β-cell reserves. The insulinotropic effects of GLP-1 are glucose dependent, thus, when blood glucose levels fall, GLP-1 no longer stimulates insulin secretion, thereby preventing the possible development of hypoglycemia (28, 60, 165-167). GLP-1 also confers glucose sensitivity to glucose-resistant β-cells in both humans and rats, thereby improving the ability of the β-cell to sense and respond to glucose (47-49). A number of recent studies also indicate that GLP-1 may promote β-cell neogenesis and proliferation (54-57). *In vitro* experiments using a pancreatic β-cell line demonstrated that GLP-1, together with glucose, was able to activate the expression of immediate early genes that encode transcription factors regulating cell proliferation and differentiation (168). In addition, GLP-1 treatment was able to reverse the age-related decline in glucose tolerance in rats (169) and to convert nonendocrine AR42J cells into cells which can produce and secrete insulin (55).

GLP-1 inhibits glucagon secretion (31-35, 170, 171) and stimulates somatostatin secretion (31, 172-174). The increase in somatostatin secretion is mediated directly via GLP-1 receptors on the somatostatin-secreting pancreatic δ-cells (175, 176), whereas the inhibitory effect of GLP-1 on glucagon secretion may be indirect, through the stimulation of insulin and somatostatin secretion, both of which inhibit glucagon secretion (177). However, GLP-1 may also inhibit glucagon secretion directly, via interaction with GLP-1 receptors which are present on pancreatic α-cells (178).

GLP-1 has also been shown to inhibit pentagastrin- and meal-induced gastric acid secretion (42, 112, 179), as well as gastric emptying (43, 180-183). The inhibition of gastric emptying by GLP-1 promotes attenuation of meal-associated increases in blood glucose levels by slowing the transit of nutrients from the stomach to the small intestine.

GLP-1 receptors and GLP-1-immunoreactive nerve fibers are detected in brain regions known to be involved in the regulation of a number of homeostatic functions, including ingestive behavior (184-191). Central administration of GLP-1 has been shown to inhibit short-term food and water intake in rodents (36-38, 41, 189, 192) and peripheral administration of GLP-1 promotes satiety and suppresses energy intake in normal, diabetic and obese humans (39, 40, 193-195). In rodents, this inhibitory effect on food intake may be mediated by a direct interaction of GLP-1 with hypothalamic centers in the CNS which modulate feeding behaviour or, alternatively, via induction of a conditioned taste aversion (196-199).

GLP-1 has also been postulated to enhance glucose disposal directly (50, 51), possibly through peripheral effects on liver, skeletal muscle and adipose tissues (53). GLP-1 was found to enhance insulin-stimulated glucose metabolism in both adipocyte cell cultures

(52) and isolated rat adipocytes (200) and to increase glucose incorporation into glycogen in both isolated rat hepatocytes (201, 202) and skeletal muscle (202, 203). However, subsequent investigations were unable to confirm these reports (204, 205) and evidence for the presence of GLP-1 receptors on these peripheral tissues is ambiguous (206-210). In addition, although GLP-1 has been shown to enhance insulin sensitivity in depancreatized dogs (211), studies in both normal (212) and type 2 (non-insulin-dependent) diabetic humans (213, 214) do not support such a role for GLP-1. Thus, a role for GLP-1 in the mediation of glucose disposal in peripheral tissues, independent of its effects on insulin and glucagon, is controversial.

GLP-1 receptors are expressed on heart cells (210, 215) and in the nucleus of the tractus solitarius in the CNS, a region which is involved in the central control of cardiovascular function (190, 216). Consistent with these observations, GLP-1 treatment increases systolic, diastolic and mean arterial blood pressure and heart rate in rats (217, 218) and also increases heart rate in conscious calves (219).

GLP-1 may also modulate the hypothalamic-pituitary axis. GLP-1 receptors have been detected on cells of the pituitary gland in the rat (190, 220) and GLP-1 was reported to stimulate cAMP formation as well as thyroid-stimulating hormone (TSH) release from cultured mouse pituitary thyrotrophs and isolated rat anterior pituitary cells (221). GLP-1 also stimulated luteinizing hormone-releasing hormone (LHRH) secretion in a rodent hypothalamic neuronal cell line and central GLP-1 administration rapidly increased plasma luteinizing hormone (LH) levels (222) and stimulated hypothalamic neuroendocrine neurons (223) in rats.

Due to its ability to lower blood glucose levels through a number of different mechanisms, GLP-1 is currently being assessed in clinical trials as a potential therapeutic agent for the treatment of diabetes. Additionally, meal-induced GLP-1 secretion was found to be impaired in type 2 diabetic patients (224, 225), emphasizing the importance of GLP-1 replacement therapy in these patients. Although the sulfonylurea drugs currently being used to treat type 2 diabetes also stimulate insulin secretion, they do not stimulate insulin gene expression and biosynthesis nor is their activity glucose dependent (4, 82). Thus, sulfonylurea treatment does not replace depleted β-cell insulin stores and it has the additional risk of potentially inducing hypoglycemia, a complication of sulfonylurea drug treatment (226). A number of studies in humans and rodents with type 2 diabetes have shown that GLP-1 treatment can enhance glucose-stimulated insulin secretion and lower fasting as well as post-prandial blood glucose levels (49, 227-230). Furthermore, subcutaneous GLP-1 treatment for up to three weeks improved post-prandial glycemic control in diabetic patients (231, 232), indicating that GLP-1 maintains its effects, despite prolonged treatment. In addition, GLP-1 treatment was also found to decrease fasting and postprandial blood glucose levels as well as the meal-related insulin requirement in type 1 (insulin-dependent) diabetic patients (233-235), likely through inhibition of glucagon secretion and gastric emptying (234). These findings suggest that GLP-1 therapy may be beneficial to both noninsulin- and insulin-dependent diabetic patients. However, a potential drawback to GLP-1 is its very short plasma half-life (less than 2min) (61), due to rapid inactivation by the ubiquitous protease DPP-IV (see below) (61, 62, 236). These findings have prompted the development of DPP-IV-resistant GLP-1 analogs (237-240) and specific DPP-IV inhibitors (241) as potential anti-diabetic agents.

(c) Metabolism and Clearance

As noted above, the half-life of circulating, biologically active GLP-1 is less than 2 min. This relatively short plasma half-life is attributed to the protease activity of DPP-IV, which is present on the surface of white blood cells in the circulation and on the vascular endothelium in the small intestine, adjacent to the sites of GLP-1 release (242-244). DPP-IV catalyzes the cleavage of GLP-1, or its amidated derivative, at the position 2 Ala residue to yield the biologically inactive peptides GLP-1(9-37) and GLP-1 (9-36)NH_2, which may function as competitive antagonists of the GLP-1 receptor *in vivo* (245, 246).

In vitro studies have shown that the neutral endopeptidase (NEP-24.11) has endoproteolytic activity on GLP-1 and thus, may also be involved in the metabolism of GLP-1 (247, 248).

The primary route of clearance of GLP-1 appears to be through the kidney (249, 250). Uremic patients have elevated levels of circulating, immunoreactive GLP-1 relative to control individuals (251) and bilateral nephrectomy or uretal ligation in rats is associated with increases in the circulating half-life of GLP-1 (249). A role for tissues other than renal, such as the liver and lung, in GLP-1 clearance, has not been clearly established (249).

(d) The GLP-1 Receptor

The GLP-1 receptor (GLP-1R) was originally cloned from a rat pancreatic islet cDNA library via functional cloning (252). The receptor has subsequently been found to be expressed in a number of different tissues including the brainstem, hypothalamus, pituitary, lung, stomach, heart, intestine, α-, β- and δ-cells of the islets, and kidney (175, 178, 188, 190, 191, 208-210, 215, 253). The presence of GLP-1 receptors in peripheral tissues such as

muscle, liver and fat is not conclusive (208, 210) and has prompted the suggestion that structural variants or a second, closely related receptor may exist (4, 254).

The GLP-1R belongs to the seven transmembrane-spanning, heterotrimeric G protein-coupled family of receptors (252), which includes the GIP receptor, among others (255). Evidence to date indicates that GLP-1 mediates its effects via a single GLP-1R that appears to be identical in all tissues examined (215). *In vitro* studies have indicated that signaling through the GLP-1 receptor is coupled to both adenylate cylase and phospholipase C and activation of the PKA and PKC pathways, respectively (163, 208, 252). GLP-1 binding to its receptor is also associated with increases in intracellular calcium levels (256-258).

Structure/function studies have shown that the N-terminal histidine residue and the C-terminal residues 34-37 of GLP-1 are essential for GLP-1R activation (259, 260). The N-terminal extracellular domain of the GLP-1R is required for GLP-1 binding (261, 262), whereas the third intracellular loop is necessary for efficient coupling of the GLP-1R to adenylyl cyclase and subsequent activation of PKA (263, 264).

Little is known about the factors that regulate GLP-1R gene expression. Studies examining GLP-1R mRNA regulation *in vitro* found that receptor mRNA levels are down-regulated in response to GLP-1, activation of PKC, high glucose or dexamethasone (265-268). Analysis of the cloned 5'-flanking sequences of the human GLP-1R gene suggests that GLP-1R expression is positively regulated by the binding of Sp1 and Sp3 transcription factors to the promoter region (269), and cell- and tissue-specific GLP-1R gene expession is negatively regulated by more distal elements (270). Recently, a distal cell-specific silencer element (DCS) was identified in the 5'-flanking sequences of the GLP-1R gene (271). The DCS was found to be responsible for repressing human GLP-1R gene expression in

fibroblast and pancreatic α-cells, but not in pancreatic β or δ cells, indicating that the DCS mediates suppression of tissue- and cell-specific GLP-1R expression (271).

In islet cell lines, the GLP-1R undergoes homologous and heterologous desensitization (272-275) and internalization (275, 276), which are both correlated with receptor phosphorylation (273-275). However, desensitization of the GLP-1R has not been observed in studies performed *in vivo* (4).

An N-terminally truncated version of lizard exendin-4, exendin (9-39), can bind to the GLP-1 receptor and function as a specific GLP-1 receptor antagonist (65, 66, 277, 278). Exendin (9-39) is commonly used as a tool to examine the physiological consequences of loss of GLP-1 R signaling in both *in vitro* and *in vivo* studies. The ability of exendin (9-39) to significantly reduce postprandial insulin levels (279) and diminish glucose tolerance and total insulin secretory response in normal rats (280) led to the conclusion that GLP-1 is a physiologically important incretin *in vivo*. In humans, exendin (9-39) treatment increases postprandial blood glucose levels (281) and blocks the insulinotropic and glucagonostatic effects of physiological doses of GLP-1 (282), thus indicating that GLP-1 is important for the maintenance of normal glucose homeostasis. Additional studies with exendin (9-39) in humans (282) and fasted baboons (283) has provided evidence that GLP-1 may have a tonic inhibitory effect on glucagon secretion. Exendin (9-39) also inhibits the extrapancreatic effects of GLP-1 on gastric emptying (46, 284, 285), heart rate and blood pressure (218, 286), food intake (36, 37), and glucose metabolism in skeletal muscle and liver (287).

(e) The GLP-1R-/- Mouse

To ascertain the relative physiological importance of GLP-1-mediated actions, mice with a targeted disruption of the GLP-1R gene (GLP-1R-/-) were generated (288). These

mice are viable, fertile and appear to develop normally (288). GLP-1R-/- mice exhibit a mild fasting hyperglycemia and abnormal glycemic excursion in response to oral glucose, which is associated with a reduction in glucose-stimulated insulin secretion (288), consistent with an essential physiological role for GLP-1 in the regulation of glucose-stimulated insulin secretion. The mild diabetic phenotype of GLP-1R-/- mice may reflect the compensatory upregulation of glucose-dependent GIP secretion and insulinotropic action that is seen in these mice (289). GLP-1R-/- mice also exhibit glucose intolerance in response to intraperitoneal (i.p.) glucose administration (288), which bypasses the incretin and gastric-emptying effects of GLP-1, suggesting that even basal GLP-1-mediated signaling is important for handling a glucose load, irrespective of the site of glucose entry. In addition, despite the observation that GLP-1 enhances β-cell responsivity to glucose, isolated islets from GLP-1R-/- mice exhibit preserved glucose responsiveness (290). Although GLP-1 has been shown to inhibit glucagon secretion and regulate peripheral glucose disposal, GLP-1R-/- mice have normal basal and post absorptive glucagon levels (291), and display normal whole-body glucose utilization (291). However, *in vitro* studies using isolated islets from wild type and GLP-1R-/- mice suggest that the presence of the GLP-1R, even in the absence of bound ligand, is essential for maintaining the glucose-dependent insulin secretory capacity of the pancreatic β-cells (292). Although this study contradicts the observation of normal glucose competence in GLP-1R-/- islets (290), subsequent studies have shown that the β-cells of GLP-1R-/- mice have undergone compensatory changes in response to genetic deletion of the GLP-1R (293). These changes include reduced basal, but enhanced GIP-stimulated cAMP production, as well as abnormalities in both basal and glucose-stimulated

cytosolic calcium oscillations, thus supporting an essential role for the GLP-1R in β-cell signal transduction (293).

Although GLP-1 has been shown to be a potent inhibitor of food intake (36-38, 41, 189, 192), GLP-1R-/- mice exhibit normal body weight and feeding behaviour (288), indicating that GLP-1R signaling is not essential for regulation of satiety and maintenance of normal body weight. Moreover, GLP-1R-/- mice maintained on a high fat diet for 18 weeks do not develop obesity (294).

In light of the finding that central GLP-1 administration stimulates TSH, LH, corticosterone, and vasopression secretion in rats (221-223), the neuroendocrine consequences of disrupted GLP-1 signaling was assessed in GLP-1R-/- mice (295). Male GLP-1R-/- mice have slight reductions in gonadal weight and females exhibit small delays in pubertal onset (295). However, both males and females reproduce normally and respond appropriately to fluid restriction (295). In addition, despite a small but significant reduction in adrenal weights in both male and female GLP-1R-/- mice, adrenal histology and basal plasma levels of corticosterone were normal (295). The circulating levels of thyroid hormone, testosterone, estradiol and progesterone were also normal in these mice (295). However, GLP-1R-/- were found to have an exaggerated corticosterone response to stress, relative to control mice (295). Taken together, these findings suggest that, although generalized neuroendocrine function is normal in GLP-1R-/- mice, GLP-1 signaling appears to be involved in the normal neuroendocrine response to stress.

1.3 Glucose-Dependent Insulinotropic Polypeptide (GIP)

(a) Biosynthesis and Secretion

In rats the GIP gene is expressed in the K cells of the intestine and in the submandibular salivary gland (296). In humans, GIP expression occurs in the intestinal K cells (297, 298), and recent studies suggest that GIP is also expressed in rodent and human stomach (299).

There is very little information regarding the factors that regulate GIP gene expression. The promoter region of the human GIP gene contains binding sites for a number of transcription factors including Sp1, AP-1 and AP-2, and transient expression assays in an insulinoma cell line indicate that GIP gene expression is regulated by two cAMP responsive elements (300). In the rat, GIP gene promoter sequences between –193 and –182 contain a functional GATA element and are responsible for regulating cell-specific gene expression (301). In rats, a glucose meal (302) and intraduodenal infusion of fats (296) have been shown to up regulate duodenal GIP mRNA levels.

Bioactive GIP (1-42) is synthesized and released from the K cells of the duodenum and proximal jejunum primarily in response to the ingestion of glucose or fat (3, 18, 303), although recent studies in rats indicate that peptones can also potently enhance GIP levels (304). In both rats and humans, mature bioactive GIP is a 42 amino acid peptide that is generated by proteolytic processing of a larger preprohormone precursor (18, 296, 305). Studies indicate that the N-terminus of GIP is important for its biological activity (306).

GIP secretion is dependent on the rate of nutrient absorption, rather than the presence of nutrients, in the small intestine (307). *In vitro* studies using cultured canine endocrine cells indicate that GIP-producing cells are responsive to the activation of adenylyl cyclase,

elevations in intracellular calcium, potassium-mediated depolarization, glucose, GRP and β-adrenergic stimulation (308).

(b) Physiological Actions

In the endocrine pancreas GIP has a well-established role as an incretin hormone (13, 14, 309, 310). In response to nutrient ingestion, GIP is released from intestinal K cells into the circulation and subsequently binds to its specific receptor on the pancreatic β-cell, resulting in augmented glucose-stimulated insulin secretion and insulin gene expression (311-313). This likely occurs via activation of PKA (314) or increases in intracellular calcium (315). GIP appears to have no effect on glucagon secretion, but it can stimulate somatostatin secretion (173).

Although GIP would appear to be a useful agent for the treatment of diabetes, studies with type 2 diabetic patients indicate that they have normal or even elevated serum levels of GIP (316-319), with the inactive truncated catabolite (GIP 3-42) as the predominant molecular form (320). Moreover, GIP has very little insulinotropic or glucagon-lowering activity in diabetic subjects (60, 321). These observations are consistent with reports that indicate that GIP gene expression is enhanced in diabetic animals, and that elevated plasma GIP levels promote chronic homologous desensitization of the GIP receptor (322). It has been suggested that such a mechanism could contribute to the impaired insulin secretion that is seen in type 2 diabetic patients (322).

In the stomach, GIP can inhibit gastric acid secretion, but only at supraphysiological doses (323). GIP has also been shown to up regulate hexose transport in the intestine (118). In the liver, GIP attenuates glucagon-stimulated hepatic glucose production (324, 325), likely through an indirect mechanism as GIP receptors have not been detected in the liver (255). In

adipose tissue, GIP stimulates fatty acid synthesis (326), enhances insulin-stimulated incorporation of fatty acids into triglycerides (327), and increases both the affinity of insulin for its receptor and the sensitivity of insulin-stimulated glucose transport (328). The signaling mechanism(s) by which GIP mediates its effects on adipocytes is currently not known (18). However, functional GIP receptors are expressed on adipocytes (255, 329, 330), and limited studies indicate that GIP binding promotes increases in the accumulation of intracellular cAMP levels in adipocyte cultures (329, 330). GIP receptor mRNA and protein are also found in normal bone and osteoblast-like cell lines (331). Treatment of osteoblast-like cells with GIP stimulates dose-dependent increases in cAMP and intracellular calcium levels and results in increased alkaline phosphatase activity and elevated collagen type I mRNA levels (331). Additionally, despite the detection of GIP receptor mRNA in a number of different tissues including the heart, adrenal cortex, testis, lung, intestine, and several regions in the brain (18, 255), the effect, if any, of GIP in these tissues remains to be determined.

(c) Metabolism and Clearance

In humans the half-life of immunoreactive GIP was determined to be approximately 20 min (15, 332-334). However, this value does not reflect the half-life of biologically active GIP, which has been estimated to be less than 2 min in rats (60) and approx 7 min and 5 min in normal and type 2 diabetic human subjects, respectively (320). The main route of clearance of GIP is through the kidney (335), and the liver appears to have no significant role in GIP clearance (336, 337).

A role for DPP-IV in the degradation of GIP (1-42) to the biologically inactive fragment GIP (3-42) has been established by *in vitro* studies using human serum (62).

Additional studies in rats and normal and diabetic humans indicate that DPP-IV is likely the primary GIP-inactivating enzyme *in vivo* (61, 320).

(d) The GIP Receptor

The GIP receptor (GIPR) was originally cloned from a rat cerebral cortex cDNA library using the polymerase chain reaction (PCR) and degenerate oligonucleotide primers corresponding to conserved amino acid residues in the transmembrane domains of members of the secretin-VIP receptor family (255). Subsequently GIPR mRNA was found to be present in the pancreas, stomach, small intestine, adipose tissue, adrenal gland, lung, pituitary, heart, testis and several regions of the brain (255, 329). Similar to the GLP-1R, the GIPR belongs to the seven-transmembrane domain, heterotrimeric G protein-coupled receptor family (255).

To date, little is known about the factors which regulate GIPR mRNA expression. Cloning and analysis of the rat GIPR gene revealed that the 5'-flanking sequences of the gene contain a number of potential regulatory elements, including a cAMP response element, an octamer binding site, three SP1 sites, as well as a transcription initiator element consensus sequence (338). However, there are no TATA or CAAT box motifs upstream of the transcription initiation site (338). In addition, cis-acting negative regulatory elements that mediate cell-specific expression may be present in the distal 5'-flanking sequences of the rat GIPR gene (338).

At the protein level, GIPR signaling is coupled to cAMP activation and increases in intracellular calcium levels (208, 256, 315, 339-344). Studies have also suggested that, in addition to cAMP, GIP may activate signal transduction via PI-3-kinase (345) or MAP kinase (MAPK) (346).

In vitro structure/function studies indicate that the N-terminal domain and the first extracellular loop of the GIPR are necessary for high-affinity binding of GIP, whereas the distal part of the N-terminal domain and the first transmembrane domain are essential for receptor activation and coupling to cAMP (347). In addition, a recent study has concluded that most of the C-terminal tail of the GIPR is not required for intracellular signaling, but that a minimum receptor length of approximately 405 amino acids is required for efficient receptor transport and plasma membrane insertion (348).

The GIPR undergoes rapid and reversible homologous desensitization (3) and specific serine residues in the C-terminal tail of the GIPR, especially serines 426 and 427, are necessary for regulating the rate of receptor internalization (348).

(e) The GIPR-/- Mouse

To determine the role of GIP-mediated signaling in the enteroinsular axis, mice with a targeted deletion of the GIPR gene (GIPR-/-) were generated (349). GIPR-/- mice exhibit normal fasting glucose and glycemic excursion in response to an intraperitoneal glucose challenge (349). However, in response to an oral glucose load, GIPR-/- display impaired glucose tolerance, which is associated with a significant reduction in insulin secretion (349). These results suggest that GIP has an important role as an incretin hormone, but GIP signaling does not appear to be essential for handling a non-enteral glucose load.

In normal rodents which are placed on a high fat diet to induce insulin resistance, meal-induced glycemic excursion remains normal, due to compensatory increases in insulin secretion. However, in GIPR-/- mice maintained on a high fat diet, the meal-induced glycemic excursion was abnormally elevated due to reduced compensatory enhancement of insulin secretion (349). These results indicate that GIP signaling has a significant role in

mediating the compensatory enhancement of insulin secretion under conditions of insulin resistance.

1.4 Dipeptidylpeptidase IV (DPP-IV)/CD26

Dipeptidylpeptidase IV (DPP-IV), also known as CD26, is a ubiquitous multifunctional glycoprotein that is expressed either on the surface of a number of different cell types, or as a soluble form in plasma (242, 243). DPP-IV belongs to the prolyl oligopeptidase family and is a serine protease which specifically cleaves dipeptides from the amino terminus of oligopeptides or proteins containing an alanine or proline residue in position 2 (i.e. X-Ala/Pro), thereby rendering them inactive or modifying their activity (242, 243). In addition to its protease activity, DPP-IV can also bind to proteins including collagen and adenosine deaminase (350, 351), and has been shown to have a role in both T-cell costimulation and tumour suppression (352-354).

DPP-IV has a fairly widespread distribution, being found in a number of tissues including kidney, lung, adrenal gland, intestine, liver, spleen, testis, pancreas and CNS, as well as on the surface of endothelial cells of blood vessels and in plasma (242, 243). DPP-IV is also expressed on activated T-helper lymphocytes and macrophages (355, 356).

GLP-1 and GIP contain an alanine residue in position two, and thus are both substrates for DPP-IV enzymatic activity. A number of studies have indicated that DPP-IV-mediated cleavage is the primary mechanism whereby GLP-1 and GIP are inactivated (61-63). DPP-IV catabolizes GLP-1 to GLP-1(9-36)NH_2 and GLP-1(9-37), which can bind, with low-affinity, to the GLP-1 receptor and may function as competitive antagonists of the GLP-1 receptor *in vivo* (63, 245, 246, 357). In human studies with normal and type 2 diabetic

patients, intravenous or subcutaneous GLP-1 was rapidly degraded (within 30 min) to GLP-1(9-36)NH_2, which accounted for more than 75% of the immunodetectable GLP-1 from these patients (358). Cleavage of GIP by DPP-IV leads to the formation of GIP (3-42), which is biologically inactive (62, 306). In *in vivo* studies with rats, greater than 50% of an intravenous bolus of GIP or GLP-1 was metabolized to its N-terminal truncated form by DPP-IV within two minutes of peptide administration (61). In contrast, N-terminal truncation was absent when these peptides were infused into a strain of rats that is DPP-IV deficient (61). Moreover, a number of studies indicate that pharmacological inhibition of DPP-IV activity can prolong the half-lives of bioactive GIP and GLP-1 (61, 359-361).

In light of the short plasma half-live of biologically active GLP-1, due to its rapid catabolism by DPP-IV, and the observation that the incretin effect of GLP-1 is preserved in type 2 diabetic patients, the use of agents which inhibit DPP-IV activity has been proposed as a potential treatment for type 2 diabetes (241, 358). In anesthetized pigs, pharmacological inhibition of greater than 90% of the DPP-IV activity with valine-pyrrolidide resulted in an increase in the levels of intact GLP-1 in both the basal (endogenous) state and following GLP-1 infusion (359). Furthermore, this increase in the half-life of bioactive GLP-1 was associated with enhancement of both the insulinotropic and glucagonostatic effects of GLP-1 (359). In rats, the DPP-IV inhibitor Ile-thiazolide increased the plasma half-life of intact, endogenous GLP-1 released in response to intraduodenal glucose, which resulted in a shortening of the time required to reach peak insulin levels and thus, a more rapid clearance of blood glucose (360). In both lean and obese Zucker rats, oral administration of the DPP-IV inhibitors Ile-thiazolide or NVP-DPP728 enhanced insulin secretion and improved glucose tolerance (362, 363). Moreover, DPP-IV inhibition in both control and high-fat fed

glucose intolerant mice increased the levels of endogenous GLP-1 and improved glucose tolerance and insulin secretion (361). Taken together, these studies indicate that DPP-IV inhibition could be a promising strategy for the treatment of diabetes.

However, DPP-IV also degrades certain cytokines and other important regulatory peptides that contain an alanine or proline in the penultimate N-terminal position, including neuropeptide Y (NPY), growth hormone releasing factor (GRF) and peptide YY (242). Furthermore, in addition to its protease activity, DPP-IV has also been implicated in the costimulation and activation of T-cells (352, 353). Thus, the use of DPP-IV inhibitors as a therapeutic treatment for diabetes could result in undesirable side effects.

As an alternative therapeutic approach, a number of DPP-IV-resistant GLP-1 analogues have been synthesized and their metabolic stability, biological activity, potency and duration of action have been assessed (237-240, 364-366). Promising results have been obtained with GLP-1-Gly8 (365) and with fatty-acid-derivatized GLP-1 analogues (239).

1.5 Exendin-4

(a) Peptide Discovery and cDNA Cloning

Most members of the glucagon superfamily of peptides, including GLP-1, have a highly conserved histidine residue at their amino terminus (His^1), as well as a phenylalanine residue at position six (367). Included in this family are two biologically active peptides, helodermin and helospectin, which were purified from the venom of Helodermatidae lizards (368-370). Thus, in an attempt to identify new bioactive peptides in lizard venom, amino-terminal sequencing was used as a strategy to screen for novel His^1 peptides (371). Using this technique, a 39-amino acid carboxy-terminal amidated peptide, designated exendin-4,

was identified and purified from the venom of a *Heloderma suspectum* lizard (64). The designation exendin-4 is derived from the observation that this peptide is found in an exocrine secretion and has endocrine activity (see below) (371).

It was determined that exendin-4 shares a 53% amino acid sequence identity with mammalian GLP-1 and subsequent studies have determined that exendin-4 is a GLP-1 receptor agonist (65, 66, 277, 278). Thus, exendin-4 can bind to the GLP-1 receptor and mimic a number of the biological activities of GLP-1, including the abilities to increase cAMP levels in guinea pig pancreatic acinar cell preparations (64) and stimulate cAMP-dependent H^+ production in rat parietal cells (277). Exendin-4 also stimulates glucose-dependent insulin secretion and increases cAMP levels and insulin gene expression in isolated rat islets and cultured islet cell lines (65). Moreover, as mentioned previously in section 1.2 (d), an exendin-4 fragment, exendin-(9-39), has been shown to be a specific GLP-1 receptor antagonist. Exendin (9-39) can block the binding and biological activities of both exendin-4 and GLP-1 (36, 37, 218, 246, 279-284, 287, 372, 373).

In light of the 53% sequence identity between GLP-1 and exendin-4, as well as the ability of exendin-4 to function as a GLP-1 receptor agonist, it was originally believed that exendin-4 may represent the reptilian GLP-1 homologue. However, the subsequent cloning and sequencing of cDNAs for both lizard proglucagon and exendin-4 demonstrated that exendin-4 is not the reptilian equivalent of GLP-1 (374). In the lizard two different proglucagon cDNAs, lizard proglucagon I and II (LPI and LPII), with unique 3'-untranslated regions were identified (374). LPI encodes glucagon and GLP-1 and its expression is restricted to the lizard pancreas (374). In contrast, LPII encodes glucagon, GLP-1 and GLP-

2 and is expressed in both the lizard pancreas and intestine (374). No mRNA transcripts corresponding to lizard proglucagon could be detected in lizard salivary gland (374).

Lizard exendin-4 is encoded by a single RNA transcript of approximately 500 nt which also encodes a 45 amino acid peptide N-terminal to the exendin sequence (designated exendin N-terminal peptide; ENTP). ENTP is followed by a pair of basic amino acids (characteristic of prohormone convertase cleavage sites), and the 40-amino acid long exendin sequence (374) (Fig. 3). The function of ENTP is currently not known but the N-terminal half of this peptide could represent a signal sequence. Lizard exendin-4 mRNA transcripts are detected only in the lizard salivary gland, and not in the pancreas or intestine (374). In addition to these studies, Southern blot analysis of lizard genomic DNA with exendin-4- and GLP-1-specific cDNA probes confirmed that lizard exendin-4 and lizard GLP-1 are distinct peptides encoded by unique lizard genes (374, 375).

The finding that proglucagon and exendin-4 are encoded by unique genes in the lizard suggests that other species, including mammals, could possess an exendin-4 gene. However, attempts by our laboratory (Baggio and Drucker, unpublished data; see Appendix 1) and others (375) to clone a mammalian exendin-4 homologue have been unsuccessful.

(b) Physiological Activities

Exendin-4 is a GLP-1 receptor agonist and has been shown to mimic the biological effects of GLP-1 in a number of different systems. Exendin-4 produces significant elevations in systolic, diastolic and mean arterial blood pressure and heart rate in rats, but its effects are more prolonged than those of GLP-1 (218), likely due to decreased degradation of exendin-4 (see below). Studies in rats have indicated that exendin-4 is approximately 90-fold more potent than GLP-1 in its ability to slow gastric emptying (67). Like GLP-1, exendin-4

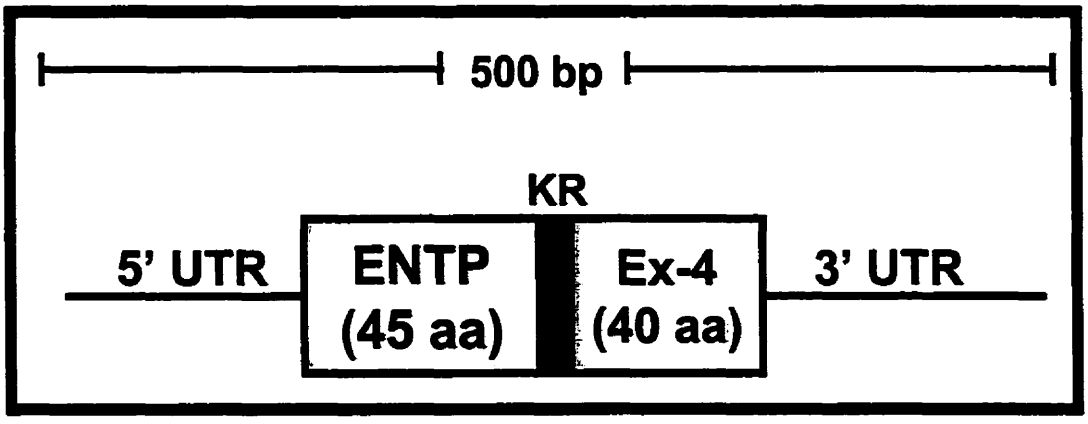


Fig. 3. Lizard proexendin-4 cDNA structure. Lizard exendin-4 is encoded by a single proexendin-4 RNA transcript of approx 500 bp. The lizard proexendin-4 cDNA encodes a 45 amino acid N-terminal peptide (ENTP), followed by a pair of basic amino acids (KR) and the 40 amino acid exendin-4 sequence (Ex-4). UTR indicates untranslated region.

increases glycogen synthase *a* activity and glucose incorporation into glycogen in both rat hepatocytes and skeletal muscle (287). Exendin-4 can also stimulate exogenous glucose uptake and metabolism in muscle (287). At the level of the pancreatic β-cell, exendin-4 binds to GLP-1 receptors and enhances glucose-stimulated insulin secretion both *in vitro* in isolated rat islets (65) and *in vivo* in rats (280). Exendin-4 also stimulates insulin gene transcription in mouse insulinoma cells (65). Relative to GLP-1, exendin-4 has been shown to be a more effective and more potent insulinotropic agent *in vivo* in rats (376) and to stimulate greater elevations in cAMP levels *in vitro* in isolated rat islets (72). Thirteen weeks of once-daily exendin-4 treatment (24 nmol/kg i.p.) significantly reduced fasting hemoglobin A_{1c} and blood glucose levels and increased fasting plasma insulin concentration in diabetic *db/db* mice (72). In diabetic *db/db* or *ob/ob* mice, acute exendin-4 treatment had a more potent and prolonged glucose lowering effect than GLP-1 (68). Treatment of Zucker diabetic *fa/fa* rats with i.p. exendin-4 twice-daily for 6 weeks led to significant dose-dependent reductions in food intake, body weight and hemoglobin A_{1c} levels, as well as an approximately 50% improvement in insulin sensitivity relative to saline-treated controls (68). In contrast to previous studies demonstrating that acute central (intracerebroventricular; ICV), but not peripheral (i.p.), GLP-1 treatment can inhibit food intake in normal rats (37), long-term subcutaneous or i.p. exendin-4 administration produced significant and sustained reductions in food intake and body weight in both normal and diabetic rats (68, 69). More recent studies have also demonstrated that, similar to GLP-1, exendin-4 stimulates β-cell neogenesis and proliferation in rats (56) and can convert AR42J cells, which have exocrine and neuroendocrine properties but lack islet hormone expression, into pancreatic endocrine cells (55).

As indicated above, a number of studies have demonstrated that exendin-4 is more potent and that its physiological effects are more prolonged relative to GLP-1. This is likely due, in part, to the longer plasma half-life of exendin-4 (18-41 min. following i.v. bolus in rats) (71), compared to GLP-1 (less than 2 min. following i.v. bolus in rats) (61). GLP-1 contains an alanine residue in position two and thus, is rapidly catabolized by DPP-IV. In addition, GLP-1 is also a potential target for the endoproteolytic activity of NEP-24.11 (247). In contrast, exendin-4 contains a glycine residue in position two and is not a substrate for DPP-IV-mediated catabolism. As well, exendin-4 has been shown to be a very poor substrate for NEP-24.11 (247). The greater potency and extended plasma half-life of exendin-4 suggest that it may be more useful than GLP-1 as a therapeutic agent for the treatment of diabetes. In light of these findings, exendin-4 is currently being evaluated in clinical trials to assess its potential as an antidiabetic agent.

1.6 Rationale, Hypotheses and Specific Goals of this Research

Do GLP-1 and GIP have distinct roles in glucose homeostasis? Incretins, the gut-derived factors that augment glucose-stimulated insulin secretion, have been shown to be essential mediators of normal glucose homeostasis. In fact, the incretin effect has been shown to account for as much as 50% of the insulin secretory response to oral glucose (8). In addition, the incretin effect is reduced or lost in type 2 diabetic patients (377), again emphasizing the importance of incretin action for glycemic control.

Thus far only two endogenous mammalian peptide hormones, GIP and GLP-1, possess the necessary activities to fulfill the requirements of an incretin hormone, and thus represent potential therapeutic agents for the treatment of diabetes. To this end,

administration of GLP-1 to type 2 diabetic patients was insulinotropic, whereas GIP was not, indicating that the incretin effect of GLP-1 is preserved in diabetic individuals.

Although GIP and GLP-1 have both been shown to augment glucose-stimulated insulin secretion, in addition to its role as an incretin, GLP-1 also contributes to the lowering of blood glucose by its additional pleiotropic effects on glucagon secretion, gastric emptying, food intake and peripheral glucose disposal. GLP-1 also has a glucose-sensitizing effect on glucose-resistant β-cells and may play a role in islet differentiation and β-cell mass expansion. The importance of these "non-incretin" effects of GLP-1 are further illustrated by studies in mice with a targeted disruption of the GLP-1 receptor gene (GLP-1R -/-). These mice exhibit mild fasting hyperglycemia and impaired glucose tolerance in response to either oral or intraperitoneal glucose challenge.

The principle action of GIP appears to be that of an incretin hormone. The biological importance of the incretin function of GIP has been supported by studies in GIP receptor -/- (GIPR-/-) mice which exhibit impaired glucose tolerance following oral glucose loading, but fasting glucose and the glycemic response to an intraperitoneal glucose challenge is normal.

Taken together, studies in the GLP-1R -/- mouse suggest that, in addition to its role as an incretin, GLP-1 may also have non-incretin effects that are required for glucoregulation. In contrast, studies with the GIP R -/- mouse indicate that the role of GIP in glucose homeostasis is restricted to that of an incretin. Thus, these observations in receptor knock out mice indicate that GIP and GLP-1 have differential roles in glucose homeostasis. However, a potential drawback to studying receptor knock out mice is that they may not accurately reflect normal physiology due to adaptive compensatory changes in regulatory systems. This is supported by the observation that although GLP-1 has been shown to inhibit

food intake in rodents and humans, GLP-1 R -/- mice display normal body weight and feeding behaviour. Moreover, GLP-1R-/- mice demonstrate upregulation of GIP secretion and action in response to oral glucose.

To avoid potential confounding adaptive changes in glucoregulation that may occur with studies in genetically modified mice, in this study we have used specific antagonists of GIP and GLP-1 action in both wild type and GLP-1R -/- mice to assess the relative contribution of these peptides to glycemic control *in vivo* and to verify our hypothesis that GIP and GLP-1 have distinct roles in glucose homeostasis.

Is the improved glucose tolerance in mice treated with pharmacological inhibitors of DPP-IV activity due to potentiation of GLP-1 action? GLP-1 is currently being evaluated in clinical trials as a potential therapeutic agent for the treatment of diabetes. However, the therapeutic potential of GLP-1 may be limited by its very short plasma half-life due, in part, to its rapid inactivation by the peptidase dipeptidylpeptidase IV (DPP-IV). Active GLP-1 is hydrolyzed by DPP-IV to yield GLP-1 (9-37/36)NH_2, which is inactive or weakly antagonistic.

DPP-IV, also known as CD26, is a serine protease belonging to the DPP-IV-like gene family. DPP-IV specifically cleaves N-terminal dipeptides from proteins containing proline or alanine in position 2. Both GLP-1 and GIP are substrates for the proteolytic activity of DPP-IV, which leads to the formation of inactive truncated versions of these peptides. A number of studies have demonstrated that pharmacological inhibition of DPP-IV activity results in elevated levels of circulating GLP-1 and improved glucose tolerance and enhanced insulin secretion in both normal and diabetic animals. These studies suggest that treatment with agents that inhibit DPP-IV activity, either alone or in conjunction with GLP-1 therapy,

may be useful for treating diabetes. However, a number of enzymes exhibit DPP-IV-like proteolytic activity (378-380), making it difficult to identify the exact targets of DPP-IV inhibitors.

Recently, a transgenic mouse line containing a targeted disruption of the CD26 gene was generated (381). CD26 -/- mice clear an oral glucose load more rapidly than wild type control littermates and they exhibit increased glucose-stimulated insulin secretion in association with increased levels of intact, biologically active GLP-1 (381).

To examine our hypothesis that the improved glucose tolerance in (a) CD26-/- mice and (b) mice treated with pharmacological inhibitors of DPP-IV activity, is largely due to potentiation of GLP-1 action, we studied glucose homeostasis in wild type and GLP-1R-/- mice treated with valine-pyrrolidide, a specific DPP-IV inhibitor.

Does long term treatment with exendin-4 lead to undesirable physiological effects? An alternative antidiabetic therapeutic approach to the use of agents which inhibit DPP-IV activity is the use of DPP-IV-resistant GLP-1 analogs. One such naturally occurring GLP-1 receptor agonist is exendin-4. As mentioned above, exendin-4 binds to the GLP-1 receptor and elicits a variety of biological effects which mimic the actions of GLP-1, including its abilities to enhance glucose-stimulated insulin secretion, regulate gastric emptying, act as a satiety factor and promote pancreatic endocrine cell differentiation and expansion of β-cell mass. As well, *in vivo* studies indicate that the insulin secretory effect of exendin-4 is more potent than that of GLP-1.

Exendin-4 is currently being evaluated in clinical trials as a potential therapeutic agent for the treatment of diabetes. However, the long term consequences of expression of exendin-4, a lizard-derived peptide, in mammalian systems remains unknown. Therefore, in

order to assess the physiological effects of chronic expression of lizard exendin-4 *in vivo*, we have generated a transgenic mouse line in which proexendin 4 expression can be regulated by induction of the mouse metallothionein I promoter.

In summary, this thesis addresses several unanswered questions regarding incretin action, including (i) the relative roles of the known incretin hormones, GIP and GLP-1, in glucose homeostasis, (ii) the potential existence of other agents, in addition to GIP and GLP-1, which can contribute to glucose homeostasis, and (iii) the physiological effects of prolonged incretin activity.

Chapter 2. Differential Roles for GIP and GLP-1 in the Control of Glucose Homeostasis

2.1 Introduction

The observation that oral glucose administration stimulates a greater increase in insulin secretion from pancreatic β-cells than an isoglycemic intravenous infusion has stimulated considerable interest in the identity of gut-derived molecules that enhance insulin secretion. The term incretin has been ascribed to factors released from the gut in response to nutrient ingestion that potentiate glucose-stimulated insulin secretion (6). To date, the two principal peptides that exhibit incretin-like activity are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Together these two peptides are thought to account for most, if not all of the incretin effect (3, 4, 382).

GIP is a 42-amino acid peptide that is synthesized in intestinal K-cells in the proximal jejunum and secreted primarily in response to the ingestion of glucose or fat (3, 4). In contrast, GLP-1, a post-translational product of the proglucagon gene (73), is released from more distally located intestinal L-cells in response to ingestion of glucose or a mixed meal (4, 29, 146, 383). Under conditions of elevated blood glucose concentrations, both GIP and GLP-1 stimulate insulin secretion and proinsulin gene transcription via specific receptors expressed on islet β-cells (163, 164, 311, 312).

The principle action of GIP appears to be the stimulation of glucose-dependent insulin secretion following enteral nutrient ingestion. Consistent with this hypothesis, GIP immunoneutralizing antisera or a GIP receptor peptide antagonist reduced insulin secretion following oral glucose challenge in rats (20, 384). The biological importance of GIP as an incretin is further illustrated by GIP receptor -/- mice that exhibit defective glucose clearance

following oral glucose loading, but normal fasting glucose and glycemic excursion after intraperitoneal glucose challenge (349). In contrast, GLP-1 R-/- mice exhibit fasting hyperglycemia and abnormal glycemic excursion in response to both oral and intraperitoneal glucose challenge (288).

Although results of studies in knockout mice may be used to infer specific physiological actions of GIP and GLP-1 for control of glucose homeostasis, disruption of incretin receptor signaling from birth may be associated with subtle developmental and adaptive changes that could modify the interpretation of physiological studies. For example, GLP-1R-/- mice exhibit abnormalities in the hypothalamic-pituitary-adrenal axis (295) and up regulation of glucose-dependent GIP secretion and enhanced sensitivity to GIP action (289), complicating the interpretation of results ascribed simply to interruption of GLP-1R signaling *in vivo*. Accordingly, to control for potential confounding developmental or adaptive changes in incretin action observed in genetically modified mice, we have assessed the importance of GIP and GLP-1 for glycemic control in wild-type, as well as GLP-1R-/-, mice using antagonists of GLP-1 and GIP action *in vivo*.

2.2 Methods

2.2.1 Animals

GLP-1 receptor -/- (288) and age-matched (6- to 8-week-old males) wild-type CD1 mice (Charles River Laboratories, Inc. Montreal, Quebec) were housed under a 12 h light, 12 h dark cycle in the Toronto General Hospital animal facility with free access to food (standard rodent chow) and water, except where noted. All wild-type mice used for these studies were acclimatized to the animal facility for several weeks prior to analysis. All

procedures were conducted according to protocols and guidelines approved by the Toronto Hospital Animal Care Committee.

2.2.2 Glucose Tolerance Tests and Measurement of Plasma Insulin Levels

Oral (OGTT) or intraperitoneal (IPGTT) glucose tolerance tests were carried out following an overnight fast (16-18 h). The GLP-1 receptor antagonist exendin (9-39)NH_2 (5 μg; California Peptide Research Inc., Napa , CA) or phosphate-buffered saline (PBS) was administered intraperitoneally 20 min prior to glucose administration, after a fasting blood glucose measurement had been obtained. Anti-GIP R antiserum (provided by Dr. Timothy Kieffer, University of Alberta, Edmonton, Canada) was raised in rabbits against a synthetic peptide containing an extracellular epitope of the GIP receptor (Gly-Gln-Thr-Thr-Gly-Glu-Leu-Tyr-Gln-Arg-Trp-Glu-Arg-Tyr-Gly-Trp-Glu-Cys) coupled to KLH (385). Immunopurified GIPR antibody (GIPR Ab) blocks GIP-mediated increases in intracellular cAMP and specifically displaces ^{125}I-GIP binding with half-maximal displacement at approximately 1 μg/ml (385). In the rat, plasma levels of GIPR Ab peak approximately 4h after i.p. injection and remain at this level for 2 days (385). When GIPR Ab is delivered at a dose of 1 μg/g body weight, the insulinotropic action of an exogenous bolus of GIP is completely abolished in rats (385). Immunopurified GIPR Ab (1 μg/g body weight) or a rabbit γ-globulin control (1μg/g body weight; Jackson Immuno Research Laboratories, Inc.,West Grove, PA) was given intraperitoneally at the onset of fasting, 16-18 hr prior to the glucose tolerance tests. For glucose tolerance tests, mice were given 1.5 mg glucose/g body weight orally through a gavage tube (OGTT) or via injection into the peritoneal cavity (IPGTT). Blood was drawn from a tail vein at 0, 10, 20, 30, 60, 90 and 120 minutes following glucose administration, and blood glucose levels were measured by the glucose

oxidase method using a One Touch Basic Glucometer (Lifescan Ltd., Burnaby, BC). Blood samples (100 µl) for measurement of insulin secretion were removed from tail veins during the 10- to 20-minute time period following oral or intraperitoneal glucose administration and immediately mixed with a 10% vol of a chilled solution containing 5000 KIU/ml Trasylol (Miles Canada, Etobicoke, Canada), 32 mM EDTA, and 0.1 nM Diprotin A (Sigma Chemical Co., St. Louis, MO). Plasma was separated by centrifugation at 4°C and stored at -80°C until assayed. Plasma samples were assayed for insulin content using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois) with mouse insulin as a standard.

2.2.3 Prolonged Exposure to Incretin Antagonists

For more protracted studies, all mice were given free access to standard rodent chow and water during the course of the experiments. Wild-type CD1 and GLP-1 receptor -/- mice were given intraperitoneal injections of either PBS or 5 µg of exendin (9-39)NH_2 in 8% gelatin. Injections were commenced at 0500 h, and each animal was given an intraperitoneal injection of the appropriate test substance every 4 h, with the last injection given 3 h prior to sacrifice, for a total of 15 h of treatment. Blood glucose levels were measured, animals were euthanized and blood was obtained by cardiac puncture. Plasma was collected from the blood samples for analysis of insulin levels (as described above). The pancreas was removed from each animal and a portion was used for RNA isolation and Northern blot analysis. The remaining portion of the pancreas was homogenized twice in 5 ml of extraction medium [1 N HCl containing 5% (vol/vol) formic acid, 1% (vol/vol) trifluoroacetic acid, and 1% (wt/vol) NaCl] at 4°C. Peptides and small proteins were adsorbed from extracts by passage through a C18 silica cartridge (Waters Associates, Milford, MA). Adsorbed peptides were eluted with 4 ml of 80% (vol/vol) isopropanol containing 0.1% (vol/vol) trifluoroacetic acid. Pancreatic

insulin levels were measured using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois) with mouse insulin as a standard. Total protein levels in extracts were determined using the Bradford method (386) with Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA). For longer term studies with GIPR Ab, wild-type CD1 and GLP-1 receptor -/- mice were given intraperitoneal injections of either rabbit γ-globulin or 1μg/g body weight of purified GIPR Ab. Only a single injection of GIPR Ab was required, because the antibody is stable in plasma for several days (385). At 18 h following administration of GIPR Ab or γ-globulin control, mice were euthanized with CO_2 and exsanguinated by cardiac puncture. Blood glucose, plasma insulin and pancreatic insulin content were determined as described above.

2.2.4 RNA Isolation & Northern Blot Analysis

Following prolonged exposure to PBS, exendin (9-39), rabbit γ-globulin or GIPR Ab, mice were euthanized with CO_2, and pancreases were removed immediately for RNA extraction by the acid-guanidinium isothiocyanate method (387). Total RNA (10 μg) was electrophoresed in a 1% (wt/vol) formaldehyde-agarose gel and transferred to a nylon membrane (Nytran Plus; Schleicher and Schuell, Keene, New Hampshire). For Northern blot analysis, the blot was hybridized to ^{32}P-labeled random-primed cDNA probes corresponding to rat proglucagon, rat insulin or 18S rRNA.

2.2.5 Statistics

Results are expressed as means ± SEM. Statistical significance was calculated by ANOVA and Student's *t*-test using INSTAT 1.12 (Graph-Pad Software, Inc., San Diego, CA). A p value < 0.05 was considered to be statistically significant.

2.3 Results

2.3.1 Effects of Exendin (9-39) on Blood Glucose and Plasma Insulin

To assess the effects of acute blockade of GLP-1R signaling *in vivo*, we used the GLP-1 receptor antagonist exendin (9-39), a truncated lizard GLP-1-related peptide that binds to and antagonizes mammalian GLP-1 receptors (65). Treatment of wild-type mice with exendin (9-39) immediately prior to oral glucose challenge produced a statistically significant increase in blood glucose excursion during the 10- to 30-minute time period following glucose administration (Fig. 4A; $p<0.05$ for saline- vs. exendin (9-39)-treated mice). Surprisingly, plasma insulin levels were not significantly different, following oral glucose loading, in saline- vs. exendin (9-39)-treated mice (Fig. 4B).

Because GLP-1R -/- mice exhibit abnormal glycemic excursion following both oral and intraperitoneal glucose challenge, these findings suggest that GLP-1-mediated signaling events are important for β-cell function and glucose disposal, independent of the site of glucose entry (288). Consistent with the importance of non-incretin actions of GLP-1 for glucoregulation, exendin (9-39) increased significantly the glucose excursion following intraperitoneal glucose challenge ($p<0.05$, saline- vs. exendin (9-39)-treated mice, from 30-120 min; Fig. 5A). Furthermore, the levels of glucose-stimulated circulating insulin were reduced significantly in exendin (9-39)-treated mice (Fig. 5B; $p<0.05$; 0.58 ± 0.02 vs. 0.47 ± 0.02 ng/ml in saline- vs. exendin (9-39)-treated mice, respectively).

Although exendin (9-39) is generally viewed as a specific GLP-1 receptor antagonist, several reports suggest that exendin (9-39) may also bind to the GIP receptor and potentially antagonize the actions of GIP (314, 315). To verify that exendin (9-39) is a specific antagonist of murine GLP-1 receptor signaling *in vivo*, we assessed the effect of exendin (9-

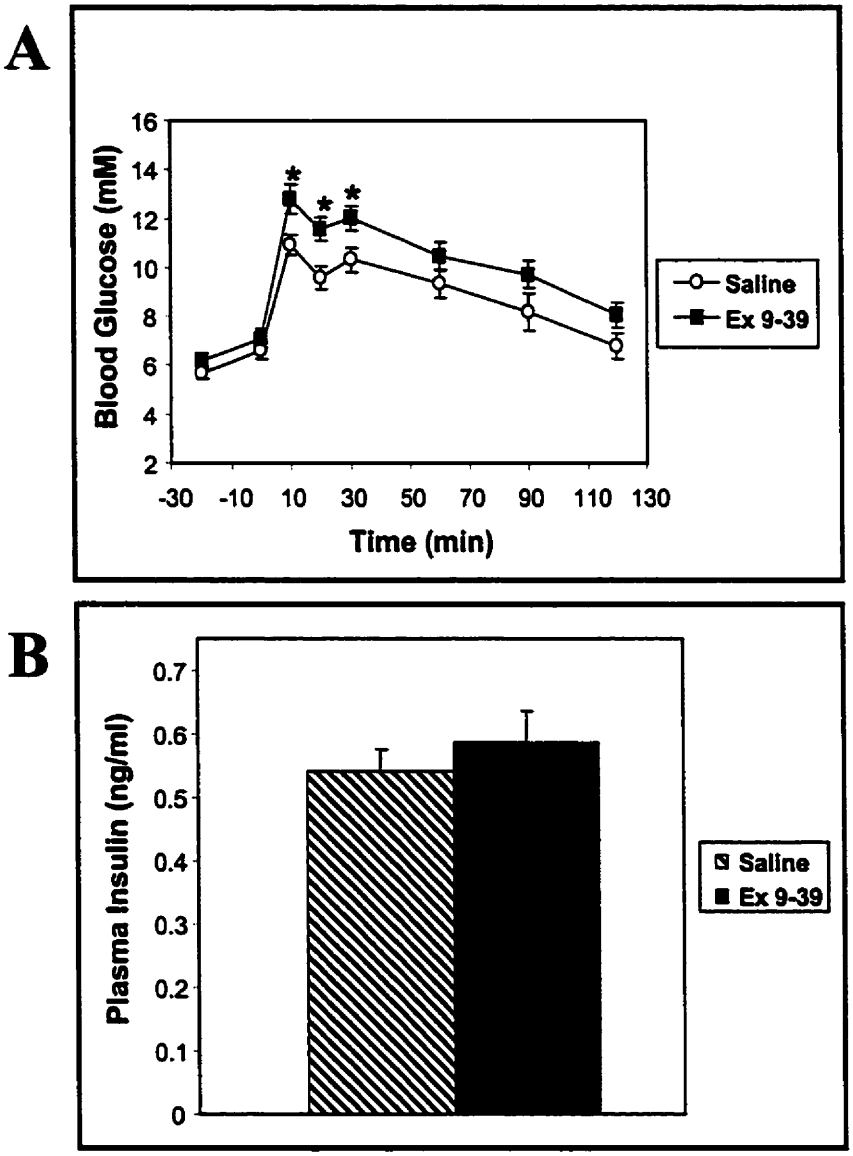


FIG. 4. Oral glucose tolerance and plasma insulin levels in wild-type mice receiving either saline or 5 μg exendin (9-39). Values are expressed as means ± SEM; n = 11-12 mice/group. *$p<0.05$ vs. control (saline). **A**: Oral glucose tolerance test in saline- or exendin (9-39)-treated wild-type males. **B**: Plasma insulin concentration at the 10-20 minute time period following oral glucose administration in saline- or exendin-(9-39)- treated wild-type males.

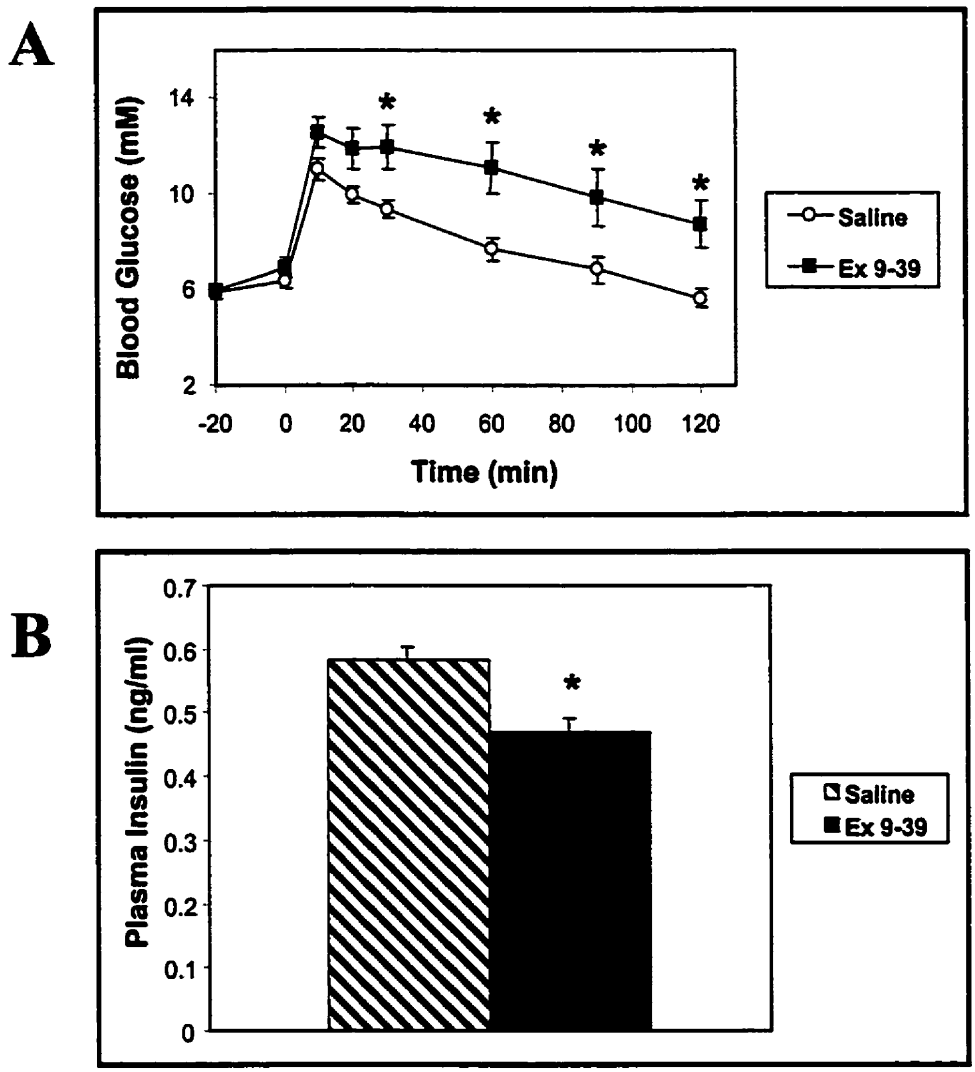


FIG. 5. Intraperitoneal (i.p.) glucose tolerance and plasma insulin levels in wild-type mice receiving either saline or 5 μg exendin (9-39). Values are expressed as means ± SEM; n = 12-13 mice/group. *$p<0.05$ vs. control (saline). **A:** Intra-peritoneal glucose tolerance test in saline- or exendin-(9-39)-treated wild-type males. **B:** Plasma insulin concentration at the 10-20 minute time period following i.p. glucose administration in saline- or exendin-(9-39)-treated wild-type males.

39) on glycemic excursion in mice with targeted genetic disruption of the GLP-1 receptor (288). Treatment of GLP-1 R -/- mice with exendin (9-39) had no statistically significant effect on the glycemic excursion following oral or intraperitoneal glucose loading (Figs. 6A and B), demonstrating the specificity of exendin (9-39) for GLP-1R receptor-mediated glucose clearance at the dose employed here *in vivo*.

2.3.2 Effects of Anti-GIP Receptor (GIPR) Antiserum (GIPR Ab) on Blood Glucose and Plasma Insulin

To ascertain the contribution of GIP action to glucose disposal following glucose loading in mice, we initially used the peptides GIP (6-30)NH_2 and GIP (7-30)NH_2, which had previously been shown to antagonize GIPR function *in vitro* and *in vivo* in rats, respectively (384, 388, 389). However, we found that these GIPR peptide antagonists had no consistent effect on glucose excursion or levels of plasma insulin in wild-type or GLP-1R-/- mice (data not shown). As an alternative, we used an immunoneutralizing antisera directed against the GIP receptor (GIPR Ab). Administration of GIPR Ab to wild-type mice prior to oral glucose challenge led to no change in fasting blood glucose levels, but a significant increase in blood glucose was detected at the 10- min time point of an OGTT (Fig. 7A; 13.1 ± 0.6 vs. 10.8 ± 0.5 mM in GIPR Ab- vs. control-treated wild-type mice, respectively, $p<0.05$). The increase in blood glucose was associated with a small but non-significant increase in plasma insulin concentration (Fig. 7B). In contrast, treatment of GLP-1R-/- mice with GIPR Ab also produced a significant increase in blood glucose (Fig. 8A; 10.4 ± 0.8 vs. 8.3 ± 0.5 mM in GIPR Ab- vs. control-treated mice, $p<0.05$) but additionally caused a significant reduction in levels of glucose-stimulated insulin (Fig. 8B; 0.31 ± 0.03 vs. 0.46 ± 0.05 ng/ml for mice receiving GIPR Ab vs. rabbit γ-globulin, respectively, $p<0.05$).

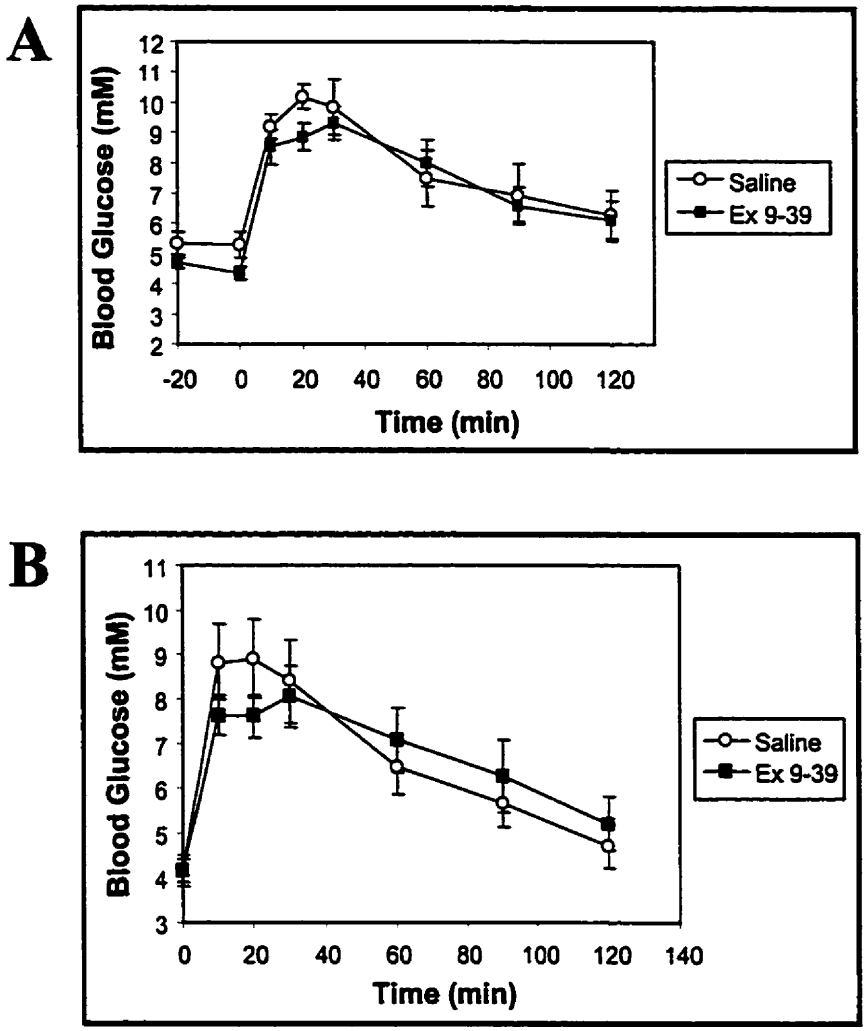


FIG. 6. Oral and intraperitoneal glucose tolerance tests in GLP-1 R -/- mice receiving either saline or 5 μg exendin (9-39). Values are expressed as means ± SEM; n = 5-6 mice/group. **A:** Oral glucose tolerance test in saline- or exendin-(9-39)- treated GLP-1 R -/- males. **B:** Intraperitoneal glucose tolerance test in saline- or exendin-(9-39)-treated GLP-1R -/- males.

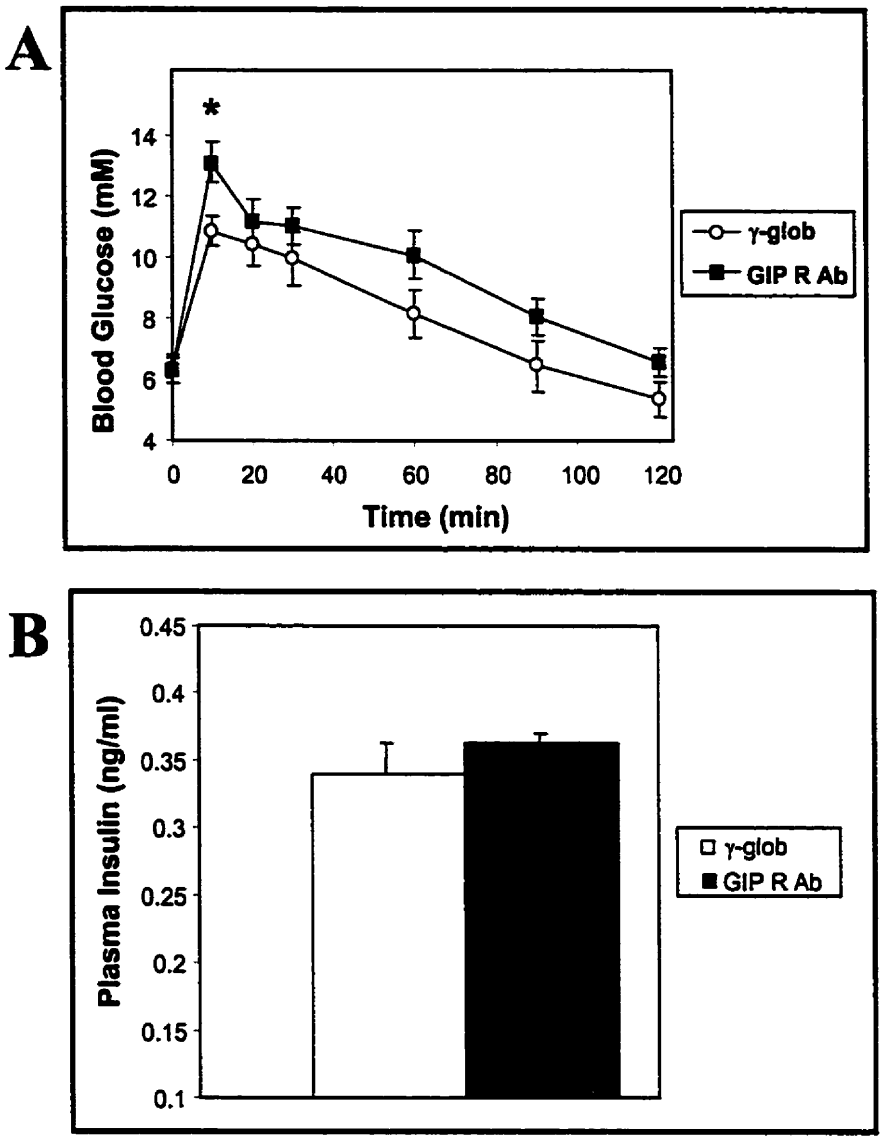


FIG. 7. Oral glucose tolerance and plasma insulin levels in wild-type mice receiving either 1 μg/g body wt rabbit gamma globulin or 1 μg/g body wt GIPR Ab. Values are expressed as means ± SEM; n = 8-10 mice/group. **A:** Oral glucose tolerance test in rabbit gamma globulin- or GIPR Ab-treated wild-type males. *$p<0.05$ vs. control (rabbit gamma globulin). **B:** Plasma insulin concentration at the 10-20 minute time period following oral glucose administration in rabbit gamma globulin- or GIPR Ab-treated wild-type males.

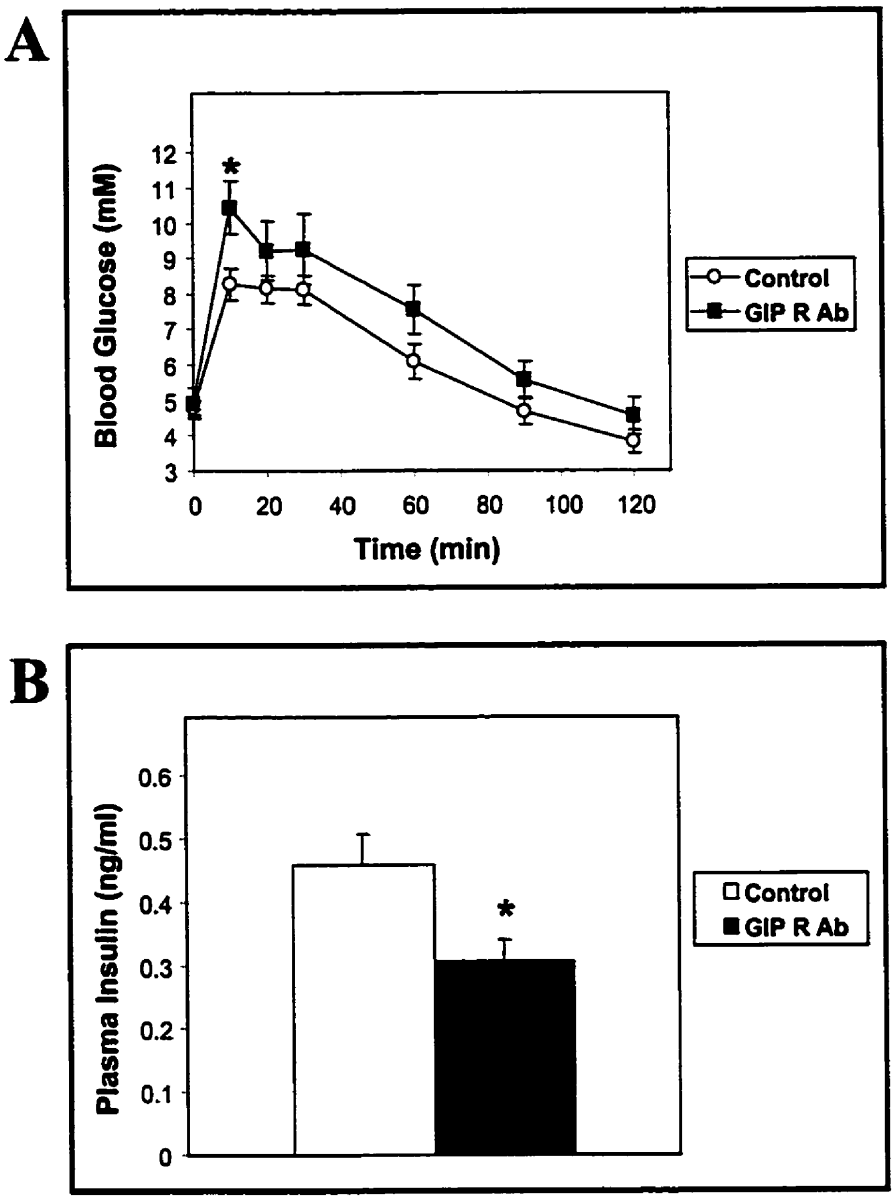


FIG. 8. Oral glucose tolerance and plasma insulin levels in GLP-1 R -/- mice receiving either 1 μg/g body wt rabbit gamma globulin or 1 μg/g body wt GIPR Ab. Values are expressed as means ± SEM; n = 7-11 mice/group. *$p<0.05$ vs. control (rabbit gamma globulin). **A:** Oral glucose tolerance test in rabbit gamma globulin- or GIPR Ab-treated GLP-1 R -/- males. **B:** Plasma insulin concentration at the 10-20 minute time period following oral glucose administration in rabbit gamma globulin- or GIPR Ab-treated GLP-1 R -/- males.

In contrast to the significant increase in blood glucose observed after intraperitoneal glucose loading and treatment with exendin (9-39) (see Fig. 5A), administration of GIPR Ab had no effect on blood glucose or plasma insulin levels after intraperitoneal glucose loading in wild-type or GLP-1R -/- mice (data not shown).

2.3.3 Prolonged Antagonism of Incretin Action

The results of these experiments demonstrated that acute antagonism of GLP-1 or GIP action produces differential effects on glycemic excursion following oral vs. intraperitoneal glucose loading. As both GLP-1 and GIP have been postulated to regulate glycemia, in part through effects on insulin biosynthesis at the level of insulin gene transcription (163, 164, 311, 312), we examined the effects of administering either exendin (9-39) or GIPR Ab on glucose control, over a more prolonged 18 h time period. Repeated administration of exendin (9-39) to wild-type mice produced a significant elevation in blood glucose (Fig. 9A, 7.1 ± 0.4 mM vs. 8.8 ± 0.4 mM for saline- vs. exendin (9-39)-treated mice, $p<0.05$) and a significant reduction in the levels of plasma insulin (Fig. 9B, 2.4 ± 0.1 vs. 1.7 ± 0.2 ng/ml for saline- vs. exendin (9-39)- treated mice, $p<0.05$). Comparable treatment of GLP-1R-/- mice with repeated injections of exendin (9-39) had no effect on either blood glucose or plasma insulin (Figs. 9C and D). In contrast to changes in glucose and insulin in mice treated with repeated administration of exendin (9-39), no significant perturbation of blood glucose or plasma insulin levels was observed following analysis of either wild-type or GLP-1R-/- mice, 18 h subsequent to administration of GIPR Ab (Fig. 10, A-D).

Despite the postulated importance of GLP-1R signaling for insulin gene transcription, no significant alterations in the levels of insulin (or proglucagon) mRNA transcripts (Figs. 11A and C) or pancreatic insulin content (Fig 12A) were detected, following repeated

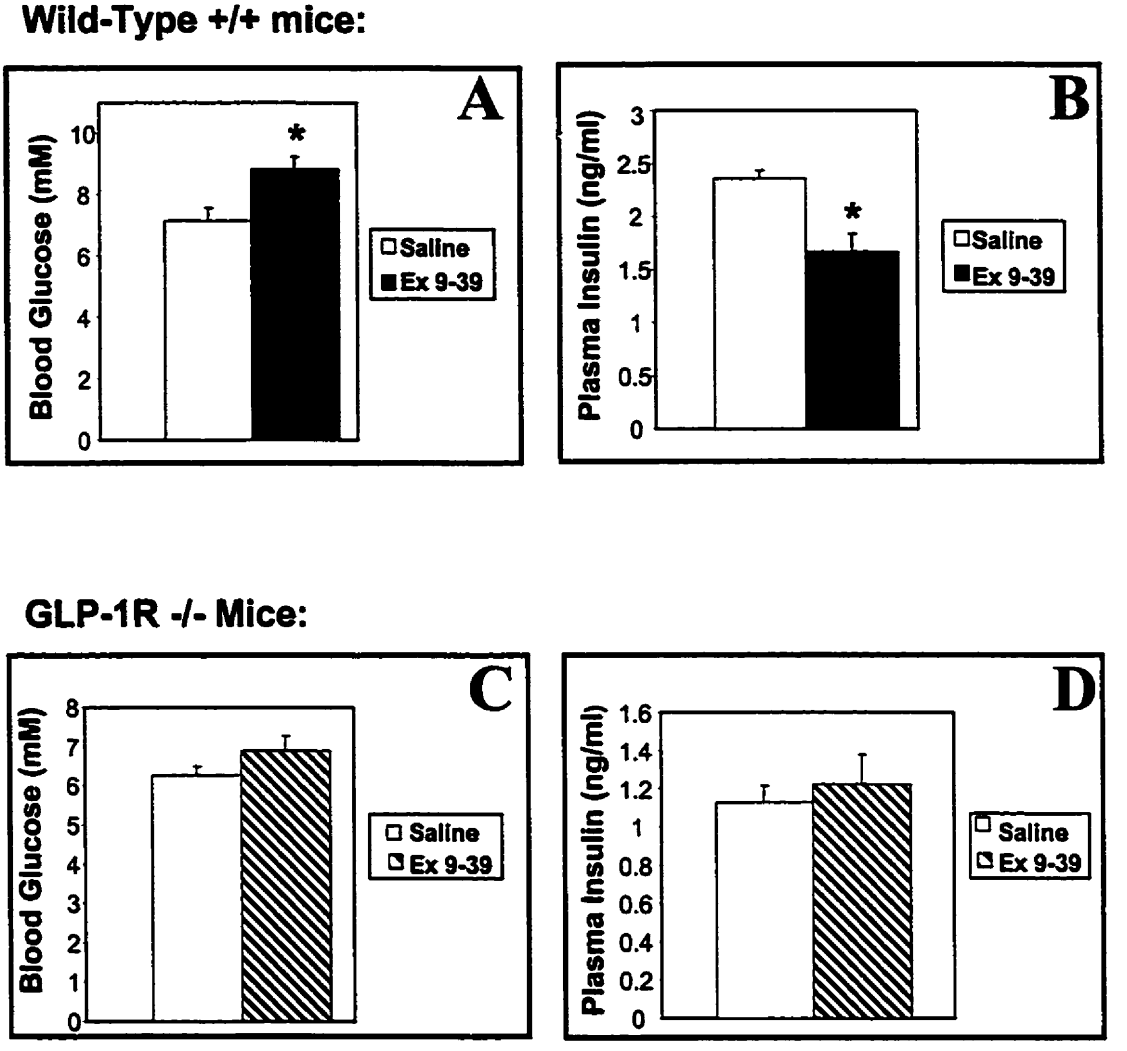
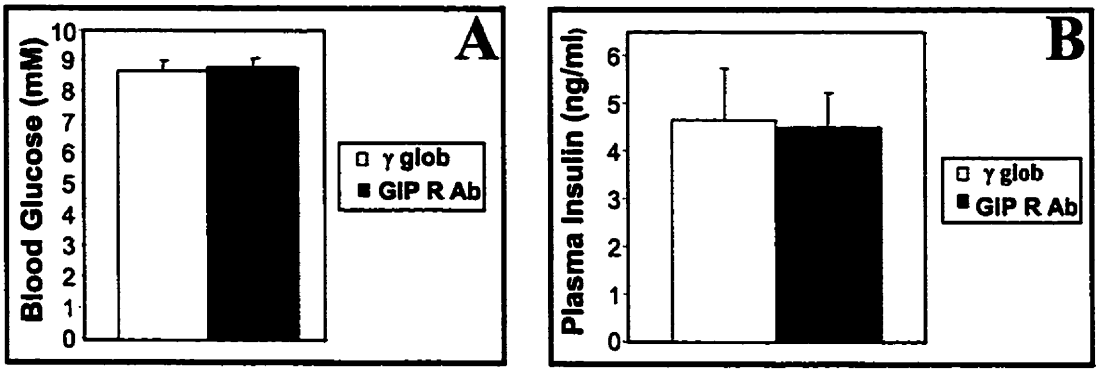


FIG. 9. Blood glucose and plasma insulin levels in wild-type and GLP-1 R -/- mice following prolonged treatment with saline or exendin (9-39). A: Blood glucose level in wild-type males treated with saline (open bar) or exendin (9-39; solid bar). Values are expressed as means ± SEM; n = 8-9 mice/group. *$p<0.05$ vs. control (saline). **B:** Plasma insulin concentration in wild-type males treated with saline (open bar) or exendin (9-39; solid bar). *$p<0.05$ vs. control (solid bar). **C:** Blood glucose level in GLP-1 R -/- males treated with saline (open bar) or exendin (9-39; hatched bar). Values are expressed as means ± SEM; n = 4 mice/group. **D:** Plasma insulin concentration in GLP-1R-/- males treated with saline (open bar) or exendin (9-39; hatched bar).

Wild-Type +/+ Mice:



GLP-1R -/- Mice:

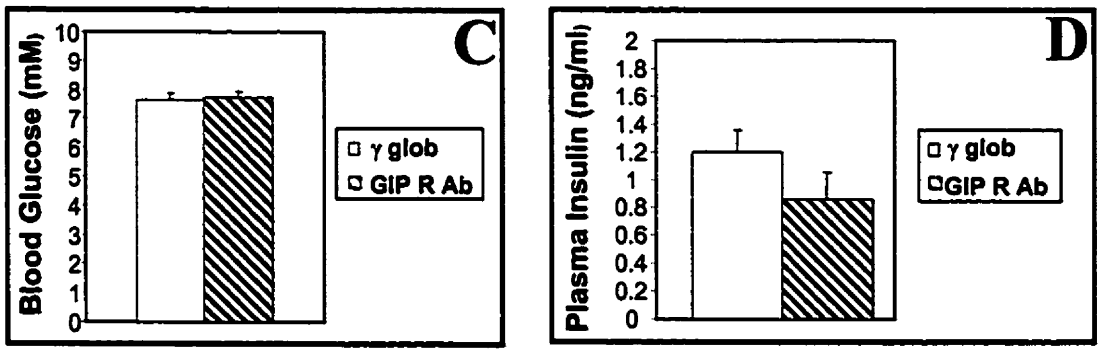


FIG. 10. Blood glucose and plasma insulin levels in wild-type and GLP-1 R -/- mice following prolonged treatment with rabbit gamma globulin or GIPR Ab. A: Blood glucose level in wild-type males treated with rabbit gamma globulin (open bar) or GIPR Ab (solid bar). Values are expressed as means ± SEM; n = 10 mice/group. **B:** Plasma insulin concentration in wild-type males treated with rabbit gamma globulin (open bar) or GIPR Ab (solid bar). **C:** Blood glucose level in GLP-1 R -/- males treated with rabbit gamma globulin (open bar) or GIPR Ab (hatched bar). Values are expressed as means ± SEM; n = 13 mice/group. **D:** Plasma insulin concentration in GLP-1 R -/- males treated with rabbit gamma globulin (open bar) or GIPR Ab (hatched bar).

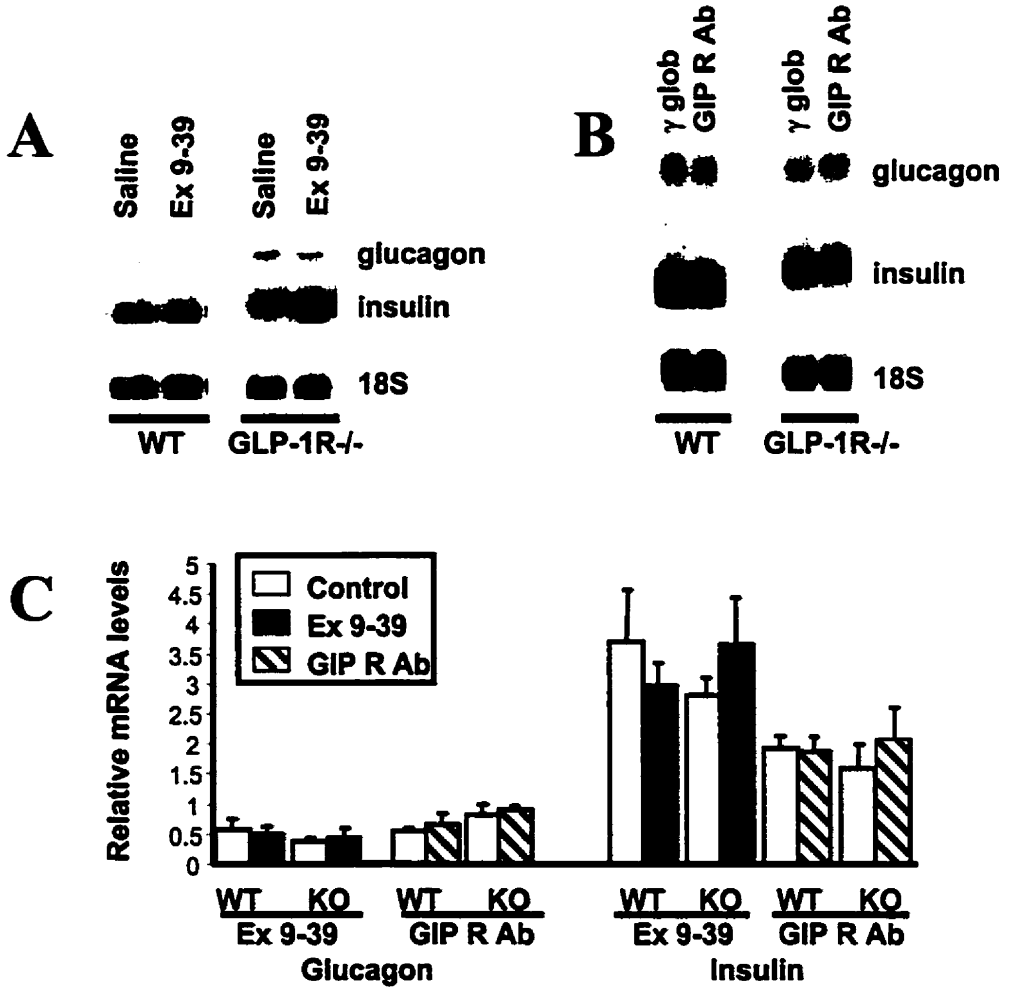


Fig. 11. Northern blot analysis of pancreatic insulin and glucagon mRNA transcripts in wild-type and GLP-1R-/- mice treated with exendin (9-39) (A) or GIPR Ab (B). Relative mRNA levels (C) for insulin or glucagon and 18S rRNA. Values are expressed as means ± SEM. n = 8 mice/group for experiments with saline and exendin (9-39) or 4-9 mice/group for experiments with γ-glob and GIPR Ab. (All quantification was carried out using the Molecular Dynamics PhosphorImager and ImageQuant™).

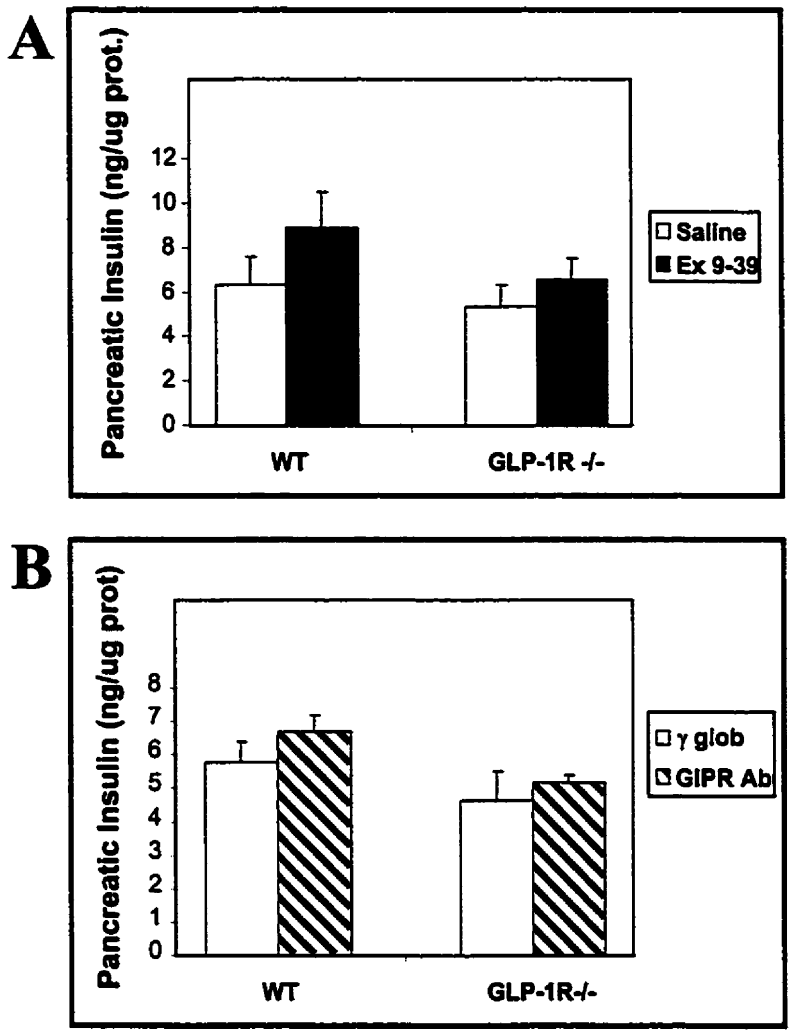


Fig. 12. Pancreatic insulin content in wild-type and GLP-1R-/- mice treated with exendin (9-39) (A) or GIPR Ab (B). Values are expressed as mean ± S.E.M. and n = 8 mice/group for experiments in A and 4-9 mice/group for experiments in B.

treatment with exendin (9-39), in either wild-type or GLP-1R -/- mice. Similarly, no significant changes in the levels of insulin mRNA or insulin content were detected in the pancreas of mice treated with GIPR Ab (Figs. 11B,C and 12B).

2.4 Discussion

Although originally identified as an incretin, GLP-1 has subsequently been shown to exhibit multiple nonincretin actions including inhibition of both glucagon secretion (31, 35) and gastric emptying (42, 181). GLP-1 also confers glucose sensitivity to glucose-resistant β-cells (47) and may also increase insulin-independent glucose disposal in peripheral tissues (51). Taken together with effects on reduction of food intake (39, 40), it appears that GLP-1 exerts both incretin- and nonincretin- mediated actions that contribute to glucose-lowering *in vivo*.

The importance of nonincretin effects of GLP-1 are further exemplified by experiments in mice with genetic disruption of GLP-1R signaling. Studies of islet function demonstrate defects in basal islet cAMP and glucose-stimulated calcium signaling in GLP-1 R -/- islets (293). The importance of basal GLP-1R signaling for β-cell function may partly explain why GLP-1R-/- mice also exhibit mild fasting hyperglycemia and abnormal glucose excursion following intraperitoneal glucose challenge (288), conditions that would not be associated with increases in levels of circulating GLP-1. Although the phenotype of impaired glucose tolerance in GLP-1R-/- can be mild and variable, analysis of large numbers of GLP-1R-/- mice of different ages demonstrates statistically significant impairment of glucose homeostasis in the fasting state and after oral and intraperitoneal glucose challenge (288, 291, 294).

Interpretation of the modest impairment of glucose tolerance after genetic disruption of the GLP-1R is complicated by the observation that GIP secretion and GIP-stimulated insulin secretion are up-regulated in GLP-1R-/- mice, suggesting that compensatory enhancement of GIP action partially modifies the phenotype of GLP-1 deficiency *in vivo* (289). Furthermore, it remains possible that potential developmental effects of GLP-1R deficiency might also modify islet and β-cell development and responsivity, hence the abnormalities in β-cell function and glucoregulation detected in GLP-1R-/- mice may not necessarily be directly correlated with acute disruption of GLP-1 action *in vivo*.

Accordingly, to eliminate confounding variables introduced by the potential contribution of developmental and adaptive changes in physiological regulatory systems, we re-examined the importance of GLP-1 and GIP action for both incretin- and nonincretin-mediated control of glycemia in wild-type mice. Our data clearly show that inhibition of GLP-1 activity during intraperitoneal glucose challenge produces abnormal glycemic excursion in wild-type mice, associated with a significant reduction in plasma insulin. Because non-enteral glucose challenge would not be expected to stimulate GLP-1 secretion, our findings strongly suggest that basal levels of circulating GLP-1 are essential for glycemic control, both in the fasting state and following glucose challenge, independent of the mode of glucose entry.

Further evidence supporting the importance of basal GLP-1 signaling for glucoregulation derives from studies using exendin (9-39) in both humans and baboons. Administration of exendin (9-39) produced significant elevations in fasting levels of both glucose and glucagon, suggesting that even basal GLP-1 signaling during the fasting state exerts a tonic inhibitory effect on glucagon secretion (281-283). The finding that glucagon

secretion is under tonic inhibitory control by GLP-1 signaling is consistent with our observation that glucose levels rise in exendin (9-39)-treated mice, without an obligatory increase in the levels of circulating insulin. Additional evidence for the importance of basal GLP-1 signaling derives from studies demonstrating that exendin (9-39) is an inverse agonist of the β-cell GLP-1 receptor and that constitutive activity of the GLP-1 receptor, even in the absence of bound ligand, is important for maintaining basal levels of cAMP and for sustaining pancreatic β-cells in a glucose competent state (292, 293).

In contrast to the importance of GLP-1 for glucose regulation and β-cell function in the fasting state, our current data strongly suggests that the role of GIP in glucose control is considerably more restricted, principally functioning as an incretin in the post-absorptive state. Disruption of GIP action during oral glucose challenge produced a significant increase in glycemic excursion in both wild-type and GLP-1R-/- mice, in association with a diminution of glucose-stimulated insulin secretion. In contrast, administration of GIPR Ab had no effect on fasting glucose or glycemic excursion after intraperitoneal glucose loading, not even in mice with loss of GLP-1 function.

Although the incretin function of GIP is well established (3, 4, 20), a role for GIP in the control of β-cell function in the fasting state is less clearly defined. Infusion of GIP produced a dose-dependent increase in plasma insulin, in fasting rats, that was attenuated by co-infusion of ANTGIP, a GIP receptor peptide antagonist (389). However, no change in fasting plasma insulin concentration was detected in rats 30 min. following subcutaneous administration of ANTGIP (389). Although treatment with ANTGIP diminished glucose-stimulated insulin secretion in rats, the effect of ANTGIP on blood glucose or insulin secretion following an intraperitoneal glucose challenge or on fasting blood glucose levels

was not reported (384, 389). Although we cannot be absolutely certain that the limitations of the immunoneutralizing GIPR Ab may affect our experimental results, the finding that GIP is primarily important for glucose clearance after enteral, but not intraperitoneal, glucose loading, is consistent with data from GIPR-/- mice. These mice exhibit normal fasting glucose, and the glycemic response to intraperitoneal glucose challenge is comparable and normal in the presence or absence of GIPR signaling (349). These findings are entirely consistent with our data showing no effect of GIPR Ab on fasting glucose or intraperitoneal glucose clearance in mice *in vivo*. Taken together, the cumulative evidence strongly suggests that the glucoregulatory actions of GIP on the β-cell are restricted to the potentiation of glucose-stimulated insulin secretion following enteral nutrient absorption.

The results of several studies have suggested an important role for GLP-1 in the regulation of insulin gene expression and insulin biosynthesis. Incubation of islet cell lines with GLP-1 increases proinsulin mRNA via activation of insulin gene transcription (163, 164). Similarly GIP increases insulin mRNA and insulin content in islet cells, via induction of insulin promoter activity and insulin gene expression (311, 312). Despite the putative importance of GLP-1 and GIP for insulin gene expression and insulin biosynthesis, we were unable to detect changes in pancreatic insulin content or insulin mRNA in wild-type mice treated with either exendin (9-39) or GIPR Ab. Furthermore, we found minimal to no changes in pancreatic insulin mRNA and insulin content in GLP-1R-/- mice (289-291). These findings, taken together with our data using exendin (9-39) or GIPR Ab, strongly suggest that GLP-1 and GIP are not essential for insulin gene expression and insulin biosynthesis in mice *in vivo*.

In summary, these studies demonstrate that GLP-1 is essential for normal glucose-stimulated insulin secretion in mice, independent of the mode of glucose entry. Although several investigators have suggested that the predominant effect of GLP-1 on glucose control resides at the level of gastric emptying (43, 390), our data clearly indicate an essential role for GLP-1 in glucoregulation independent of nutrient entry via the gastrointestinal tract. In contrast, GIP plays a more limited role in glucose homeostasis, with GIP actions restricted to the classical incretin function of potentiating nutrient-stimulated insulin secretion. The wider spectrum of GLP-1 actions on gastric emptying, β-cell function, glucagon secretion, food intake and islet growth suggest that GLP-1 is likely to exhibit more potential, compared to GIP, as a therapeutic agent for the treatment of diabetes.

Chapter 3. Inhibition of DPP-IV Activity Improves Glucose Tolerance and Insulin Secretion in GLP-1R-/- Mice

3.1 Introduction

Dipeptidyl peptidase IV (DPP-IV), also known as CD26, is a multifunctional, ubiquitously expressed glycoprotein that can be found either anchored to cell surfaces, or as a soluble protein in plasma (242, 243). In humans and rodents the DPP-IV cDNA encodes a polypeptide of approx. 766 amino acids (242, 391) and the solubilized glycoprotein consists of two identical subunits of approx. 120 kDa each (242, 392). The 6 N-terminal amino acids of DPP-IV/CD26 comprise its very short cytoplasmic tail, with the majority of the protein, including the C-terminal catalytic domain, located extracellularly (242, 243, 392). The soluble form of DPP-IV/CD26 lacks the cytoplasmic tail and transmembrane domains (243).

In the rat, the most abundant levels of DPP-IV/CD26 activity are found in the kidney (393) and brush-border membranes of intestinal enterocytes (242). High levels of DPP-IV/CD26 activity are also found in the lung, adrenal glands, liver, parotid gland, spleen and testis (242). More recently, DPP-IV/CD26 activity has also been detected in the secretory granules of α-cells in the pancreatic islets (394). Additionally, DPP-IV/CD26 is also located on the endothelial cells of blood vessels (395), activated T-helper lymphocytes (355), subsets of macrophages (356), and on mammary, skin and synovial fibroblasts (396-398). In the rodent CNS, DPP-IV/CD26 has been detected in the choroid plexus (399), in the median eminence (400), on astrocytes in the cerebellum and spinal cord, and in nerve perineurium (401, 402). However, whether the DPP-IV/CD26 found in the CNS is catalytically active remains to be determined (403-405). Soluble DPP-IV/CD26 activity is found at high levels in seminal fluid, with lower levels detected in plasma and cerebrospinal fluid (243).

DPP-IV/CD26 appears to be involved in a number of biological processes. It can act as a binding protein for adenosine deaminase (351), as well as for collagen and fibronectin and thus, may be important for cell-matrix interactions (243, 350). DPP-IV/CD26 has also been shown to be involved in T-cell activation and proliferation and consequently, appears to play a role in immune regulation (353). In addition, DPP-IV/CD26 is a serine protease that preferentially cleaves dipeptides from the N-terminus of polypeptides which contain either a proline or alanine residue in position 2 (*i.e.* X-Pro/Ala, where X is any amino acid) (406, 407). Significantly, DPP-IV/CD26-mediated hydrolysis has been shown to alter the biological specificity or receptor selectivity of a number of mammalian regulatory peptides including substance P, NPY, peptide YY, growth hormone releasing hormone (GRH), as well as certain chemokines (62, 408-413).

The two most widely recognized incretin hormones, GLP-1 and GIP, are also substrates for the proteolytic activity of DPP-IV/CD26 (61, 62). DPP-IV/CD26-mediated hydrolysis of biologically active GIP and GLP-1 yields, respectively, GIP (3-42), which is inactive (306, 310), and GLP-1 (9-36)NH_2, which is inactive and may also function as a competitive antagonist of the GLP-1R *in vivo* (34, 245, 260, 357, 414). Furthermore, it has been suggested that DPP-IV/CD26 may be the primary enzyme responsible for the degradation and short plasma half-life of GLP-1 and GIP *in vivo* (61, 62, 236). Recent *in vivo* studies in the pig have shown that intact GLP-1 newly secreted from the intestine is rapidly degraded to GLP-1 (9-37/36)NH_2 as it enters the DPP-IV/CD26-containing blood vessels which drain the intestinal mucosa (244).

While both GIP and GLP-1 are potential therapeutic agents for the treatment of diabetes, only GLP-1 has been shown to retain its incretin effect in diabetic patients (60).

However, due to the short metabolic half-life of GLP-1, therapeutic efforts have focused on the development of DPP-IV-resistant GLP-1 analogues or, alternatively, the development of specific DPP-IV inhibitors. Such reagents would simultaneously prolong the biological activity of GLP-1 and minimize the accumulation of the antagonistic metabolite GLP-1 (9-37/36)NH_2.

Pharmacological inhibition of DPP-IV/CD26 activity has been shown to increase both endogenous and exogenous levels of biologically active GLP-1 and to improve glucose tolerance and enhance insulin secretion in a number of normal and type 2 diabetic animal models (359-363). However, although DPP-IV/CD26 is presumed to be the major enzyme responsible for the degradation of GLP-1 and GIP *in vivo* (61, 62, 236), additional enzymes which exhibit DPP-IV/CD26-like activity have been identified (378-380). Thus, the specific molecular targets of DPP-IV/CD26 inhibitors are not clear.

In order to elucidate the physiological function of DPP-IV/CD26 and determine its role in glucoregulation, mice harboring a targeted inactivation of the DPP-IV/CD26 gene were generated (CD26-/-) (381). These mice, which are fertile and appear to develop normally, exhibit normal fasting blood glucose levels. However, they are able to clear an oral glucose load more rapidly than wild-type littermates and exhibit significant increases in glucose-stimulated insulin levels in association with increased levels of intact biologically active GLP-1 (381). In addition, intact biologically active GLP-1 or GIP was completely resistant to N-terminal degradation when incubated in plasma derived from CD26-/- mice (381). Moreover, treatment with valine-pyrrolidide, a pharmacological inhibitor of DPP-IV/CD26 enzymatic activity, resulted in improved glucose tolerance in wild-type mice but no effect in CD26-/- mice, indicating that valine-pyrrolidide mediates glucose clearance

specifically via suppression of DPP-IV/CD26 activity (381). Taken together, the studies performed with the CD26-/- mouse suggest that the proteolytic activity of DPP-IV/CD26 has an essential role in glucoregulation.

The precise mechanism by which DPP-IV/CD26 activity modulates blood glucose levels is unknown, but likely involves regulation of the activity of GLP-1, and possibly GIP or other substrates as well. Thus, in order to determine whether DPP-IV inhibitors lower blood glucose exclusively through GLP-1-dependent mechanisms, we examined the effects of DPP-IV/CD26 inhibition in GLP-1R-/- mice using the pharmacological inhibitor valine-pyrrolidide. GLP-1R-/- mice are glucose intolerant due to a lack of GLP-1 signaling which results in insufficient levels of glucose-stimulated insulin secretion (288). It was hypothesized that, if GLP-1 alone is responsible for the observed improvements in glucose tolerance and insulin secretion in CD26-/- mice, pharmacological inhibition of DPP-IV/CD26 activity in GLP-1R-/- mice would have no effect on glucose tolerance or insulin secretion.

3.2 Methods

3.2.1 DPP-IV Inhibition

Valine-pyrrolidide (val-pyr) was provided by Dr. Nicolai Wagtmann (Novo Nordisk, Denmark). It is a stable and highly selective competitive inhibitor of DPP-IV activity (244, 359), that is essentially inactive against other proteolytic enzymes (359). Lyophilized val-pyr was freshly dissolved at 4.12 mg/ml in water and injected i.p. into mice at a dose of 20.6 mg/kg, 30 min prior to initiation of glucose tolerance tests.

3.2.2 Mice

GLP-1 receptor -/- and age-matched (6- to 8-week-old males) wild-type CD1 mice (Charles River Laboratories, Inc. Montreal, Quebec) were housed under a 12 h light, 12 h dark cycle in the Toronto General Hospital animal facility with free access to food (standard rodent chow) and water, except where noted. All wild type mice used for these studies were acclimatized to the animal facility for several weeks prior to analysis. All procedures were conducted according to protocols and guidelines approved by the Toronto Hospital Animal Care Committee.

3.2.3 Glucose Tolerance Tests and Measurement of Plasma Insulin Levels

Oral (OGTT) or intraperitoneal (IPGTT) glucose tolerance tests were carried out following an overnight fast (18 h). The DPP-IV inhibitor val-pyr (20 mg/kg of a 4.12 mg/ml stock solution) or vehicle (water) was administered intraperitoneally 30 min prior to glucose administration, after a fasting blood glucose measurement had been obtained. For glucose tolerance tests, mice were given 2.0 g glucose/kg body weight orally through a gavage tube (OGTT) or via injection into the peritoneal cavity (IPGTT). Blood was drawn from a tail vein at 0, 30, 60, 120 and 180 minutes following glucose administration, and blood glucose levels were measured by the glucose oxidase method using a One Touch Basic Glucometer (Lifescan Ltd., Burnaby, BC). Blood samples (100 µl) for measurement of plasma insulin levels were removed from tail veins during the 10- to 20-minute time period following oral or intraperitoneal glucose administration and immediately mixed with a 10% vol of a chilled solution containing 5000 KIU/ml Trasylol (Miles Canada, Etobicoke, Canada), 32 mM EDTA, and 0.1 nM Diprotin A (Sigma Chemical Co., St. Louis, MO). Plasma was separated by centrifugation at 4°C and stored at -80°C until assayed. Plasma samples were assayed for

insulin content using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois) with mouse insulin as a standard.

3.2.4 Statistics

All results are expressed as means ± SEM. Statistical significance was calculated by ANOVA and Student's *t*-test using INSTAT 1.12 (Graph-Pad Software, Inc., San Diego, CA). A p value < 0.05 was considered to be statistically significant.

3.3 Results

3.3.1 Effects of DPP-IV/CD26 Inhibition on Oral Glucose Tolerance

In CD26-/- mice an oral glucose load is cleared more rapidly than in wild type control mice (381). This accelerated glucose clearance is associated with increased levels of intact, biologically active GLP-1 and enhanced insulin secretion (381). To determine whether the improved oral glucose tolerance observed in CD26-/- mice is mediated entirely by GLP-1-dependent mechanisms, we treated wild-type and GLP-1R-/- mice with the pharmacological DPP-IV/CD26 inhibitor val-pyr, prior to an oral glucose tolerance test. Surprisingly, inhibition of DPP-IV/CD26 activity during an oral glucose challenge had no effect on the rate of glucose clearance in wild-type mice (Fig. 13A), but it did produce a notable, although not statistically significant, increase in the plasma insulin concentration (Fig. 13B). However, in GLP-1R-/- mice, inhibition of DPP-IV/CD26 activity with val-pyr resulted in a significant improvement in oral glucose clearance ($p<0.05$ for vehicle- vs. val-pyr-treated GLP-1R-/- mice at 30 and 60 min following oral glucose; Fig. 14A), which was associated

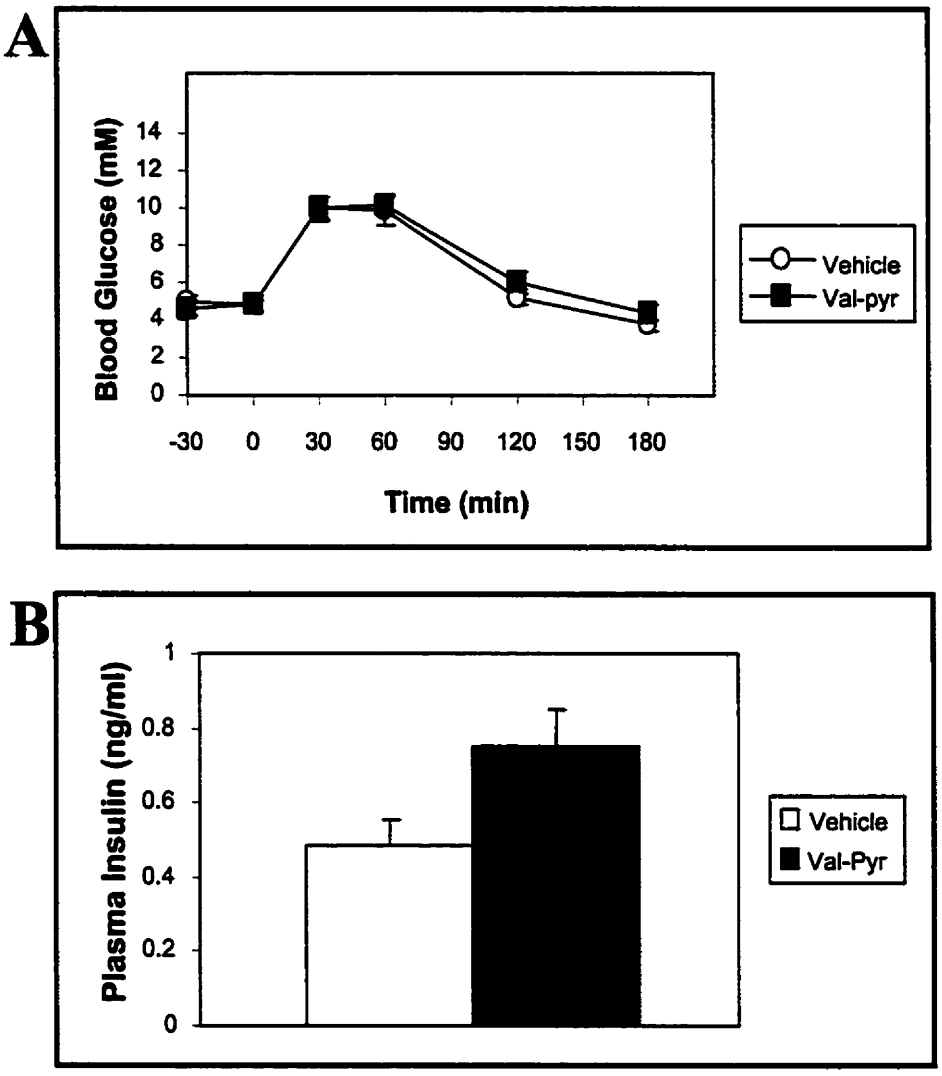


Fig. 13. Oral glucose tolerance and plasma insulin levels in wild-type mice receiving either vehicle (water) or 20.6 mg/kg val-pyr. Values are expressed as means ± SEM; n = 8-10 mice/group. **A:** Oral glucose tolerance test in vehicle- or val-pyr-treated wild-type males. **B:** Plasma insulin concentration at the 10-20 min period following oral glucose administration in vehicle- or val-pyr-treated wild type males.

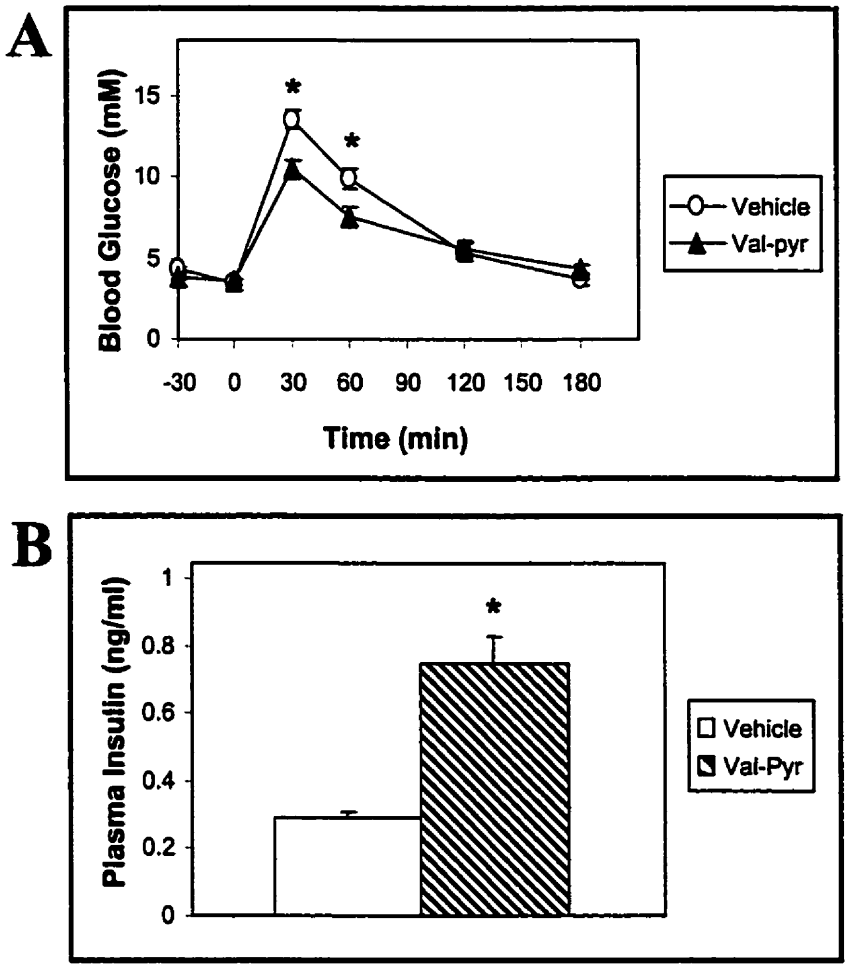


Fig. 14. Oral glucose tolerance and plasma insulin levels in GLP-1R-/- mice receiving either vehicle (water) or 20.6 mg/kg val-pyr. Values are expressed as means ± SEM; n = 9-11 mice/group. *$p<0.05$ vs. control (water). **A:** Oral glucose tolerance test in vehicle- or val-pyr-treated GLP-1R-/- males. **B:** Plasma insulin concentration at the 10-20 min period following oral glucose administration in vehicle- or val-pyr-treated GLP-1R-/- males.

with enhanced insulin secretion (0.29±0.02 ng/ml in vehicle-treated vs. 0.74±0.08 ng/ml in val-pyr-treated GLP-1R-/- mice; $p<0.05$; Fig. 14B). The observed improvement in oral glucose tolerance and insulin secretion in GLP-1R-/- mice following val-pyr treatment, despite lack of GLP-1R signaling, suggests that DPP-IV/CD26 does not mediate its effects on blood glucose levels exclusively through GLP-1-dependent mechanisms.

3.3.2 Effects of DPP-IV/CD26 Inhibition on Intraperitoneal Glucose Tolerance

In addition to its role as an incretin hormone, GLP-1 has also been shown to be essential for clearing an intraperitoneal glucose load (288). Administration of glucose into the peritoneal cavity bypasses the incretin effect, as well as any effects on gastric emptying. Thus, GLP-1 is important for handling a glucose load, independent of the site of glucose entry (288). To determine if DPP-IV/CD26-mediated glucoregulation also involves nonincretin substrates, including and/or in addition to GLP-1, wild-type and GLP-1R-/- mice were treated with val-pyr prior to an intraperitoneal glucose tolerance test. Treatment with val-pyr prior to an intraperitoneal glucose challenge had no effect on glucose tolerance or plasma insulin concentration in wild-type mice (Figs. 15A and B). However, inhibition of DPP-IV/CD26 activity in GLP-1R-/- mice significantly decreased the glycemic excursion following the intraperitoneal glucose load ($p<0.05$ for vehicle- vs. val-pyr-treated GLP-1R-/- mice at 30 and 60 min following intraperitoneal glucose; Fig. 16A). Moreover, the plasma insulin concentration was significantly increased in the val-pyr-treated GLP-1R-/- mice (0.33±0.02 ng/ml in vehicle-treated vs. 0.44±0.02 ng/ml in val-pyr-treated GLP-1R-/- mice; $p<0.05$; Fig. 16B). These results suggest that DPP-IV/CD26-mediated regulation of blood glucose is likely independent of effects on gastric emptying and involves additional

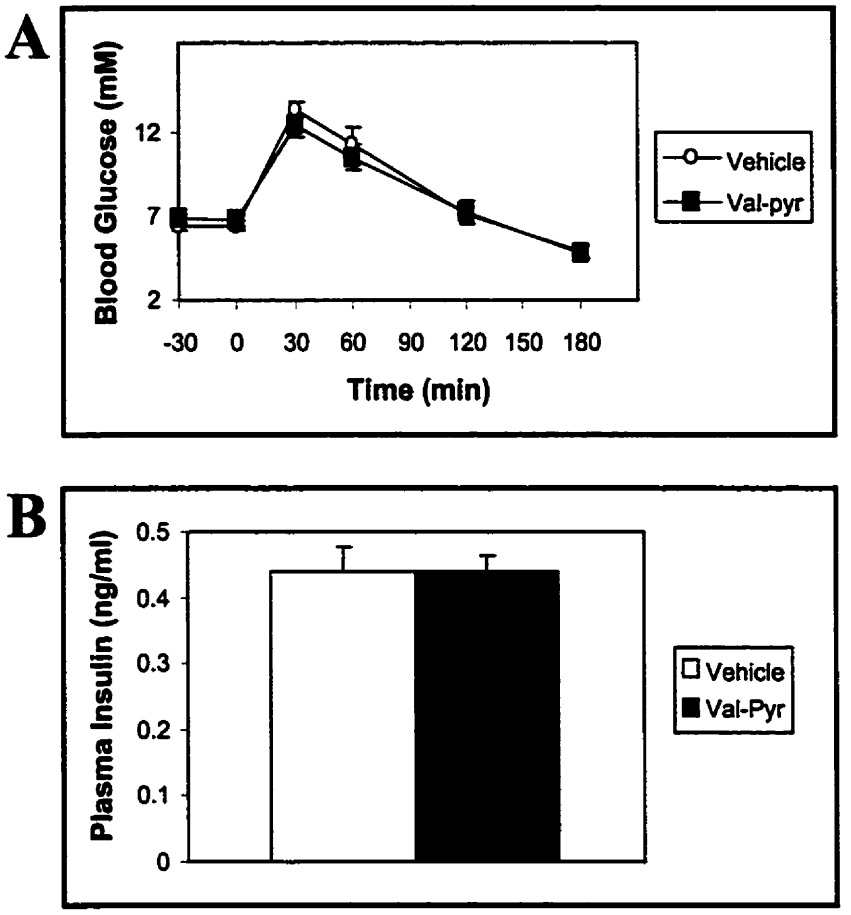


Fig. 15. Intraperitoneal (i.p.) glucose tolerance and plasma insulin levels in wild-type mice receiving either vehicle (water) or 20.6 mg/kg val-pyr. Values are expressed as means ± SEM; n = 8-10 mice/group. **A:** i.p. glucose tolerance test in vehicle- or val-pyr-treated wild-type males. **B:** Plasma insulin concentration at the 10-20 min time period following i.p. glucose administration in vehicle- or val-pyr-treated wild-type males.

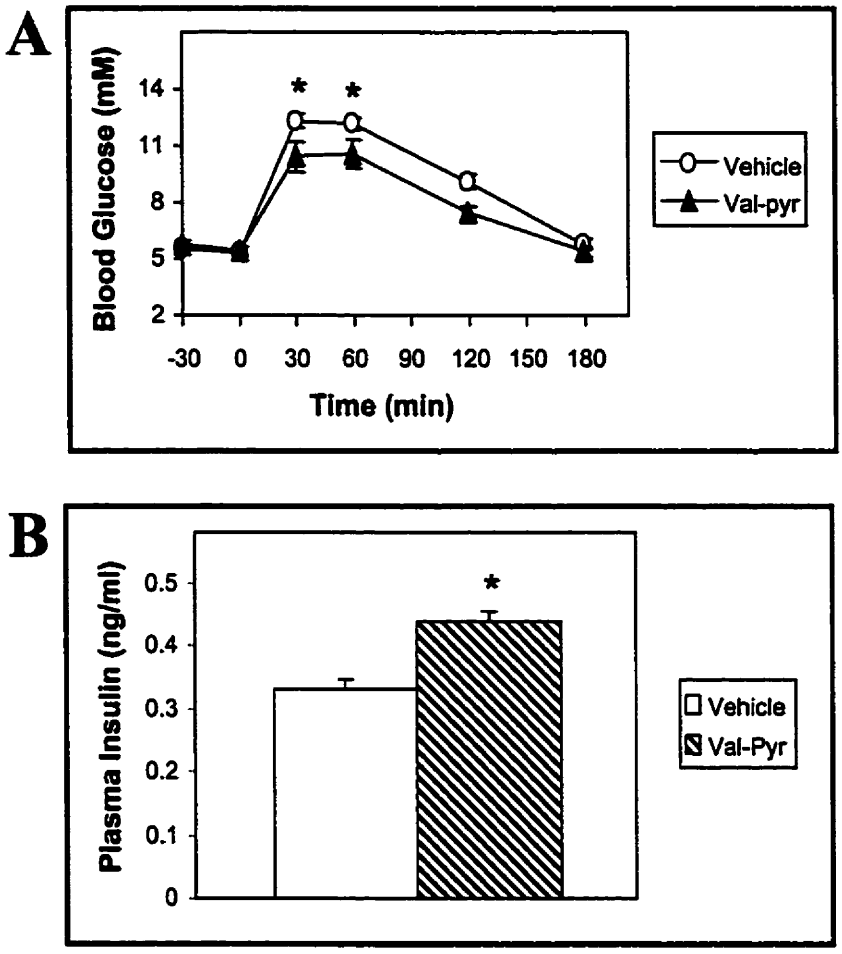


Fig. 16. Intraperitoneal (i.p.) glucose tolerance and plasma insulin levels in GLP-1R-/- mice receiving either vehicle (water) or 20.6 mg/kg val-pyr. Values are expressed as means ± SEM; n = 8-14 mice/group. *$p<0.05$ vs. control (water). **A:** i.p. glucose tolerance test in vehicle- or val-pyr-treated GLP-1R -/- males. **B:** Plasma insulin concentration at the 10-20 min time period following i.p. glucose administration in vehicle- or val-pyr-treated GLP-1R-/- male mice.

mechanisms, and not simply inhibition of endogenous GLP-1 degradation. Hence, these results indicate that the effects of substrates other than GLP-1 play an important role in mediating DPP-IV/CD26-regulated glucose control.

3.4 Discussion

Recent studies have shown that mice with a targeted inactivation of the DPP-IV/CD26 gene are able to clear a glucose load more rapidly than their wild-type littermates and that this improved glucose tolerance is associated with increased plasma insulin levels. These results indicate that the proteolytic activity of DPP-IV/CD26 plays an important role in the regulation of blood glucose levels (381). However, the exact mechanism by which DPP-IV/CD26 modulates glucoregulation is uncertain. A number of studies in pigs and both normal and diabetic rodents have shown that DPP-IV/CD26 inhibition results in increased levels of intact, biologically active endogenous GLP-1 (359-361, 363). Moreover, in CD26-/- mice, the improved glucose tolerance and increased insulin secretion were associated with increased levels of intact, biologically active GLP-1 (381). Thus, DPP-IV/CD26 likely mediates its effects on blood glucose levels by regulating the activity of GLP-1, although other substrates, including GIP, could also be involved.

In this study, wild type and GLP-1R-/- mice were treated with val-pyr, a specific DPP-IV/CD26 inhibitor, to determine if GLP-1-dependent mechanisms were entirely responsible for mediating the effects of DPP-IV/CD26 on blood glucose regulation.

Surprisingly, val-pyr treatment had no effect on blood glucose levels in wild-type mice. Similarly, in studies performed on anesthetized pigs, inhibition of DPP-IV/CD26 activity with val-pyr produced a significant increase in plasma insulin concentration, but had

no effect on blood glucose levels (359). Moreover, treatment of wild-type C57BL/6J mice with val-pyr, or administration of the DPP-IV inhibitor NVP-DPP728 in lean Zucker rats, prior to oral glucose tolerance tests, produced only mild improvements in glucose tolerance, despite marked increases in plasma insulin levels (361, 363). Since recent studies have suggested that glucagon may also be a substrate for DPP-IV/CD26's proteolytic activity (415), the lack of improved glucose tolerance in response to DPP-IV/CD26 inhibition could be due to increased levels of endogenous biologcally active glucagon.

Inhibition of DPP-IV/CD26 activity in GLP-1R-/- mice resulted in improved glucose tolerance and increased plasma insulin levels in response to either an oral or i.p. glucose challenge. These results indicate that the improved glucose tolerance and elevated insulin levels in CD26-/- mice may not be mediated exclusively through GLP-1 activity, and that DPP-IV/CD26-mediated regulation of other insulinotropic substrates is important for controlling blood glucose levels. One substrate that is likely involved in the non-GLP-1-mediated effects of val-pyr is GIP. GIPR-/- mice display impaired glucose tolerance and significant reductions in glucose-stimulated insulin secretion in response to an oral glucose challenge (349), indicating that GIP plays an essential role in glucose homeostasis following enteral glucose challenge. In addition, DPP-IV/CD26 has been shown to be the primary enzyme responsible for GIP degradation *in vivo* (61) and intact GIP was completely resistant to N-terminal cleavage when incubated in plasma obtained from CD26-/- mice (381). Furthermore, GLP-1R-/- mice exhibit upregulation of glucose-dependent GIP secretion and insulinotropic action (289) and, thus, may be hypersensitive to the increased levels of intact GIP that result from DPP-IV/CD26 inhibition. Enhanced sensitivity to GIP action could also explain why val-pyr treatment resulted in improved glucose tolerance and increased insulin

secretion in GLP-1R-/- mice in response to an i.p. glucose load. Although studies performed with both GIPR-/- and wild-type mice treated with a GIPR antagonist suggest that GIP activity is not essential for clearing an i.p. glucose load (349, 416), it is plausible that elevated levels of intact GIP in val-pyr-treated GLP-1R-/- mice could unmask additional GIP effects, including non-incretin-like actions. However, in order to accurately assess the involvement of GIP in DPP-IV/CD26-mediated glucoregulation, the levels of glucose-stimulated endogenous intact GIP must be measured in the GLP-1R-/- mice, in the presence and absence of val-pyr. Additionally, administration of a GIPR antagonist to GLP-1R-/- mice, in conjunction with val-pyr treatment, would allow us to evaluate the relative contribution of GIP to the improved glucose tolerance and enhanced insulin secretion that was observed in these mice.

From our current studies it is clear that the DPP-IV/CD26-regulated pathway of blood glucose control involves substrates other than GLP-1. As GIP is the most likely candidate peptide to be involved in the GLP-1-independent, DPP-IV/CD26-mediated regulation of blood glucose, studies aimed at evaluating the effects of val-pyr treatment on glucose homeostasis in GIPR-/- mice are warranted.

Since DPP-IV/CD26 activity inactivates a number of biologically important peptides, it is also feasible that other DPP-IV/CD26 substrates, in addition to GLP-1 and possibly GIP, contribute to the improved glucose tolerance and elevated insulin levels seen with val-pyr treatment. With respect to blood glucose regulation, peptide histidine isoleucine (PHI) has been shown to stimulate insulin secretion in mice (417, 418) and is a target for DPP-IV/CD26-mediated proteolytic cleavage (242). Moreover, pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide, has been shown to be a potent *in vivo*

stimulator of glucose-induced insulin secretion in both mice (419) and humans (420). Although the PACAP amino acid sequence contains N-terminal His-Ser, and thus does not conform to the consensus X-Pro or X-Ala motif recognized by DPP-IV/CD26 (62), studies have also shown that, despite its relatively restricted substrate specificity, DPP-IV/CD26 will cleave certain peptides that contain residues other than Pro or Ala in position 2, albeit at much slower rates (408, 421). In addition, others have shown that glucagon, a polypeptide which contains His-Ser at its N-terminus, can be degraded by DPP-IV/CD26 proteolytic activity (415). The relative contribution of insulin secretagogues such as PHI and PACAP, if any, to the improved glucose tolerance and increased insulin secretion in val-pyr treated mice remains to be determined.

Inhibition of DPP-IV/CD26 activity has been shown to improve glucose tolerance and augment glucose-stimulated insulin secretion in a number of rodent models of type 2 diabetes (361-363) and, thus, has been proposed as a relevant therapy for the treatment of type 2 diabetes (241). However, given the variety of substrates that are cleaved by DPP-IV/CD26, its role as an immune modulator, and the existence of other enzymes which exhibit DPP-IV/CD26-like activity, the use of pharmacological agents that inhibit DPP-IV/CD26 activity in humans may be associated with undesirable side effects. Nonetheless, CD26-/- mice appear healthy, are fertile, and tolerate complete loss of DPP-IV/CD26 activity without any reported side effects, other than the observed effects on glucose metabolism (381). Also, mice with a targeted disruption of the fibroblast activation protein (FAP) gene, which encodes an enzyme that possesses DPP-IV/CD26-like activity, are fertile and show no evidence of developmental defects (422). In addition, a strain of DPP-IV/CD26-deficient

Fischer rats that arose as a result of a spontaneous mutation in the CD26 gene, is phenotypically normal (423).

In conclusion, the current study demonstrates that DPP-IV/CD26 inhibition improves glucose tolerance and enhances insulin secretion in the glucose intolerant GLP-1R-/- mouse. Although additional studies are required to assess the consequences of long-term inhibition of DPP-IV/CD26 activity *in vivo*, the results of this study and others indicate that DPP-IV/CD26 inhibitors may be practical and valuable agents for the treatment of type 2 diabetes.

Chapter 4. Sustained Expression of Exendin-4 does not Perturb Glucose Homeostasis, β-cell mass or Food Intake in Metallothionein-Exendin-4 Transgenic Mice

4.1 Introduction

Glucagon-like peptide-1 (GLP-1), a product of the proglucagon gene is released from gut endocrine cells and potentiates glucose-dependent insulin secretion (29). GLP-1 also regulates gastric emptying, food intake, glucagon secretion and islet proliferation, and hence is currently under investigation as a therapeutic agent for the treatment of diabetes (29). However, a significant limitation to GLP-1 therapy in diabetic subjects is the short biological half-life of this peptide (61, 63, 358), limiting its ability to control blood glucose for an extended period of time. These considerations have prompted the investigation of strategies designed to prolong the duration of GLP-1 action *in vivo* (237, 241).

Exendin-4, a peptide structurally related to but distinct from GLP-1 (374), was originally purified from the venom of a *Heloderma suspectum* lizard (64, 424). Subsequent characterization of exendin-4 activity demonstrated that the lizard peptide was a potent agonist for the mammalian GLP-1 receptor (GLP-1R) (64, 65, 68, 277, 424). Exendin-4 exhibits a much longer *in vivo* half-life and prolonged duration of action (68), rendering it more potent for protracted stimulation of GLP-1 receptor signaling and sustained improvement in glucose homeostasis *in vivo*. Despite the structural homology of lizard exendin-4 and mammalian GLP-1, a mammalian exendin-4 gene has not yet been identified (374, 375).

The finding that exendin-4 represents a potent GLP-1-like analogue has prompted studies of exendin-4 activity in normal and diabetic rodents. Exendin-4 potentiates glucose-stimulated insulin secretion and lowers blood glucose in both rats and mice (56, 57, 68, 69,

72). Exendin-4 also inhibits food intake, raising the possibility that chronic exendin-4 treatment may decrease satiety and promote weight loss *in vivo* (68, 69). Furthermore, recent studies demonstrate that exendin-4 administration leads to induction of pancreatic endocrine cell differentiation, islet proliferation, and expansion of β-cell mass (55-57).

Although the biological activities of exendin-4 and GLP-1 have been examined in numerous short term studies, limited information is available regarding the physiological actions of these peptides in experimental paradigms characterized by prolonged exposure to increased levels of GLP-1R agonists. To assess the physiological effects of chronic expression of lizard exendin-4 *in vivo*, we have generated transgenic mice in which lizard exendin-4 expression is under the control of the inducible mouse metallothionein-I (MT-I) promoter. The physiological characterization and metabolic effects of sustained exendin-4 expression in mice *in vivo* are reported in the following sections.

4.2 Methods

4.2.1 MT-Exendin Transgene Construction and Generation of Transgenic Mice

To generate the MT-Exendin transgene, a 492 bp cDNA encoding lizard proexendin-4 (374) (see Fig. 3) was cloned into the *Bgl II* site of the pEV142 expression vector (425), under the control of an inducible mouse MT-I promoter. A 1.9 Kb *Eco RI* fragment containing the MT-Exendin transgene was electro-eluted from a 1% (wt/vol) agarose gel and further purified on an Elutip-d column (Schleicher and Schuell, Keene, NH). Transgenic mice were generated by Chrysalis, DNX Transgenic Sciences, Princeton, N.J. on a C57BL/6 x SJL genetic background. All mice used in these studies were 16–20 weeks old. Control animals were age- and sex-matched transgene-negative mice from the same litter or family.

For induction of MT-I promoter activity, drinking water was supplemented with 25 mM $ZnSO_4$ for a minimum of 72 h. All procedures were conducted according to protocols and guidelines approved by the Toronto Hospital Animal Care Committee.

4.2.2 Plasma Extraction

Blood samples were obtained by cardiac puncture and mixed with 10% (vol/vol) TED (500 KIU/ml Trasylol, 1.2 mg/ml EDTA and 0.1 mM Diprotin A). Plasma was collected by centrifugation at 4°C and mixed with two volumes of 1% (vol/vol) trifluoroacetic acid, pH 2.5. Peptides and small proteins were adsorbed from plasma extracts by passage through a C18 silica cartridge (Waters Associates, Milford, MA). Adsorbed peptides were eluted with 4 ml of 80% (vol/vol) isopropanol containing 0.1% (vol/vol) trifluoroacetic acid. Total protein levels in plasma extracts were determined using the modified Bradford method (386) with Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine γ-globulin as a standard.

4.2.3 High Pressure Liquid Chromatography (HPLC) and Radioimmunoassay (RIA)

HPLC was performed on a Waters system using a C18 μBondapak column. Radioimmunoassay for exendin-4-like immunoreactivity was carried out using a rabbit anti-exendin-4 antiserum (Cocalico Biologicals Inc., Reamstown, PA), synthetic exendin-4 (California Peptide Research Inc., Napa, CA) as standard, and ^{125}I-exendin-4, prepared by the chloramine T method (130, 426).

4.2.4 Glucose Tolerance Tests and Measurement of Plasma Insulin Levels

Oral (OGTT) or intraperitoneal (IPGTT) glucose tolerance tests were carried out following an overnight fast (16-18 h). Glucose (1.5 mg /g body weight) was administered orally through a gavage tube (OGTT) or via injection into the peritoneal cavity (IPGTT).

Blood was drawn from a tail vein at 0, 10, 20, 30, 60, 90 and 120 minutes following glucose administration and blood glucose levels were measured by the glucose oxidase method using a One Touch Basic Glucometer (Lifescan Ltd., Canada). Blood samples (100 µl) for measurement of insulin secretion were removed from tail veins during the 10- to 20-minute time period following oral or intraperitoneal glucose administration and immediately mixed with a 10% vol of a chilled solution containing 5000 KIU/ml Trasylol (Miles Canada, Etobicoke, Canada), 32 mM EDTA, and 0.1 nM Diprotin A (Sigma Chemical Co., St. Louis, MO). Plasma was separated by centrifugation at 4°C and stored at -80°C until assayed for insulin content using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois), with mouse insulin as a standard.

4.2.5 Measurement of Food and Water Intake

For feeding studies, mice were fasted for 18 h and then placed into individual cages containing preweighed rodent chow, with free access to water. At the indicated time points, the chow was re-weighed and total food intake (g/g body wt) was calculated. Food intake was monitored for a total of 24 h. For drinking studies, mice were water deprived for 13 h and then placed into individual cages containing preweighed water bottles, with free access to food. At 0.5, 1, 2 and 24 h the water bottles were re-weighed and water intake (ml) was determined.

4.2.6 Histology and Immunohistochemistry

The pancreas was removed, fixed overnight in either 10% buffered formalin or 4% paraformaldehyde and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin using standard protocols. Immunostaining for insulin and glucagon was carried out as previously described (81, 107, 427).

4.2.7 Estimation of β-Cell Mass

The entire pancreas was removed, weighed, fixed in acidic formalin and paraffin embedded. Paraffin blocks were sectioned and a set of 6–9 sections from each pancreas was sampled by systematic uniform random sampling (SURS). The sampled sections were immunostained for insulin using guinea-pig anti-insulin (Dako, Denmark) as primary antibody (1:100 dilution) and rabbit anti-guinea-pig immunoglobulin (Dako, Denmark) as secondary antibody (1:50 dilution). Antibody binding was visualized by 3,3-diaminobenzidine and sections were counterstained by Meyers hematoxylin. The volume fraction of β-cells within tissue blocks was estimated according to the principle of Delesse (428). The sections were examined using an Olympus BH-2 microscope equipped with a video camera and connected to a computer with C.A.S.T.-grid software (Olympus, USA). Sampling within sections was also performed by SURS. A coherent double-lattice grid was used for point counting. Sampling and grid density was calibrated such that approximately 100-200 points hitting β-cells and approximately the same number of points hitting pancreas were counted per pancreas (429). All estimates of β-cell mass were determined in a blinded manner.

4.2.8 Streptozotocin (STZ) Studies

Six-week-old C57BL/6 male mice were used for these studies. Streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO) was freshly dissolved at 10 mg/ml in 0.1M sodium citrate buffer (pH 5.5). On each day of the experiment all mice were fasted for 3 h (8:30 am-11:30 am) and then their blood glucose levels were measured from a tail vein sample using the glucose oxidase method and a One Touch Basic Glucometer (Lifescan Ltd., Canada). On day 0, following the 3 h fast and measurement of blood glucose, half of the mice were treated

with a single intraperitoneal dose of STZ (200 mg/kg), while the other half served as controls and were given 0.1M sodium citrate alone. On days 2-11, following the 3 h fast and measurement of blood glucose, control and STZ-treated mice received a single i.p. dose of either saline or 24 nmol/kg exendin-4 (California Peptide Research Inc., Napa, CA). On the last day of the experiment (day 11), following a final blood glucose measurement, the mice were euthanized and exsanguinated by cardiac puncture. Plasma was collected from the blood samples for measurement of insulin levels (as described above). The pancreas was removed from each animal and homogenized twice in 5 ml of extraction medium [1 N HCl containing 5% (vol/vol) formic acid, 1% (vol/vol) trifluoroacetic acid, and 1% (wt/vol) NaCl] at 4°C. Peptides and small proteins were adsorbed from extracts by passage through a C18 silica cartridge (Waters Associates, Milford, MA). Adsorbed peptides were eluted with 4 ml of 80% (vol/vol) isopropanol containing 0.1% (vol/vol) trifluoroacetic acid. Pancreatic insulin levels were measured using a rat insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois) with mouse insulin as a standard. Total protein levels in extracts were determined using the Bradford method (386) with Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA).

4.2.9 Statistics

Results are expressed as means ± SEM. Statistical significance was calculated by ANOVA and Student's *t*-test using INSTAT 1.12 (Graph-Pad Software, Inc., San Diego, CA). A p value <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Generation of MT-Exendin Transgenic Mice and Transgene Expression

A 1.9 Kb fragment (Fig. 17A) containing: (i) 770 bp of the mouse MT-I promoter, including 5'-flanking and exon 1 sequences (430, 431); (ii) the 492 bp lizard proexendin-4 cDNA (374); and (iii) 625 bp of the human growth hormone gene containing the polyadenylation signal and 3'-flanking sequences (432) was used for the generation of MT-Exendin (MT-Ex) mice. Transgenic mice were identified by Southern blot analysis (Fig. 17B). Male and female MT-Ex transgenic mice were viable, fertile and appeared to develop normally.

Northern blot analysis detected transgene RNA expression in several tissues including heart, duodenum, jejunum, ileum, colon, stomach, brain, pancreas, and adipose tissue (Fig. 18 and data not shown). Tissue and plasma extracts from MT-Ex mice were prepared and analyzed by radioimmunoassay for exendin-4-like immunoreactivity (Ex 4-IR) using exendin-4 antiserum (performed by Feisal Adatia in Dr. Patricia Brubaker's laboratory in the Department of Physiology at the University of Toronto). The exendin-4 antiserum used for these studies does not cross-react with glucagon, glicentin, oxyntomodulin, gastric inhibitory polypeptide (GIP), vasoactive intestinal polypeptide (VIP), or glucagon like peptides 1 and 2 (GLP-1 and GLP-2), nor does it require a free N-terminus for binding (Dr. Patricia Brubaker, personal communication). In wild-type non-transgenic mice, basal levels of Ex 4-IR were less than 27 pg/ml (Fig. 19A). In contrast, basal plasma levels of Ex 4-IR were 434 ± 39 and 330±84 pg/ml in male and female transgenic mice, respectively (Fig 19A), and induction of transgene expression with zinc treatment resulted in a further ~ 2.5-fold increase in the circulating levels of Ex 4-like IR in both male and female mice (Fig. 19A).

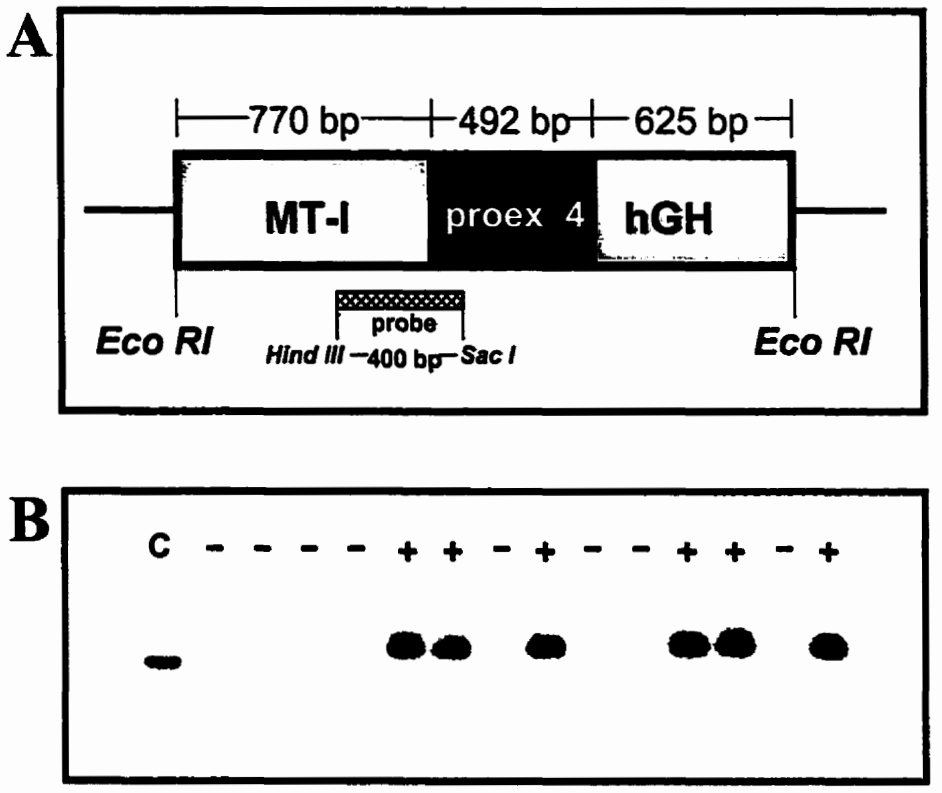


Fig. 17. Structure of the MT-Exendin transgene and Southern blot analysis of genomic DNA from transgenic mice. A: The *Heloderma suspectum* proexendin-4 cDNA was cloned into the pEV142 expression vector, downstream of an inducible mouse metallothionein-I promoter (MT-I) and upstream of 3'-flanking sequences from the human growth hormone (hGH) gene. The 1.9 Kb *Eco RI* fragment containing the MT-Exendin transgene was purified and used to generate transgenic mice. The portion of the transgene that was used as a probe to identify transgenic mice is indicated by the cross-hatched bar. **B:** Transgenic mice were identified by Southern blot analysis of genomic DNA digested with *Hind III* and *Sac I* by using a fragment derived from the MT-Exendin transgene as a probe. Transgene-negative mice are indicated by (-) and MT-Exendin transgene-positive mice are indicated by (+). In the first lane (C), the DNA fragment corresponding to the probe sequence was separated in the gel and served as a positive control.

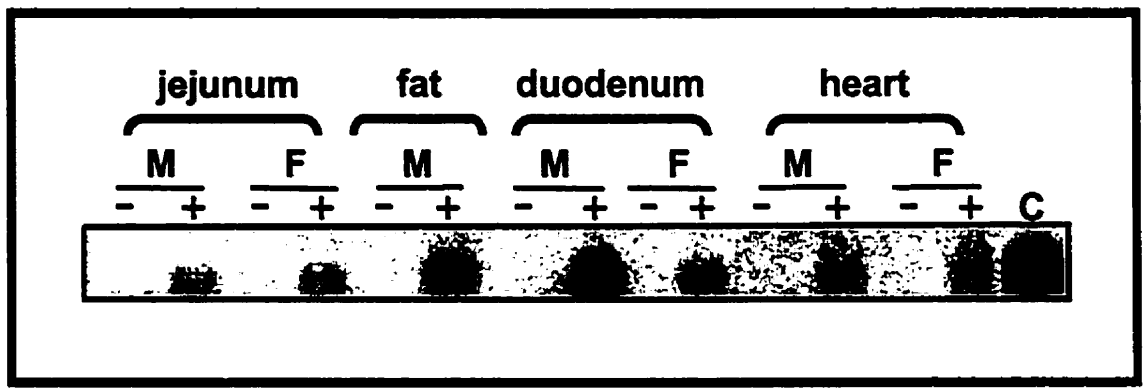


Fig. 18. MT-Exendin transgene expression in adult mouse tissues. Northern blot analysis of total RNA (10 μg) isolated from male (M) and female (F) wild-type control (-) or MT-Exendin transgenic (+) mouse tissues. In the last lane (C), mRNA (25 ng) isolated from lizard salivary gland was separated in the gel and served as a positive control. The blot was hybridized with a ^{32}P-labeled cDNA fragment corresponding to the lizard proexendin-4 cDNA.

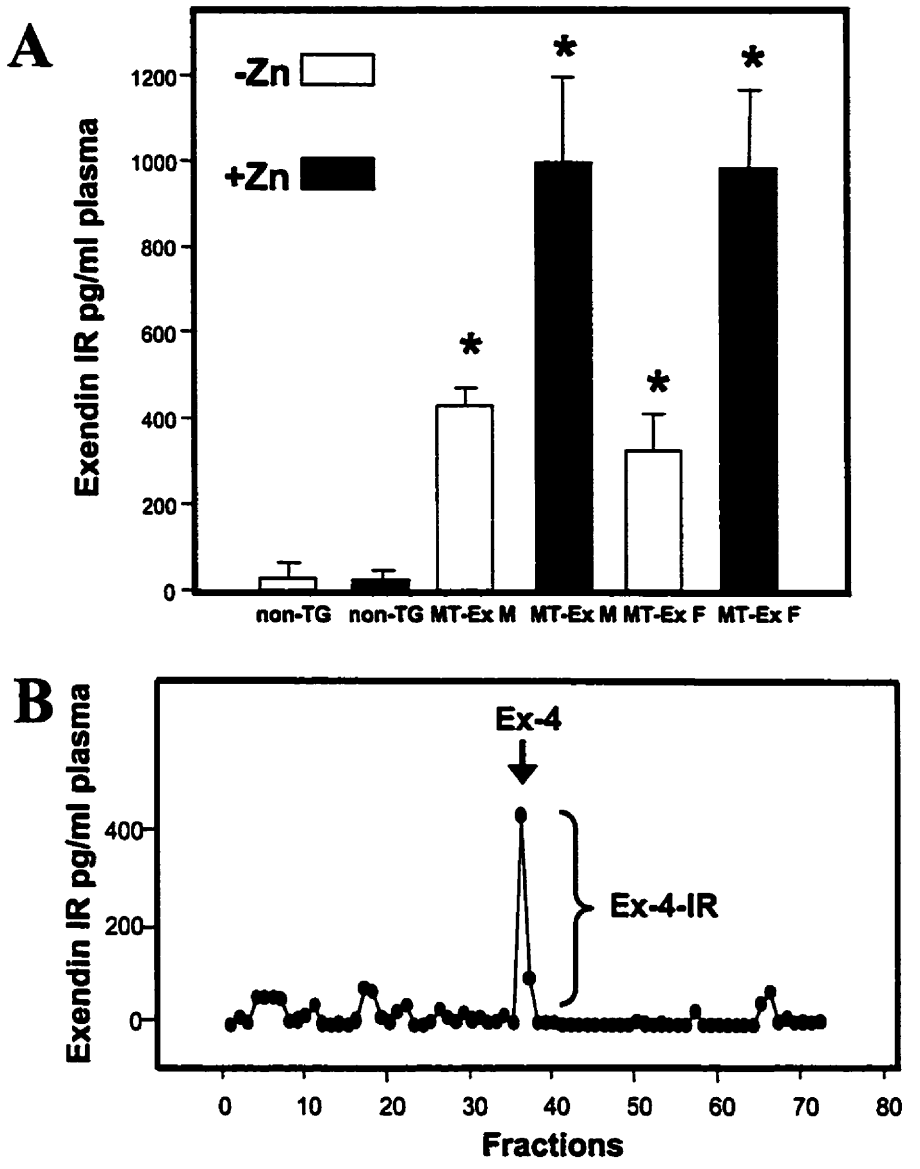


Fig. 19. Detection of exendin-4-like immunoreactivity in the plasma of MT-Exendin transgenic mice. A: RIA for detection of exendin-4-like immunoreactivity (Exendin IR) in plasma from wild-type control littermates (non-TG) and transgenic male (MT-Ex M) or female (MT-Ex F) mice. Mice were given either standard drinking water (-Zn, open bar) or water supplemented with 25 mM $ZnSO_4$ (+Zn, solid bar) to up-regulate transgene expression. Zinc supplementation was for a period of 72 hr. Values are expressed as means ± SE. *$p<0.05$, transgenic vs. control. **B:** HPLC elution profile of exendin-4-like immunoreactivity (Ex-4-IR) extracted from the plasma of a 4-month-old zinc-treated MT-Exendin male mouse. The elution position of synthetic exendin-4 is indicated by the arrow.

To determine whether proexendin was both processed appropriately and secreted into the circulation, HPLC and radioimmunoassay analyses were carried out to characterize the molecular forms of circulating exendin-4-IR (performed by Feisal Adatia in Dr. Patricia Brubaker's laboratory). The major exendin-immunoreactive peptide detected in plasma extracts from MT-exendin-4 transgenic mice eluted at the same position as synthetic exendin-4 (Fig. 19B). Significant amounts of exendin-4-immunoreactivity eluting in the same position as synthetic exendin-4 were also detected in the testes and adrenal glands of transgenic mice (data not shown).

4.3.2 *In Vivo* Effects of Exendin-4 on GLP-1 Receptor-Dependent Physiological Endpoints

Although the biological properties of exendin-4 have been examined in acute administration studies and following once daily administration regimens in rodents (56, 57, 72), the long term consequences of increased circulating exendin-4 on GLP-1 receptor-dependent actions has not been examined.

As GLP-1 receptor signaling is essential for control of fasting blood glucose, glucose clearance and glucose-stimulated insulin secretion (29), we examined these parameters in control and MT-Ex transgenic mice. Fasting blood glucose levels were normal in MT-Ex mice under conditions of either basal or induced transgene expression (Figs. 20 and 21). Despite clearly detectable levels of circulating exendin-4 immunoreactivity, blood glucose excursion and glucose-stimulated insulin was comparable in wild-type control and MT-Ex transgenic mice following either oral (Fig. 20A) or intraperitoneal glucose challenge (Fig. 21A). In contrast, induction of transgene expression with zinc treatment resulted in a

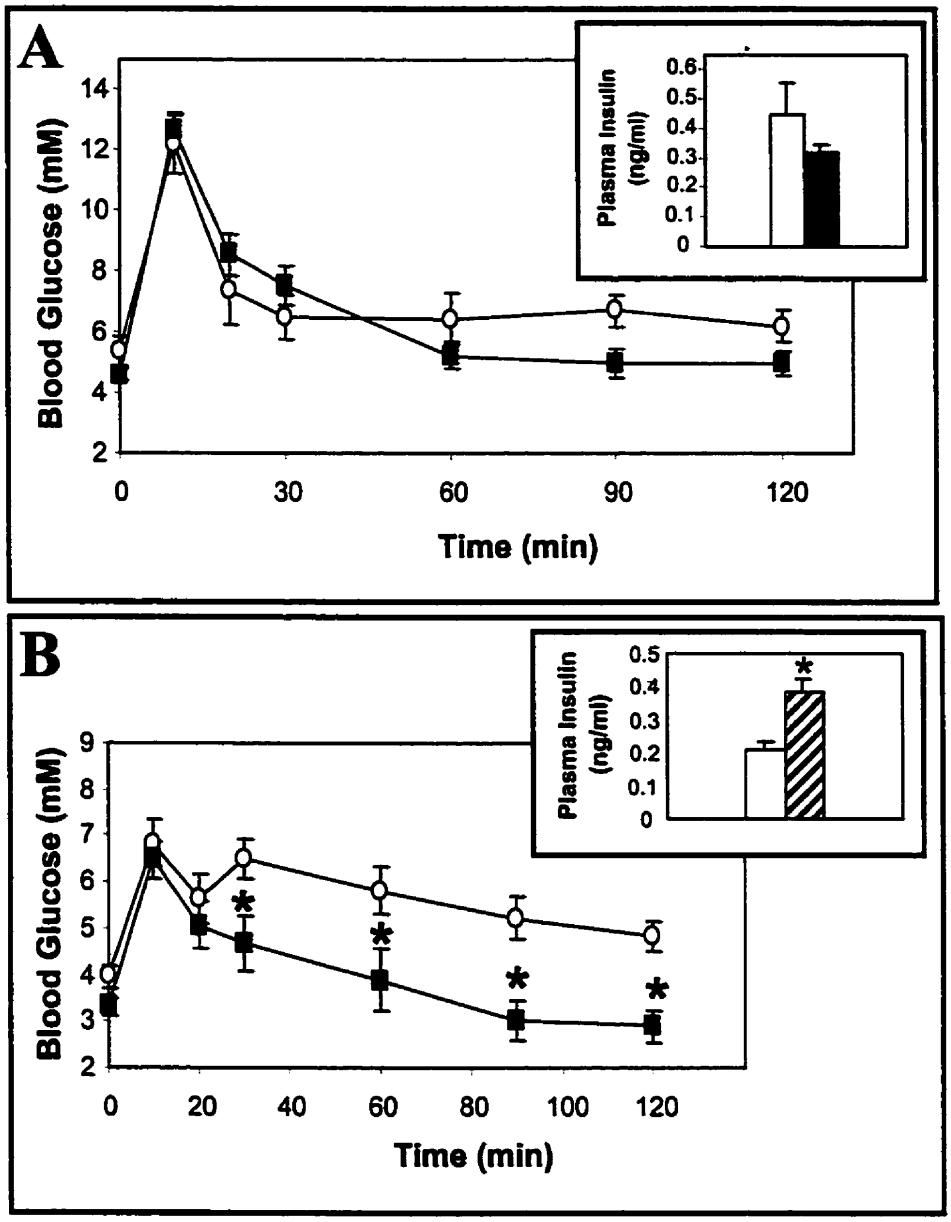


Fig. 20. Oral glucose tolerance and plasma insulin levels in control and MT-Exendin transgenic female mice. Values are expressed as means ● SEM, n = 8-12 mice/group. *$p<0.05$, transgenic vs. control mice. **A:** Oral glucose tolerance in control (open circles) and MT-Exendin (solid squares) mice. Plasma insulin concentrations (inset) following oral glucose in control (open bar) and MT-Exendin (solid bar) mice were measured in plasma obtained at the 10-20 min time point following oral glucose. **B:** Oral glucose tolerance in control (open circles) and MT-Exendin (solid squares) mice following treatment with 25 mM $ZnSO_4$ to up-regulate transgene expression. Plasma insulin concentrations (inset) in control (open bar) and MT-Exendin (hatched bar) mice were obtained at the 10-20 min time point following oral glucose.

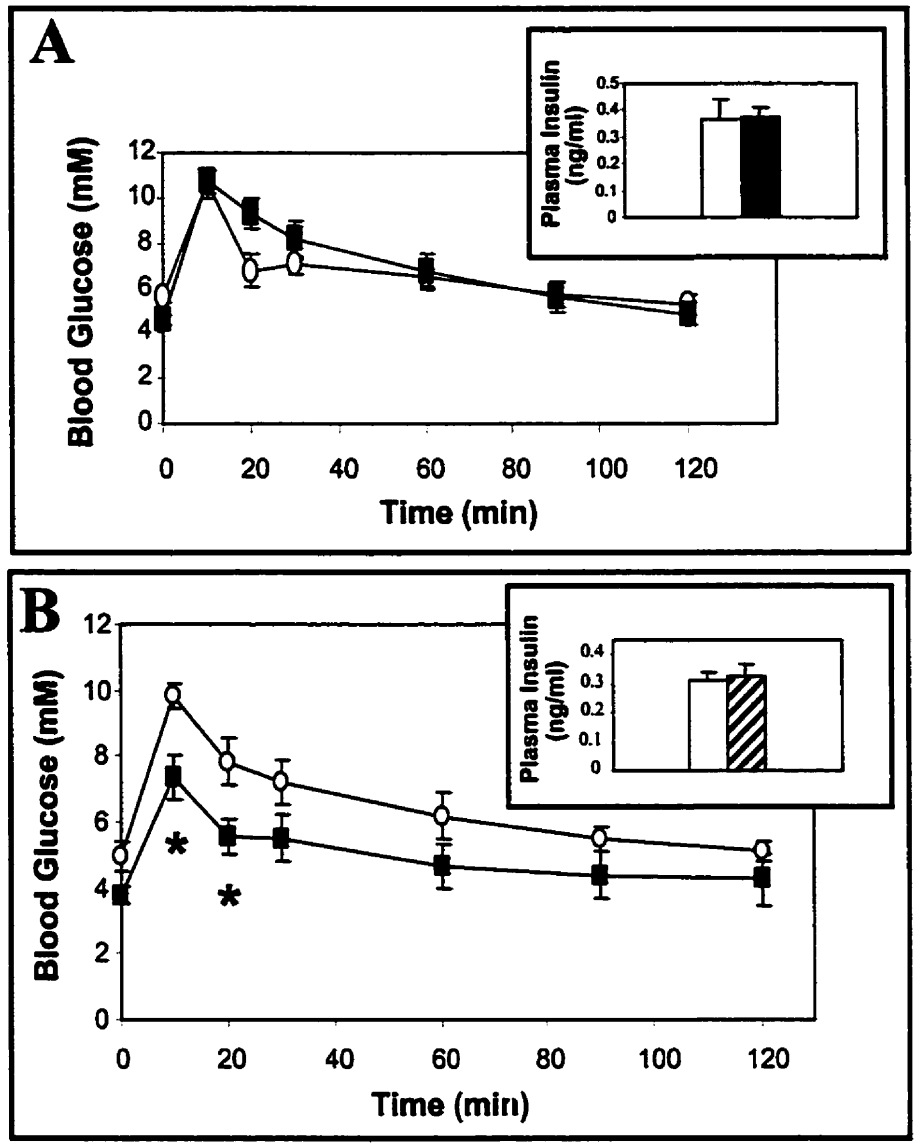


Fig. 21. Intraperitoneal glucose tolerance and plasma insulin levels in control and MT-Exendin transgenic female mice. Values are expressed as means ± SEM, n = 8-10 mice/group. *$p<0.05$, transgenic vs. control mice. **A:** Intraperitoneal (i.p) glucose tolerance in control (open circles) and MT-Exendin (solid squares) mice. Plasma insulin concentrations (inset) following i.p. glucose in control (open bar) and MT-Exendin (solid bar) mice were measured in plasma obtained at the 10-20 min time point following i.p. glucose. **B:** Intraperitoneal (i.p.) glucose tolerance in control (open circles) and MT-Exendin (solid squares) mice following treatment with 25 mM $ZnSO_4$ to up-regulate transgene expression. Plasma insulin concentrations (inset) in control (open bar) and MT-Exendin (hatched bar) mice were obtained at the 10-20min time point following i.p. glucose.

significant reduction in the glycemic excursion in response to oral (Fig. 20B) and intraperitoneal (Fig. 21B) glucose loading. The reduced glycemic excursion was associated with a significant increase in plasma levels of glucose-stimulated insulin after oral but not intraperitoneal glucose challenge (0.38 ± 0.04 ng/ml vs. 0.21±0.02 ng/ml, for insulin in MT-Ex transgenic vs. control mice respectively; $p<0.05$; Fig. 20B inset).

The physiological importance of GLP-1 receptor signaling for CNS control of food intake and body weight remains unclear. Administration of intracerebroventricular (ICV) GLP-1 or exendin-4 inhibits short term feeding, while repeated administration of the GLP-1R antagonist exendin (9-39) increases food intake and promotes weight gain in rats (36, 41). In contrast, mice with complete disruption of GLP-1R signaling do not exhibit defects in feeding control or body weight homeostasis (288, 294). Basal levels of exendin-4 expression had no effect on short (2 hr) or long (24 hr) term food intake (Fig. 22A and B) in MT-Ex mice. However, up regulation of transgene expression following zinc treatment lead to a small, but significant reduction in short term (2hr) food intake (0.026±0.003 g/g body weight in MT-Ex transgenic vs. 0.034±0.001 g/g body weight in control mice; $p<0.05$; Fig. 22C and D). Basal levels of transgene expression were also associated with a significant reduction in short term (up to 2hr) water intake (Fig. 23A and B). In contrast to recent studies demonstrating weight loss in exendin-4-treated rats (69), no significant differences in body weight, relative to non-transgenic littermates, were observed in MT-Ex mice at 4-8, 16 or 20 weeks of age (data not shown).

Increasing evidence suggests that both GLP-1 and exendin-4 stimulate β-cell replication and neogenesis, enhance islet size, and promote differentiation of pancreatic precursor cells into islet cells (54-57). To examine the effects of transgene expression on

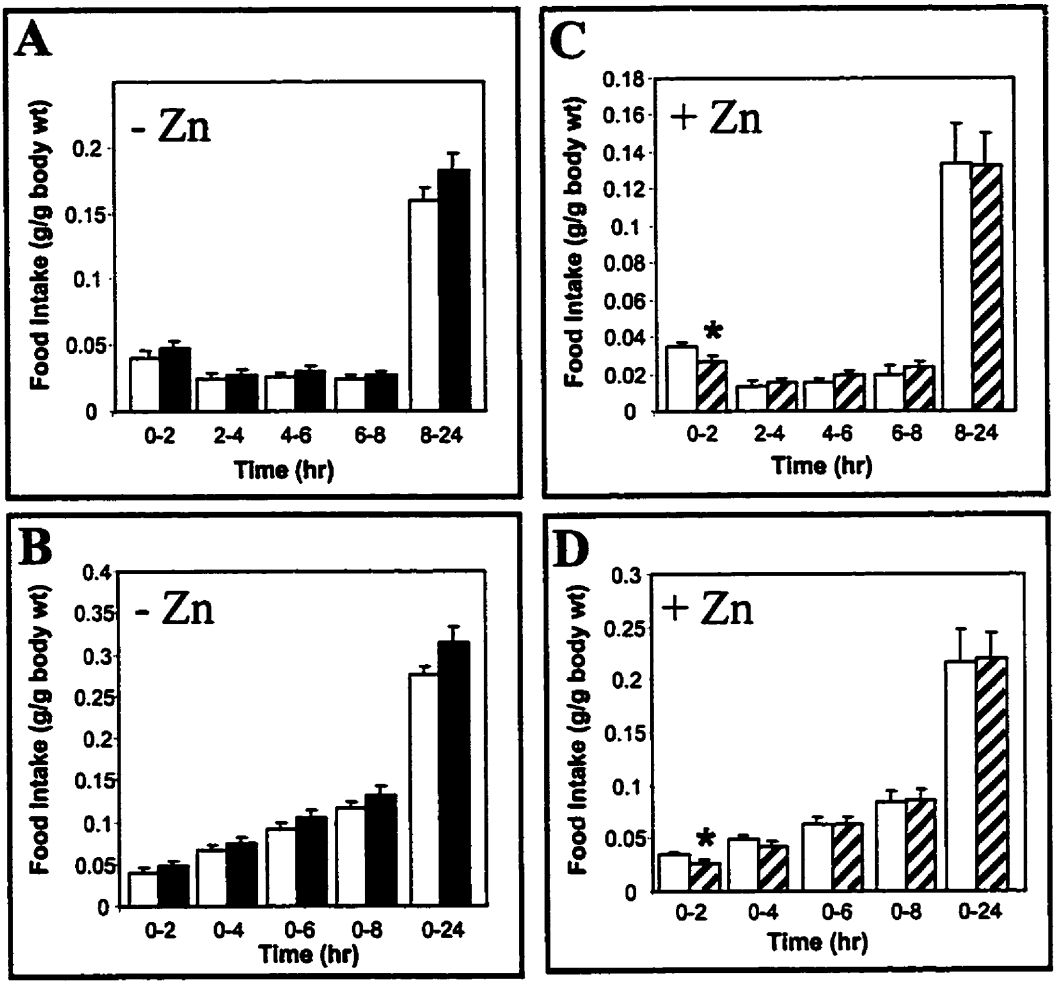


Fig. 22. Food intake in control and MT-Exendin mice. Following an overnight fast, food intake was monitored during specific time intervals **(A)** and **(C)**, as well as cumulatively **(B)** and **(D)**, for a total period of 24 hr in control (open bar) and MT-Exendin (solid or hatched bars) mice. +Zn denotes mice treated with zinc supplementation as described in section 4.2.5. Values are expressed as means ± SEM; n = 6 mice/group. *$p<0.05$, transgenic vs. control mice.

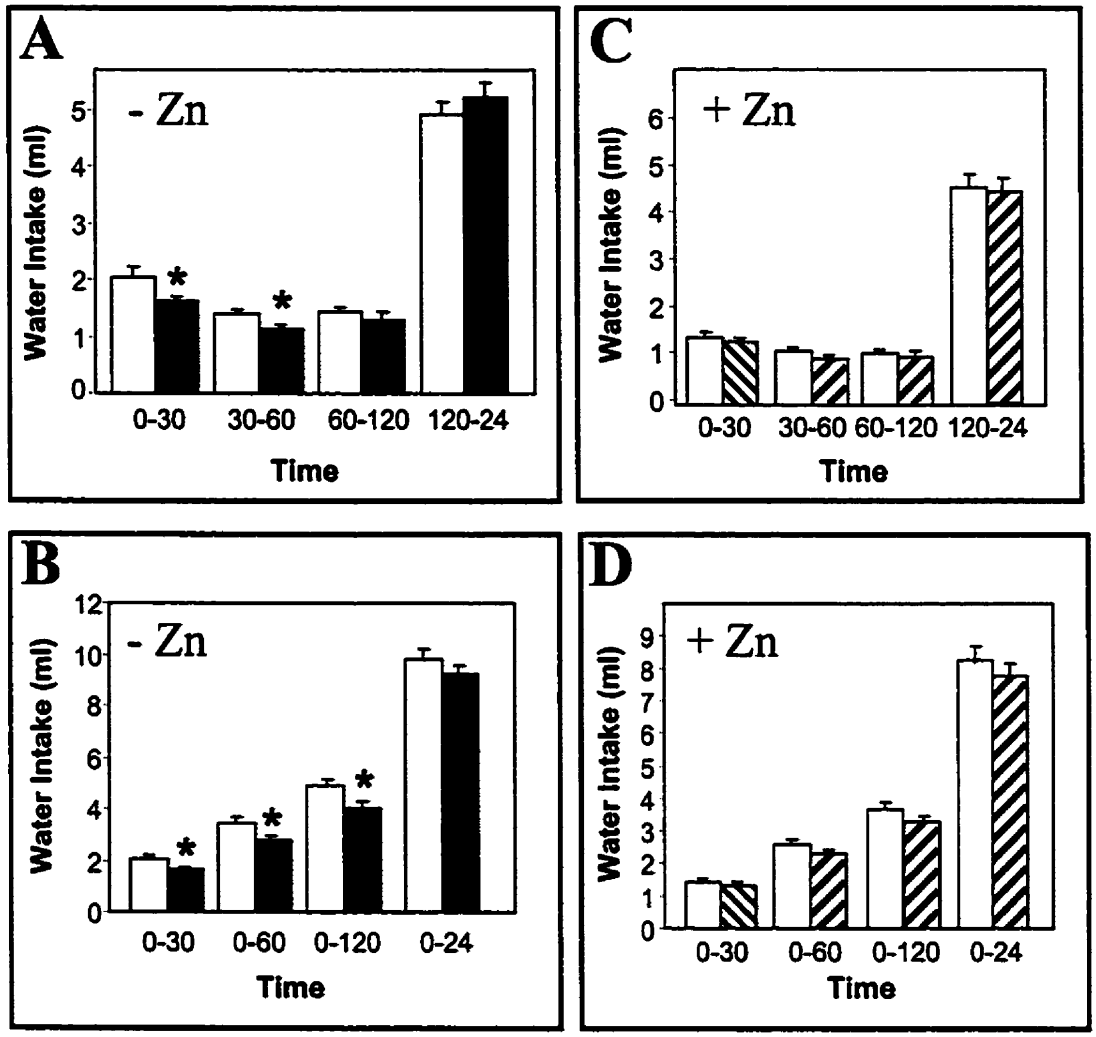


Fig. 23. Water intake in control and MT-Exendin mice. Following a 13 h period of water deprivation, water intake was monitored during specific time intervals (A) and (C), as well as cumulatively (B) and (D), for a total period of 24 h in control (open bars) and MT-Exendin transgenic (solid bars) mice. +Zn denotes mice treated with zinc supplementation as described in section 4.2.5. Values are expressed as means ± SEM; n = 5-7 mice/group. *p<0.05, transgenic vs. control mice.

islet growth, we examined pancreata from MT-Ex transgenic mice. Islet histology appeared normal and comparable in transgenic and wild-type control mice, with no gross evidence of islet neogenesis or abnormal distribution of endocrine cell types within the islets (Fig. 24A and B). Furthermore, quantitative analysis demonstrated no differences in β-cell mass in MT-Ex transgenic compared to wild-type control mice (carried out by Troels Bock at the Bartholin Institute in Denmark) (Fig. 24C).

To determine whether hyperglycemia was required for exendin-4-mediated induction of islet proliferation and enhanced β-cell mass, wild-type C57BL/6 mice were rendered hyperglycemic following administration of a single, large dose of streptozotocin and groups of diabetic mice were treated daily with intraperitoneal injections of saline or exendin-4 (24 nmol/kg). Daily exendin-4 treatment resulted in a significant improvement in fasting blood glucose levels in diabetic mice, but had no effect on fasting blood glucose in non-diabetic animals (Fig. 25A). Levels of plasma or pancreatic insulin were not detectable in saline- or-exendin-4-treated diabetic mice. In non-diabetic mice, exendin-4 treatment significantly increased fasting plasma insulin levels (2.56±0.16 ng/ml in exendin-4-treated vs. 1.8±0.22 ng/ml in saline treated controls; $p<0.05$; Fig. 25B) but had no statistically significant effect on pancreatic insulin content (Fig. 25C). Furthermore, evidence of increased islet size or islet neogenesis was not detected in a non-quantitative histological assessment of pancreata following exendin-4 treatment in diabetic mice (Fig. 26).

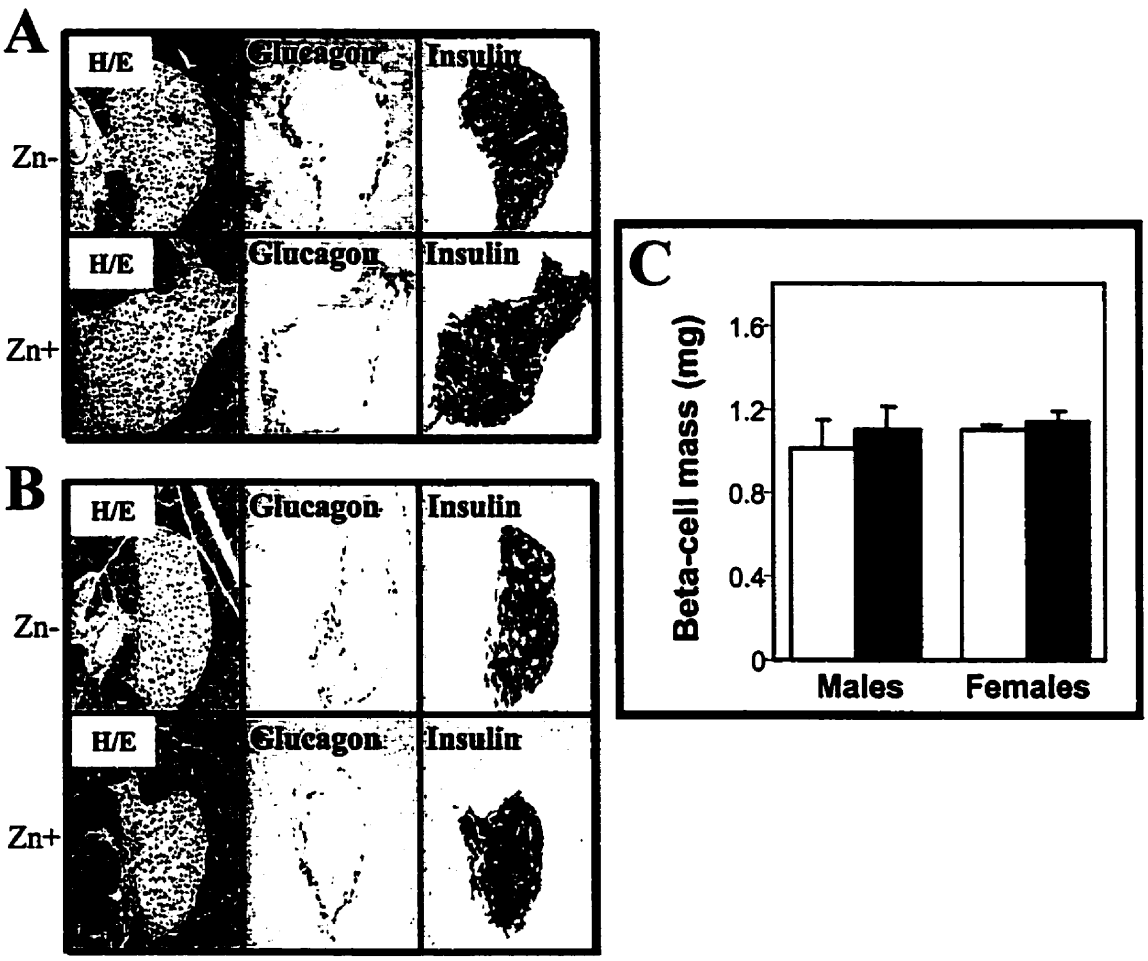


Fig. 24. Normal islet morphology and β-cell mass in MT-Exendin transgenic Mice. Hematoxylin and eosin (H/E) and immunohistochemical staining for glucagon and insulin in the pancreatic islets of control **(A)** and MT-Exendin transgenic **(B)** mice. Pancreata were obtained from control and transgenic animals that were given either standard drinking water (Zn-), or water supplemented with 25 mM $ZnSO_4$ (Zn+) for 5-7 days to up-regulate transgene expression. **C:** β-cell mass in control (open bars) and MT-Exendin transgenic (solid bars) mice. Values are expressed as means ± SEM; n = 3-8 mice/group. All mice were maintained on water supplemented with 25 mM $ZnSO_4$ for 5-7 days to up-regulate transgene expression.

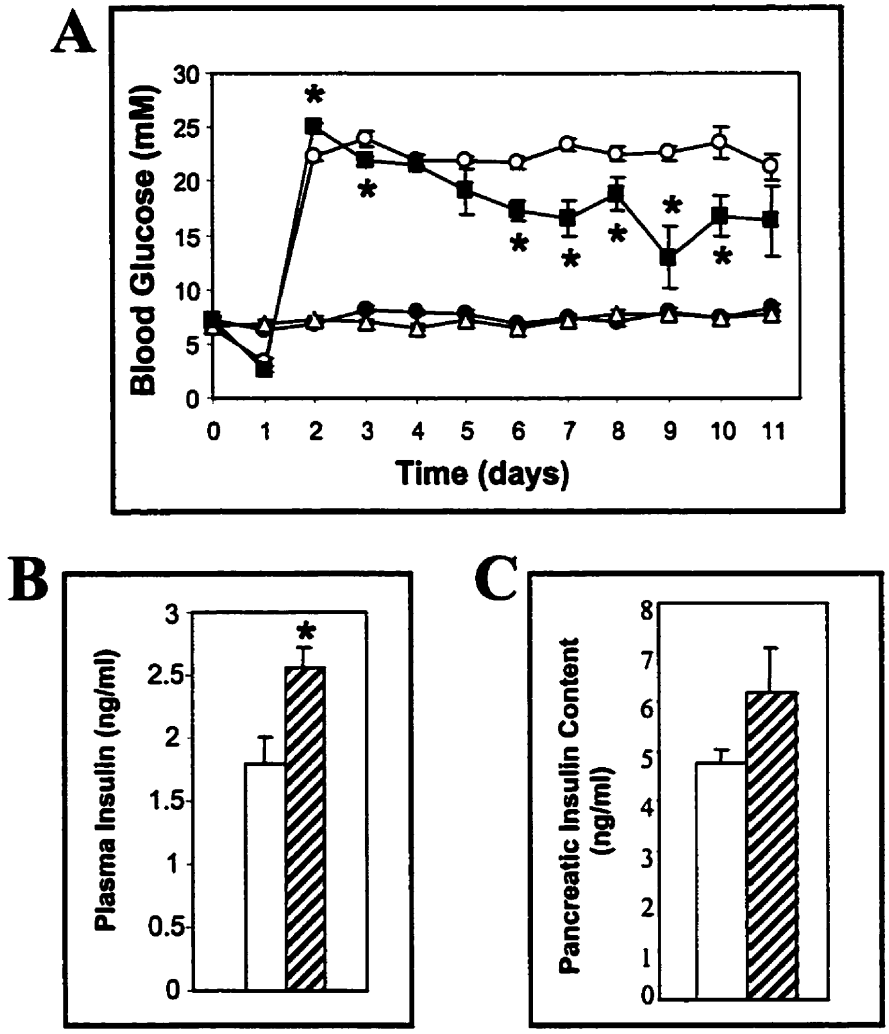


Fig. 25. Effects of exogenous exendin-4 on fasting blood glucose, plasma insulin and pancreatic insulin content in diabetic and non-diabetic C57BL/6 mice. **A:** Fasting blood glucose levels in mice treated with saline (solid circles), exendin-4 (open triangles), streptozotocin (STZ) alone (open circles), or STZ plus exendin-4 (solid squares). Values are mean ± SEM; n = 5-7 mice/group. *$p<0.05$, STZ plus exendin-4-treated vs. STZ-treated mice. Plasma insulin **(B)** and pancreatic insulin content **(C)** on day 11 of exendin-4 treatment in saline (open bar) or exendin-4-treated (hatched bar) non-diabetic mice. Detection Limits for insulin were 156 pg/ml in plasma and 39 pg/ml in pancreatic tissue samples.

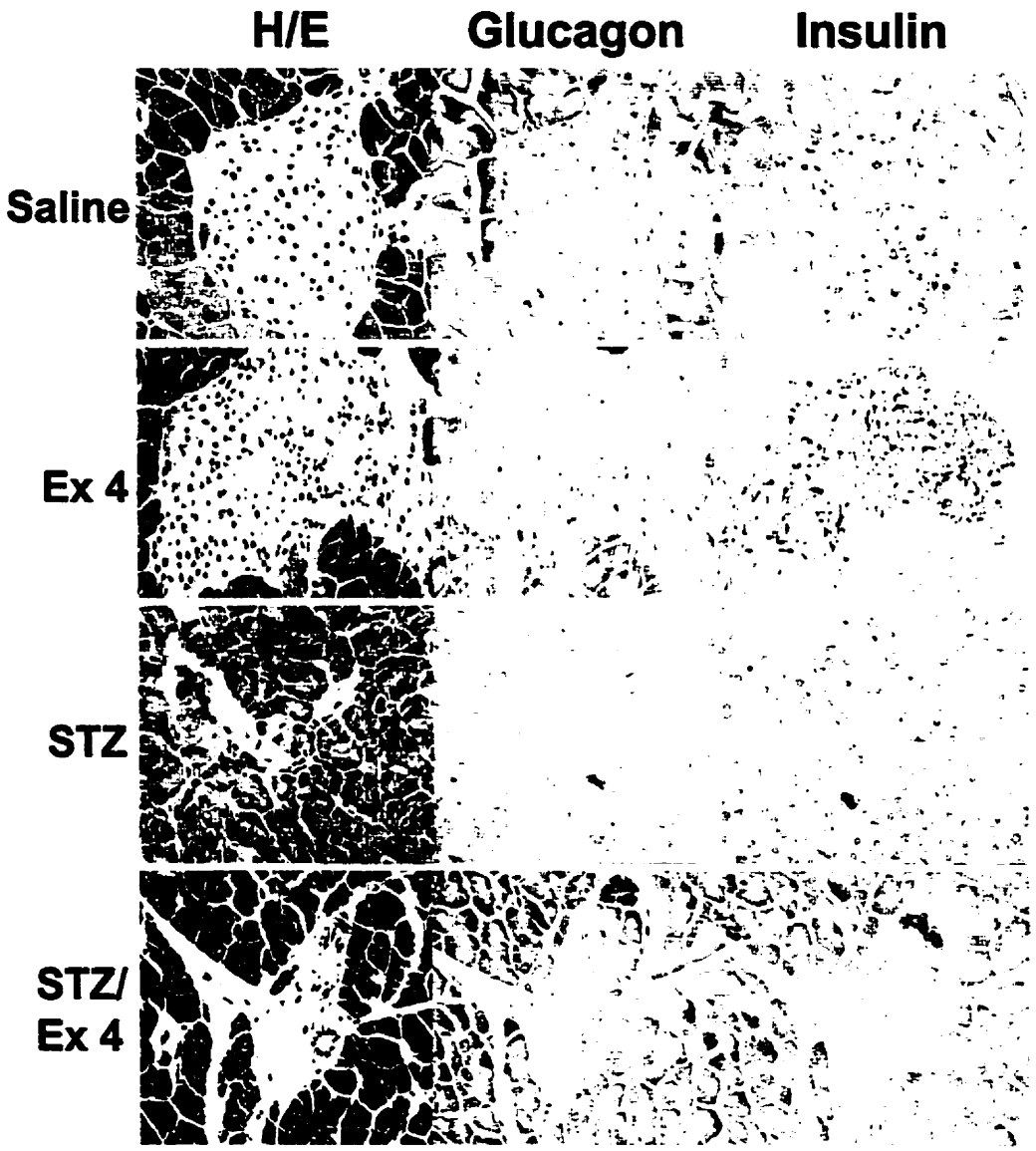


Fig. 26. Exendin-4 treatment has no effect on islet morphology in normal or diabetic C57BL/6 mice. Hematoxylin/eosin (H/E) and immunohistochemical staining for glucagon and insulin in the pancreatic islets of C57BL/6 mice treated with either saline, exendin-4 (Ex 4), streptozotocin (STZ), or streptozotocin plus exendin-4 (STZ/Ex 4).

4.4 Discussion

The observation that GLP-1 exhibits a very short plasma half-life due to its rapid degradation by dipeptidyl peptidase-IV (DPP-IV) (61, 63) has prompted a search for DPP - IV-resistant GLP-1 analogues that exhibit longer durations of action and enhanced potency *in vivo*. Several GLP-1 analogues have now been reported that exhibit improved potency in both normal and diabetic rodents (237, 238, 240, 366). Furthermore, fatty acid derivatives of GLP-1 may also result in enhanced albumin binding and more prolonged bioactivity *in vivo* (239). The naturally occurring lizard exendin-4 peptide is not a substrate for DPP-IV, and consequently exhibits a much longer half-life and greater potency *in vivo* (68, 72, 424).

GLP-1 and exendin-4 have been administered daily to humans and diabetic rodents for periods of up to several weeks (57, 69, 72, 169, 231), however the long term consequences of more prolonged exendin-4 administration have not been examined. Although cell-based delivery systems for GLP-1 and exendin-4 have been proposed (433), there is little information available on the viability or efficacy of this strategy in rodents *in vivo*. The generation of mice expressing lizard proexendin-4 provides an opportunity to assess the safety and feasibility of continuous exendin-4 delivery in mice *in vivo*. Even though studies of the molecular determinants of proexendin-4 processing have not yet been reported, the finding of detectable levels of circulating exendin-4 in MT-Ex transgenic mice is consistent with the correct processing and secretion of the lizard proexendin precursor in murine tissues *in vivo*. Furthermore, the levels of circulating bioactive exendin-4 detected in MT-Ex mice are much higher than plasma levels of GLP-1 (29) and within the range or higher than plasma levels of exendin-4 that were able to decrease blood glucose in diabetic

db/db mice (68, 434). Hence, our current observations cannot simply be attributed to a failure to achieve sufficient levels of bioactive exendin-4 *in vivo*.

Exogenous GLP-1/exendin-4 treatment has been shown to reduce both fasting and postprandial blood glucose levels and enhance glucose-stimulated insulin secretion in both human and rodent studies (4, 29, 72, 170, 227, 228, 376, 435, 436). In complementary studies, mice with a targeted disruption of the GLP-1 receptor gene exhibit mild fasting hyperglycemia (288) and immunoneutralization or blockade of GLP-1 action increases fasting blood glucose in baboon, rodent and human studies (280, 282, 283). These findings implicate an important role for basal GLP-1 signaling, even in the fasting state, for control of glucose homeostasis. Although basal levels of circulating exendin-4 were clearly detectable in MT-Ex mice, fasting blood glucose was normal, even upon further induction of transgene expression with zinc treatment. Furthermore, hypoglycemia was not observed in MT-Ex mice despite further induction of transgene expression with zinc. As exendin-4 has been estimated to be up to at least 5000 times more potent than GLP-1 with respect to glucose lowering *in vivo* (68), our findings of normoglycemia in MT-Ex mice further emphasize the glucose-dependence of GLP-1R signaling for glucoregulation *in vivo* (4, 29).

Although incretins such as GIP and GLP-1 have been proposed as possible treatments for patients with diabetes, short term infusion of GIP has been associated with diminished effectiveness in diabetic patients (437) and desensitization of the GIP receptor in diabetic rats *in vivo* (322). Both homologous and heterologous desensitization of GLP-1 receptor signaling has also been observed in islet cell lines *in vitro* (272, 274, 438). However, daily administration of exendin-4 to diabetic mice for 13 weeks reduced levels of blood glucose and glycosylated hemoglobin and increased plasma insulin (72), demonstrating that a single

daily exendin-4 injection does not produce significant desensitization *in vivo*. The results of our studies in MT-Ex transgenic mice extend these observations by demonstrating that, despite continuous exposure to transgene-derived exendin-4 for several months, acute induction of transgene expression in older mice led to enhanced glucose clearance and significantly increased levels of glucose-stimulated insulin following oral glucose challenge. These findings suggest that ongoing continuous exposure to exendin-4 is not associated with significant impairment of GLP-1 receptor-dependent actions such as loss of the glucose-lowering effects of exendin-4 *in vivo*. However, whether β-cell desensitization to GLP-1R agonists will prove to be an issue in long term human studies cannot be inferred from our current studies in MT-Ex mice.

The physiological importance of GLP-1 receptor signaling for control of food and water intake remains unclear (439), however a large number of studies have demonstrated that exogenous administration of GLP-1 or exendin-4 clearly reduces food intake. In rodents, ICV administration of GLP-1 reduced short- but not long-term food and water intake (36-38, 40), whereas peripheral GLP-1 administration inhibited water intake, but had no effect on feeding (37). However, a recent study found that acute central (via ICV) or peripheral (via sub-cutaneous) administration of GLP-1 or exendin-4 resulted in a reduction in food intake (for at least 4 hr) in Zucker obese rats (440). Moreover, additional studies have shown that daily i.p. exendin-4 treatment can reduce short-term, but not long-term, food intake in rodents (69, 72). In both normal and type 2 diabetic humans, intravenous administration of GLP-1 was found to promote satiety and reduce energy intake (39, 40).

Although chronic ICV administration of exendin (9-39) increased feeding and weight gain in rats (41), we found no evidence for dysregulation of food intake or body weight in

MT-Ex transgenic mice. The effects of exendin-4 on food intake may be related to the mode of exendin-4 delivery and the relative changes in the levels of systemic exendin-4. Rats treated with a single daily i.p. dose of exendin-4 exhibited no significant changes in food intake or body weight after the first few days of exendin-4 administration, whereas twice daily exendin-4 dosing led to a more sustained reduction in food intake and body weight (69). In contrast, basal transgene expression of exendin-4 in MT-Ex mice was associated with a significant reduction in short term water intake. However, only induced, but not basal exendin-4 expression was associated with a significant reduction in short term food intake. These findings have implications for future studies designed to deliver therapeutic levels of exendin-4 that promote sustained reductions in food intake and body weight over a long term treatment period.

Several experiments implicate a role for exogenous exendin-4 in the induction of β-cell neogenesis and proliferation. Treatment of pancreatic AR42J cells with exendin-4 induced differentiation into insulin-secreting islet cells (55), and exendin-4 stimulated β-cell replication and neogenesis, enhanced ductal pdx-1 expression in the islets and improved glucose control in rats and mice (56, 57). In contrast, we observed no differences in islet morphology or β-cell mass in normoglycemic MT-Ex transgenic mice. The finding of normal islet histology in MT-Ex transgenic mice may reflect the need for additional metabolic conditions, such as hyperglycemia, to promote islet neogenesis following activation of GLP-1R signaling. Alternatively, ductal and islet cells chronically exposed to exendin-4 may compensate by downregulating the GLP-1R-dependent signaling pathways leading to increased islet proliferation. Taken together, our data suggest that sustained

exposure to circulating exendin-4 alone in normoglycemic transgenic mice is not sufficient for induction of islet proliferation or neogenesis.

To address the possibility that hyperglycemia or a different mode of exendin-4 delivery is required for induction of islet cell proliferation, we examined glucose regulation, pancreatic insulin content and islet histology in normal and diabetic mice treated with daily administration of exogenous exendin-4. Although a small improvement in blood glucose was observed in exendin-4-treated diabetic mice, we did not observe any changes in islet histology after 10 days of exendin-4 administration. Hence, in contrast to the results of recent studies (56, 57), our results from MT-Ex transgenic mice and exogenous exendin-4 administration to diabetic mice demonstrate that increased levels of exendin-4 alone are not sufficient to stimulate meaningful changes in β-cell mass or islet neogenesis.

As exendin-4 and long acting GLP-1 analogues have generated considerable interest as potential therapeutic agents for the treatment of diabetes, several questions about the safety and efficacy of these molecules remain unanswered. Our analyses of MT-Ex mice demonstrate that although bioactive exendin-4 is liberated following transgene expression, sustained reductions in food intake or body weight, or induction of islet proliferation are not invariable consequences of continual exendin-4 expression in the mouse. Given the central importance of these biological actions for the potential treatment of diabetes, MT-Ex mice represent a useful new model for analysis of the physiological consequences of long-term activation of GLP-1 receptor signaling *in vivo*.

Chapter 5. Discussion and Future Directions

Incretin hormones have proven to be essential regulators of glucose homeostasis. Studies utilizing GIPR-/- and GLP-1R-/- mice have indicated that the two most widely recognized incretin hormones, GIP and GLP-1, contribute to glucose lowering via both similar and distinct mechanisms. These findings are corroborated by our studies which utilized specific receptor antagonists to inhibit GIP and GLP-1 action *in vivo*. Although our studies were limited by the efficacy and tissue bioavailability of our receptor antagonists, the results obtained with exendin (9-39) and GIPR Ab treatment support the observations in the GLP-1R-/- and GIPR-/- mice, respectively, by an independent approach. Thus, in addition to its role as an incretin, GLP-1 also has non-incretin effects that contribute to glucose lowering, including glycemic control by basal levels of circulating GLP-1 that is independent of the site of glucose entry. In contrast, the role of GIP in glucose homeostasis appears to be restricted to its incretin function.

The non-incretin effects of GLP-1 on glucoregulation could be mediated by any one or a combination of its abilities to suppress glucagon secretion or enhance peripheral glucose disposal. Although previous studies have reported that GLP-1 has a direct, insulin-independent effect on glucose disposal in both normal and diabetic humans (50, 51), more recent studies do not support such a role for GLP-1 (441, 442). In addition, whole-body glucose utilization was similar in wild-type and GLP-1R-/- mice, under both basal and hyperinsulinemic conditions (291). Taken together, these studies indicate that GLP-1 likely does not regulate blood glucose levels by enhancing glucose uptake in peripheral tissues. Thus, the observed increase in the glucose excursion in response to exendin (9-39) treatment in our studies is likely not due to an effect on glucose uptake in the periphery.

Administration of exendin (9-39) to humans or baboons results in elevations in the fasting levels of both glucose and glucagon, indicating that basal levels of GLP-1 have a tonic inhibitory effect on glucagon secretion (282, 283). Although we did not examine the effects of exendin (9-39) treatment on fasting glucose or glucagon levels, or on glucose-stimulated glucagon secretion, it is possible that the increased glucose excursion that was observed in mice treated with exendin (9-39) could be due to loss of the inhibitory effect of GLP-1 on glucagon secretion. However, GLP-1R-/- are reported to have normal fasting and postprandial glucagon levels (291). Our studies require the measurement of plasma glucagon levels in order to determine whether elevations in glucagon were responsible for the abnormal glucose excursion in response to exendin (9-39) treatment. One barrier to these experiments is that concurrent measurements of blood glucose, insulin and glucagon levels require considerable amounts of blood and therefore it would be difficult to simultaneously measure all of these parameters in a small animal.

Despite a reported role for GIP and GLP-1 in the regulation of insulin gene expression and insulin biosynthesis *in vitro* (163, 164, 311, 312), we found that inhibition of GIP- or GLP-1 receptor signaling had no effect on insulin mRNA levels or insulin biosynthesis. Although these results suggest that GIP and GLP-1 are not essential for insulin gene expression and biosynthesis in mice, it is also possible that our treatment period of 18 h was insufficient to induce such effects. Thus, in order for us to observe changes in insulin gene expression and biosynthesis, a more protracted treatment with the receptor antagonists may be required.

Since the results of our studies with the incretin receptor antagonists parallel the findings in the GIPR-/- and GLP-1R-/- mice, future studies aimed at clarifying the necessity

of GIP and GLP-1 for glucose homeostasis would utilize the GIPR/GLP-1R double knock-out mouse, which is currently being generated by our laboratory. The levels of fasting blood glucose, as well as the glucose excursion and plasma insulin levels in response to oral and intraperitoneal glucose challenges would be measured to determine if loss of both GIP and GLP-1 receptor signaling produces additional perturbations in glucose homeostasis.

A number of reports have shown that DPP-IV-mediated regulation of incretin hormone activity has important consequences with respect to glucose homeostasis. Consequently, new anti-diabetic therapeutic strategies are directed at the synthesis of DPP-IV-resistant incretin analogues or the generation of compounds which inhibit DPP-IV activity. It has been established that DPP-IV is the primary enzyme responsible for the degradation and inactivation of GLP-1, and a number of *in vivo* studies have shown that inhibition of DPP-IV activity is associated with increased levels of intact, biologically active endogenous GLP-1. However, the improved oral glucose tolerance and increased plasma insulin levels that we observed in GLP-1R-/- mice treated with val-pyr, a specific DPP-IV inhibitor, indicate that DPP-IV regulation of glucose homeostasis can be mediated by GLP-1-independent mechanisms. We suggested that the incretin hormone GIP, also a substrate for DPP-IV proteolytic degradation, is likely involved in the non-GLP-1-mediated effects of DPP-IV inhibition. However, we also observed improved glucose clearance and elevated plasma insulin levels in val-pyr-treated GLP-1R-/- mice during an IPGTT. Since previous studies have indicated that GIP activity is not essential for clearing a non-enteral glucose load, it is quite possible that the activities of other DPP-IV substrates contribute to the improved glucose tolerance and enhanced insulin levels in these mice. A number of insulin secretagogues, including PHI, VIP and PACAP, have been shown to be (e.g. PHI) or are

potential candidates (e.g. VIP, PACAP) for DPP-IV-mediated degradation. It is possible that, like GIP, the levels and activities of these additional peptides are also up-regulated in GLP-1R-/- mice. Thus, the improvements in glucose tolerance and insulin secretion in the val-pyr-treated GLP-1R-/- mouse could be attributed to compensatory upregulation of other insulin secretagogues and/or DPP-IV-mediated enhanced insulin secretagogue activity. Until the levels of these other peptides are measured in val-pyr-treated GLP-1R-/- mice, these inferences remain purely speculative. Future studies that would help to delineate the role of GIP in the mediation of GLP-1-independent DPP-IV-regulated glucose control would include an examination of the effects of DPP-IV inhibition in the GIPR/GLP-1R double knock-out mouse. Alternatively, simultaneous treatment of CD26-/- mice with GIPR and GLP-1R antagonists, prior to glucose tolerance tests, could provide insight as to the relative importance of GIP activity for GLP-1-independent DPP-IV-mediated glucoregulation.

The use of DPP-IV inhibitors has been proposed as a treatment for type 2 diabetes. However, given that the incretin hormones are not the only substrates for DPP-IV proteolytic activity, and that, in addition to its role as a regulatory protease, DPP-IV functions as a binding protein and immune modulator, the use of agents that inhibit DPP-IV activity may be associated with adverse side effects in humans. Moreover, thus far, only short-term studies have been used to examine the effects of DPP-IV inhibition. Although the apparent healthiness of DPP-IV/CD26-/- mice and rats indicates that long-term disruption of DPP-IV activity is not associated with severe adverse effects, these animals may have undergone compensatory or adaptive changes in response to the loss of DPP-IV activity during development. For example, DPP-IV-deficient Fisher rats have been shown to have an unexpectedly normal glucose excursion and insulin response to oral glucose, which could be

attributed to the observed compensatory reductions in GIP secretion and pancreatic desensitization to the effects of GIP in these animals (423). Thus, additional studies are warranted to assess the effects of long-term inhibition of DPP-IV activity *in vivo*. For more protracted studies, the DPP-IV inhibitor pro-pro-diphenyl-phosphonate, which has a half-life of approx 8 days in rabbits (443), would likely be a useful reagent for evaluating the long-term effects (beneficial or adverse) of DPP-IV inhibition *in vivo*.

As an alternative strategy to the use of DPP-IV inhibitors, DPP-IV-resistant GLP-1 analogues are being developed as anti-diabetic therapeutics. To this end, exendin-4, a very potent and naturally occurring GLP-1 receptor agonist is currently being evaluated in clinical trials as a potential therapeutic agent for the treatment of type 2 diabetes. The long-term consequences of expression of a non-mammalian peptide was evaluated using our exendin-4-expressing transgenic mouse (MT-Ex). Induction of proexendin transgene expression in MT-Ex mice resulted in a significant reduction in the glucose excursion in response to both oral and intraperitoneal glucose challenges, thus mimicking the effects of GLP-1 treatment. Similar to results obtained with GLP-1 treatment in rodents, transgene induction resulted in a significant reduction in short-term, but not long-term food intake in MT-Ex mice. However, recent studies examining the effects of daily peripheral injections of exendin-4 in rats have found that, although single daily doses of exendin-4 had no significant effect on food intake after the first few days of treatment, doubling the dose of exendin-4 by administering the peptide twice a day resulted in sustained reductions in food intake and body weight. As MT-Ex transgene expression was induced only for a period of 72 h prior to our food intake analysis, it is possible that this amount of time was not sufficiently long to elevate exendin-4 to levels that would affect feeding behavior.

In a recent study by Greig *et al.*, once daily injections of exendin-4 to diabetic mice for a period of 13 weeks was able to achieve long-term improvements in blood glucose and plasma insulin levels, however there were no long-lasting effects on food intake or body weight (72). Similarly, we found no significant differences in body weight in MT-Ex transgenic mice, relative to control littermates, even up to 20 wks of age. These findings are in contrast to the weight reduction observed in rats treated twice daily with exendin-4. Since the MT-Ex mice are not continuously maintained on drinking water supplemented with zinc, it is possible that continuous up-regulation of MT-Ex transgene expression is required to produce the levels of exendin-4 that are necessary to promote weight reduction.

Although recent studies have shown that exendin-4 can induce β-cell neogenesis and proliferation in rats and mice (56, 57), we observed no differences in islet morphology or β-cell mass in MT-Ex mice, despite more prolonged (5-7 days) induction of transgene expression. Although the levels of circulating exendin-4 detected in MT-Ex mice were similar to levels of exogenous exendin-4 that were shown to reduce blood glucose levels in diabetic *db/db* mice (434), they are still lower than the amounts that were used in the above mentioned studies in mice and rats. Thus, it is possible that the circulating levels of exendin-4 in MT-Ex mice were not sufficient to promote β-cell neogenesis and proliferation. Alternatively, the lack of effect of long-term exposure to exendin-4 on islet morphology and β-cell mass in MT-Ex mice could be due to compensatory changes in pancreatic islets and ducts as a result of continuous exposure to exendin-4 during development.

We also speculated that hyperglycemia may be required in order for exendin-4 to stimulate significant changes in β-cell mass or islet proliferation. Nonetheless, we did not observe any changes in islet histology following 10 days of exogenous exendin-4 treatment

in diabetic C57BL/6 mice. For these studies we used a single high dose of streptozotocin which effectively eliminated virtually all of the β-cells in these mice. Thus, the diabetic mouse model used in our studies differs from the partial pancreatectomy rat model of type 2 diabetes, in which 5-10% of the pancreas remains intact and exendin-4 treatment stimulates pancreas regeneration and β-cell neogenesis and proliferation (56). An alternative approach would be to examine the effects of exogenous exendin-4 treatment on mice treated with a lower dose of streptozotocin, which should induce a milder form of diabetes and prevent complete loss of β-cells.

Future studies using the MT-Ex transgenic mouse model would include examination of the effects of *in vivo* exendin-4 expression, at both basal and induced levels, on other GLP-1-dependent parameters. Exogenous treatment with either GLP-1 or exendin-4 has been shown to increase blood pressure and heart rate in rats, with the effects of exendin-4 being more prolonged (218). If exendin-4 is going to be used to clinically treat diabetes, it would be crucial to establish whether prolonged exposure to this peptide is associated with elevations in heart rate and blood pressure. Also, GLP-1 signaling appears to be important for the neuroendocrine response to stress. Thus, it would be of interest to evaluate the effects of long-term exendin-4 activity on the hypothalamic-pituitary-adrenal axis. In addition, GLP-1 has been shown to stimulate insulin gene transcription and biosynthesis *in vitro*, and exendin-4 treatment was found to enhance pancreatic expression of the transcription factor IDX-1 (56). IDX-1 is required for early pancreatic development and also interacts with the insulin gene promoter to augment glucose-stimulated insulin gene transcription (444). Hence, an observed upregulation of insulin and/or IDX-1 mRNA levels in our MT-Ex mice

would implicate an important role for exendin-4 in the regulation of insulin gene transcription *in vivo*.

In conclusion, incretin hormones are potential therapeutic agents for the treatment of diabetes. Thus, it is essential to understand their precise physiological functions. It is also important to identify the factors that influence their degradation, so that more effective strategies can be developed to prolong their therapeutic activities. Additionally, because of potential toxic or adverse reactions, the effects of prolonged incretin exposure/treatment must be evaluated. In our studies we have shown that the incretin hormones GIP and GLP-1 have differential roles in glucoregulation. Our results also raise the possibility that peptides other than GIP and GLP-1 may be important mediators of glucose homeostasis. Finally, using a transgenic mouse model, we have shown that sustained GLP-1 receptor signaling, mediated by exendin-4 expression, is not associated with any apparent adverse effects.

Appendix 1

Attempts to Identify a Mammalian Exendin-4 Homologue

Attempts to identify and clone a mammalian exendin-4 homologue included several approaches:

1) Northern blot analysis using the entire lizard proexendin-4 cDNA sequence as a probe to screen 5 μg of poly $(A)^+$ RNA from a number of different mouse tissues (liver, pancreas, lung, hypothalamus, salivary gland, adrenal gland) under low stringency conditions failed to detect any cross-hybridizing transcripts.

2) Several cDNA libraries (human and mouse heart, human skeletal muscle, human pancreas, mouse spleen, mouse testis) were screened using the entire lizard proexendin-4 cDNA sequence as a probe under low stringency conditions. These tissues were suspected to express an exendin-4-like transcript based on immunohistochemical analysis of mouse embryos using an exendin-4 antiserum. Although several hybridizing plaques were identified, subsequent purification and sequencing of these clones did not identify a mammalian exendin-4 homologue.

3) The above mouse and human cDNA libraries were also screened by PCR and Southern blot analyses using degenerate exendin-4 oligonucleotides as primers. Using this approach, no mammalian exendin-4-like sequences could be identified.

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