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CHARACTERIZATION OF MICE WITH A NULL MUTATION IN THE GLUCAGON-LIKE PEPTIDE-1 RECEPTOR GENE

by

Sonya M. Cook

A thesis submitted in conformity with the requirements
for the Degree of Master of Science,
Institute of Medical Science,
University of Toronto

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ABSTRACT

Characterization of mice with a null mutation in the glucagon-like peptide-1 receptor gene. Sonya M. Cook, Institute of Medical Science, University of Toronto. Master's of Science, 1998.

Glucagon-like peptide-1 (GLP-1) regulates blood glucose through stimulation of glucose-dependent insulin secretion, inhibition of gastric emptying, suppression of glucagon secretion, and the induction of satiety, as well as through potential actions on peripheral insulin sensitivity. We demonstrate here that the absence of GLP-1 signaling impairs the capacity of GLP-1R-/- mice to respond to agents associated with the induction of insulin resistance *in vivo*. Furthermore, despite reports that GLP-1 may influence peripheral insulin sensitivity, GLP-1R-/- mice were unresponsive to treatment with an insulin-sensitizing thiazolidinedione agent, suggesting GLP-1R-/- mice possess normal peripheral insulin sensitivity. Additionally, our studies show that GLP-1R+/- mice exhibit defective oral glucose tolerance in association with reduced glucose-stimulated insulin levels, supporting an essential role for GLP-1 in glucose homeostasis *in vivo*. Inhibition of DPP-IV using the chemical agent Pro-boroPro improved oral glucose tolerance in both wildtype and GLP-1R-/- mice, further implicating a role for this enzyme in incretin degradation *in vivo*. Additional studies undertaken here suggest that GLP-1R-/- mice exhibit an altered sensitivity to leptin, and furthermore, that disruption of GLP-1 signaling produces behavioural changes and perturbations in fluid homeostasis *in vivo*. Taken together, these studies further implicate a key role for GLP-1 not only in glucose homeostasis but also in the central regulation of behaviour and neuroendocrine function *in vivo*.

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ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Dr. Dan Drucker, for guiding me through two rewarding years of basic research. I also owe many thanks to Dr. Louise Scrocchi, who not only generated the GLP-1R-/- mouse, making these studies possible, but also took the time to teach me many of the laboratory techniques used throughout this thesis.

Further, I thank the additional members of my supervisory committee, Dr. M. Wheeler and Dr. P Brubaker, for their guidance, constructive criticism, and probing questions.

I would also like to thank my fellow graduate students, Laurie Baggio and Julie Lovshin, for their support, as well as my family and friends. Many thanks are also owed to Bernardo Yusta, for his inspiring enthusiasm for science, Mary Hill, for her support and patience, and Robin Boushey, for his advice and guidance. Thanks also to Andrew Arifuzzman who assisted with the behavioural studies in Chapter 7.

Finally, I would like to thank the University of Toronto, Banting and Best Diabetes Centre, and Novo Nordisk for financial support.

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Chapter 1 Introduction

Although insulin secretion is primarily regulated by blood glucose levels, gastrointestinal peptides are also known to influence insulin release. The term "incretin" was introduced in 1929 to describe humoural activity of the gut postulated to augment pancreatic insulin secretion (1). With the development of the radioimmunoassay in the 1960s, it became possible to measure plasma insulin levels (2). This facilitated the observation that insulin levels following oral glucose intake are greater than those following intravenous glucose administration, despite the achievement of nearly identical blood glucose levels by each route (3). This discrepancy in plasma insulin levels following oral versus intravenous glucose administration was attributed to the *incretin effect* (3).

Many gastrointestinal regulatory peptides have been investigated for incretin activity. Glucose-dependent insulinotropic peptide (GIP) was the first gut hormone shown to possess insulin-releasing properties at physiologically relevant concentrations (3). In accordance with its role as an incretin, GIP is released from intestinal K cells in response to the oral intake of glucose or fatty acids, and promotes pancreatic insulin secretion in a glucose-dependent manner (4). Unfortunately, non-insulin-dependent diabetes mellitus (NIDDM) patients show a diminished response to GIP, rendering it ineffective as a potential treatment for NIDDM (5). However, immunoneutralization of gut extracts to eliminate endogenous GIP activity, as well as the use of a competitive peptide antagonist for GIP receptor blockade, revealed that GIP accounts for only a portion of the incretin effect *in vivo* (6,7).

The potent glucose-dependent insulinotropic action of glucagon-like peptide-1 (GLP-1) was first demonstrated in 1987 (8,83). GIP and GLP-1 act

additively on the endocrine pancreas (9) but, contrary to GIP, the potent insulin-releasing actions of GLP-1 are preserved in NIDDM patients (5).

1.1. Intestinal Proglucagon Gene Expression

The proglucagon gene is expressed in the A cells of the pancreatic islets, the L cells of the intestinal mucosa, and in some regions of the brain (10,11). In mammals, an identical proglucagon mRNA is produced in each tissue and translated into a common 160 amino acid precursor (10,11). The rat proglucagon gene promoter contains a functional cAMP response element (CRE) (12), and experiments utilizing fetal rat intestinal cell (FRIC) cultures are consistent with a role for the PKA pathway in stimulation of proglucagon gene transcription in enteroendocrine cells (13).

Interestingly, transgenic mice containing 1.3 kb of the rat proglucagon 5' flanking sequences fused to the coding region of SV40 large T antigen exhibit transgene expression in pancreatic A cells and the brain, but not in the intestine (15). This suggests that the DNA sequences specifying islet and brain proglucagon expression are insufficient for intestinal proglucagon expression in vivo (15). Notably, transgenic mice containing 2.2 kb of rat proglucagon 5' flanking sequences fused to the coding region of SV40 large T antigen display transgene expression in the pancreas, intestine, and brain. These results support the existence of an enteroendocrine cell-specific enhancer in the region between -1.3 kb and -2.2 kb (16). Additional investigations are required in order to identify transcription factors which are specifically involved in regulation of proglucagon gene expression in enteroendocrine L cells.

1.2 Post-translational Processing of Intestinal Proglucagon

The 160 amino acid proglucagon precursor polypeptide undergoes differential post-translational processing in pancreatic and intestinal tissue (11). Glicentin, oxyntomodulin, GLP-1 and GLP-2 are the major proglucagon-derived peptides (PGDPs) produced in the intestinal L cell (4). Each peptide is flanked by a pair of basic amino acids, indicative of prohormone cleavage sites (17). Prohormone convertase 1/3 (PC1/3) is necessary (and sufficient) for processing proglucagon into its intestinal products (17). Notably, PC1/3 also cleaves GLP-1(1-37) to form the biologically active GLP-1(7-37) (17). Although the majority of GLP-1 is amidated in the intestinal L cell to form GLP-1(7-36)NH2 (18), both GLP-1(7-36)NH2 and GLP-1(7-37) are equipotent and exhibit similar half-lives (19). A role for PC2 in processing proglucagon in the pancreatic islets to produce glucagon as a bioactive product has also been described (20,21).

1.3 Secretion of GLP-1 from the Intestinal L Cell

Enteroendocrine L cells are open-type intestinal cells located primarily in the distal ileum and colon (22,23). The L cell is stimulated by activation of PKA-, PKC-, and calcium-dependent pathways (24,25). Since the L cell is an open-type endocrine cell, it is modulated by luminal contents in addition to neurotransmitters and circulating factors (22,26). Luminal GLP-1 secretagogues include glucose (35), oral galactose (28), and long-chain monounsaturated fatty acids (29). Notably, nutrient intake stimulates the synthesis as well as the secretion of the PGDPs (30), and all PGDPs are thought to be released in a one-to-one ratio (18,31).

Despite the location of the L cells in the distal ileum and colon, GLP-1 plasma levels increase rapidly following a meal in humans, returning to basal levels (0.4-7 pM) within 90 minutes (28,32,33). Although the autonomic

nervous system and additional humoural mediators have been implicated in the stimulation of PGDP secretion in rats (34,35), this has not yet been verified in humans (36).

1.4 The GLP-1 Receptor

The GLP-1 receptor (GLP-1R) was initially cloned from a rat pancreatic islet cDNA library (37), and is expressed predominantly in the pancreatic islets (B and D cells) (38,39) and the lung (37). Low-level expression of the GLP-1R has been reported in the stomach, intestine, kidney, and brain (40,41), as well as in adipose tissue, liver, and skeletal muscle, although these latter findings are controversial (40-45). Evidence to date suggests that GLP-1 transduces its effects through a single receptor which is a member of the seven transmembrane domain G-protein-coupled secretin/glucagon receptor family (37).

Both the N-terminal and the C-terminal ends of GLP-1(7-37/36NH2) have been shown to be essential for GLP-1R activation (46). The third intracellular loop of the GLP-1R is believed to be necessary for coupling of the receptor to adenylyl cyclase and subsequent activation of the PKA pathway through increased cAMP levels (47). Additionally, GLP-1 binding to its receptor is associated with membrane depolarization and increased intracellular calcium (48). Evidence also suggests that GLP-1 couples to phospholipase C (PLC), resulting in lipid hydrolysis and PKC activation (40). Furthermore, studies have supported a role for PKC in GLP-1R phosphorylation, which may result in homologous desensitization of the receptor (49,50).

Exendin-4(1-39), a peptide isolated from the venom of *Heloderma suspectum,* binds to the GLP-1R with high affinity and exerts agonistic activity (51), whereas the amino-terminally truncated form, exendin (9-39), has been

shown to function as a high affinity antagonist at the GLP-1R (52,51). Injection of rats with exendin(9-39) results in deteriorated postprandial glucose tolerance with associated decreases in plasma insulin levels (54). However, the use of exendin(9-39) as a tool to define the insulinotropic and extrapancreatic effects of GLP-1 is confounded by evidence that exendin (9-39) also functions as an antagonist at the GIP receptor (55).

1.5 Biological Activities of GLP-1

GLP-1 possesses potent glucose-dependent insulinotropic activity (8,36,56,57). In all mammalian species to date, GLP-1 only exerts stimulatory effects on insulin secretion at elevated glucose concentrations, providing a safeguard against GLP-1-induced hypoglycemia (57). GLP-1 also reportedly increases the number of ß cells in islet cultures which are able to respond to glucose stimulation (58). Importantly, GLP-1 stimulates proinsulin gene transcription and proinsulin biosynthesis, thereby contributing to replenishment of ß cell insulin stores and protection against ß cell exhaustion (59,60).

GLP-1 possesses several other biological activities which complement its insulinotropic actions by contributing to glucose lowering, including glucose-dependent suppression of glucagon secretion (61). GLP-1 is known to stimulate somatostatin secretion via GLP-1Rs expressed on D cells (62), and it has been suggested that the inhibitory effect of GLP-1 on glucagon secretion is secondary to this stimulation of somatostatin release (63). However, GLP-1Rs are also expressed on A cells (62), supporting a direct role for GLP-1 in the suppression of glucagon secretion. Interaction of GLP-1 with its receptor is known to increase cAMP levels and result in PKA activation, changes which are associated with increased glucagon secretion (14). Consistent with this, GLP-1 was shown to increase glucagon secretion from isolated rat alpha cells (64).

However, GLP-1 directly inhibited glucagon secretion in the InR1-G9 alpha cell line (65). Further studies are needed to clarify the mechanisms underlying the inhibitory effect of GLP-1 on glucagon secretion.

A central effect for GLP-1 was demonstrated when intracerebroventricular injection of GLP-1 was shown to potently inhibit feeding in fasted rats (72). Cell bodies expressing preproglucagon mRNA have been detected in the brain stem and hypothalamus (10,73), and GLP-1R mRNA and immunoreactivity, as well as GLP-1 binding, has been found in many brain regions, including the telencephalon, diencephalon, brain stem, and spinal cord (74-76). While additional studies are needed to clarify the role of GLP-1 in neuronal physiology, the widespread distribution of GLP-1 receptor expression in the brain suggests that GLP-1 functions not only as a satiety factor, but may possess additional actions in distinct areas of the CNS.

Additional actions of GLP-1 include inhibition of gastric acid secretion and gastric emptying, which may contribute to glucose-lowering by decreasing the rate of nutrient absorption (66). Although GLP-1 has been reported to stimulate glucose uptake in adipocytes and increase glycogen synthesis in liver and muscle (67,68,69), these results are largely unconfirmed (70). Nonetheless, several studies have reported that GLP-1 lowers blood glucose levels in both IDDM and NIDDM patients (26,71), suggesting that GLP-1 may be therapeutically useful in the treatment of diabetes (26).

1.6 Metabolism of GLP-1

The half-life of biologically active GLP-1 is estimated to be less than 2 min *in vivo* (77). The N-terminal histidine of GLP-1, which is essential for its biological activity, is susceptible to cleavage at the penultimate alanine residue

by vascular and plasma dipeptidyl peptidase-IV (DPP-IV) (77,78), resulting in the production of biologically inactive GLP-1(9-37/36NH2) (79).

1.7 Generation of Mice with a Null Mutation in the Glucagon-like Peptide-1 Receptor Gene (GLP-1R-/-)

As a complementary approach for defining the physiological role of GLP-1 in glucose homeostasis, the mouse GLP-1 receptor gene was isolated and homologous recombination in mouse embryonic stem (ES) cells was utilized to generate mice with a null mutation in both GLP-1 receptor alleles (80). The GLP-1R-/- mice are viable and develop normally. Surprisingly, GLP-1R-/- mice demonstrate no evidence of abnormal body weight or feeding behaviour despite the proposed role of GLP-1 in satiety (80). However, they exhibit an abnormal glycemic excursion following an oral glucose challenge in association with diminished levels of circulation insulin (80). Unexpectedly, GLP-1R-/- mice also display glucose intolerance following intraperitoneal (IP) glucose administration, which avoids the stimulatory effects of enteral glucose on incretin secretion (80). The observation that glucose handling is abnormal in GLP-1R-/- mice irrespective of the site of glucose entry implicates a key role for GLP-1 in the control of blood glucose *in vivo*.

1.8 Characterization of Mice with a Null Mutation in the Glucagon-like Peptide-1 Receptor Gene

As previously mentioned, in addition to its insulinotropic properties, GLP-1 has been implicated in the glucose-dependent suppression of glucagon secretion and potentiation of glucose uptake in peripheral tissues. Additionally, GLP-1 has been shown to stimulate insulin gene transcription, and may be involved in the maintenance of ß cells in a glucose-responsive state.

Phenotypical characterization of GLP-1R-/- mice provides an opportunity to define the physiological consequences of disrupted GLP-1 signaling on these aspects of glucose homeostasis. In view of the finding that GLP-1 functions as a satiety factor within the hypothalamus, and additionally, that both GLP-1 and its receptor are expressed in the brain, characterization of the GLP-1R-/- mouse may assist in further defining the role of GLP-1 in the central nervous system.

The thesis to follow investigates several aspects of GLP-1 activity within the GLP-1R-/- mouse model. Initially, GLP-1R-/- and wildtype mice were treated with dexamethasone and growth hormone, two agents known to induce insulin resistance in vivo, to determine the role of GLP-1 signaling in the ß cell response to chronic hyperglycemia and/or insulin resistance. In view of the proposed actions of GLP-1 in stimulation of peripheral insulin-dependent glucose metabolism, GLP-1R-/- mice were subsequently administered a thiazolidinedione insulin-sensitizing agent and investigated for improvements in glucose tolerance.

Plasma and vascular dipeptidyl peptidase-IV (DPP-IV) rapidly hydrolyzes both GLP-1 and GIP in vivo to produce biologically inactive metabolites. We administered an inhibitor of DPP-IV to GLP-1R-/- and wildtype mice in order to assess the effects of postulated increases in the biological half-lives of GLP-1 and GIP on glucose tolerance and glucose-stimulated insulin secretion. Furthermore, to determine whether a partial reduction in GLP-1 receptor expression is also associated with abnormal glucose tolerance and reduced glucose-stimulated insulin secretion, we investigated these parameters in heterozygous GLP-1R+/- mice.

The absence of GLP-1-mediated stimulation of insulin secretion and insulin gene expression may render GLP-1R-/- mice more sensitive to the inhibitory actions of leptin. In order to more fully define the role of leptin at the

level of the ß cell and in glucose homeostasis, we investigated the effects of short-term leptin administration on insulin gene expression, plasma insulin levels, and glucose homeostasis in wildtype and GLP-1R-/- mice.

Finally, in view of recent reports implicating a role for GLP-1 in activation of hypothalamic neurons involved in the regulation of fluid balance and behavioural responses, we investigated the ability of GLP-1R-/- mice to adapt to water restriction and, additionally, evaluated the response of GLP-1R-/- and wildtype mice to stress.

Chapter 2 Impaired ß Cell Response to Dexamethasone and Growth Hormone in GLP-1R-/- Mice

2.1 Introduction

The term "incretin" was introduced in 1929 to describe the activity of a gut-derived factor postulated to augment the endocrine secretion of the pancreas. Subsequent studies indeed demonstrated that plasma insulin levels following oral intake of glucose were greater than those observed following intravenous glucose infusion, consistent with the incretin effect (2,81). Many gastrointestinal regulatory peptides have been investigated for putative insulinotropic and hence incretin activity. Glucose-dependent insulinotropic peptide (GIP), present in the duodenum and upper jejunum, was the first gut peptide shown to stimulate insulin secretion at physiologically relevant concentrations (4). However, elimination of endogenous GIP activity through immunoneutralization of gut extracts (6) and the use of a competitive GIP antagonist for GIP receptor blockade has shown that GIP accounts for only a portion of the incretin effect *in vivo* (7).

A large body of evidence now supports the importance of glucagon-like peptide-1 (GLP-1) as a second key physiologically relevant incretin (4). GLP-1 is secreted from intestinal endocrine cells in response to nutrient ingestion, and stimulates insulin secretion in a glucose-dependent manner (8,36,56). Infusion of a lizard exendin (9-39) peptide that functions as a GLP-1 receptor antagonist has shown that GLP-1 receptor blockade is associated with increased blood glucose and diminished levels of glucose-stimulated insulin secretion, consistent with the physiological relevance of GLP-1 in vivo (54,82,83).

To examine the importance of GLP-1 receptor signaling for long term control of insulin secretion, mice with a targeted disruption in the GLP-1

receptor gene were generated. GLP-1R-/- mice are viable and exhibit mild fasting hyperglycemia in association with reduced insulin secretion following oral glucose challenge (80). Despite the putative importance of GLP-1 for inhibition of glucagon secretion (61,84), increased levels of circulating glucagon were not detected in GLP-1R-/- mice (80).

In addition to its acute effects on stimulation of glucose-dependent insulin secretion, GLP-1 stimulates proinsulin gene expression via an increase in gene transcription (59,60). These observations suggest that GLP-1 may also be important for maintaining insulin biosynthesis, in part via its positive effect on maintaining normal levels of proinsulin mRNA. Consistent with this hypothesis, GLP-1 administration to aging 22 month-old Wistar rats improved glucose tolerance and increased the levels of circulating insulin and both islet insulin content and pancreatic insulin RNA (85).

Although GLP-1R-/- mice exhibit both fasting hyperglycemia and glucose intolerance following either oral or intraperitoneal glucose challenge, the diabetes in GLP-1R-/- mice is relatively mild, suggesting that the level of plasma glucose is maintained reasonably well in the absence of GLP-1 signaling (80). Nevertheless, these studies were carried out in young, non-stressed mice, and it is possible that the lack of GLP-1 receptor function might be more critical for the ß cell response to chronic hyperglycemia and/or insulin resistance. To test the hypothesis that GLP-1 signaling is important for the ß cell response to insulin resistance, we have now studied glucose tolerance and insulin biosynthesis in wildtype and GLP-1R-/- mice treated with dexamethasone and growth hormone, agents known to be associated with induction of insulin resistance in vivo (86-89).

2.2 Materials and Methods

2.2.1 Mice

GLP-1R-/- and age- and sex-matched wildtype CD1 control mice (Charles River, Toronto, Canada) were housed under a light-dark cycle of 12 h in the Toronto Hospital Animal Facility, and received Lab Rodent Diet (PMI Feeds, Inc., St. Louis, MO) and tap water ad libitum. Male mice, 8-10 weeks old at the start of each experiment, were used for this study which was carried out under guidelines approved by the Toronto Hospital Animal Welfare Committee.

2.2.2 Dexamethasone and Growth Hormone Treatment

One group of 8-10 week-old male GLP-1R-/- and CD1 +/+ mice (n=5 per treatment group) received a single daily intraperitoneal injection of 100 ul saline containing 20 ug dexamethasone sodium phosphate (Pharmascience Inc., Montreal, Canada) and 24 ug human growth hormone (Protropin, Genentech Canada Inc., Burlington, ON) for one week. Age- and sex-matched wildtype and GLP-1R control animals were given injections of saline alone.

A second group of 8-10 week-old male GLP-1R-/- and wildtype CD1 +/+ mice (n=5 per treatment group) were given a single daily intraperitoneal injection of 100 ul ml saline containing 20 ug dexamethasone, or 24 ug human growth hormone, or combined dexamethasone and human growth hormone, for four weeks. Age- and sex-matched wildtype and GLP-1R-/- controls received injections of saline alone for four weeks.

2.2.3 Glucose Tolerance and Insulin Determinations

Following a 12-16 h fast, mice were challenged with 1.5 mg D-glucose per gram body weight by gastric gavage. Blood was withdrawn from the tail vein at 0, 10, 20, 30, 60, 90, and 120 min following glucose administration.

Blood glucose levels were measured by the glucose oxidase method using a One Touch Basic Glucometer (Lifescan Ltd., Canada). Blood samples for insulin measurements were withdrawn from the tail vein during the period between 20-30 minutes following oral glucose load into chilled tubes containing a 10% volume of 5000 KIU/ml Trasylol (Miles Canada, Etobicoke, Canada): 1.2 mg/ml EDTA: 0.1mM Diprotin A (Sigma Chemical Co., St. Louis, MO). The plasma was separated by centrifugation at 4°C and stored at -20°C until assayed for insulin content. The insulin content of all plasma samples, except those obtained from mice receiving four weeks of either growth hormone or dexamethasone alone, was quantified using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard. The insulin content of the plasma samples obtained from mice receiving four weeks of either dexamethasone or growth hormone injections was determined using a rat insulin ELISA kit (Crystal Chem, Inc., Chicago, IL) with mouse insulin as a standard.

2.2.4 Tissue Collection and RNA Isolation

Following the oral glucose tolerance test (OGTT), the mice were euthanized with CO_2 and the pancreas was immediately removed. The head of the pancreas was snap frozen and stored at -80°C for subsequent extraction and insulin content quantification. Total cellular RNA was isolated from the tail of the pancreas by the acid-guanidium thiocyanate method (90).

2.2.5 Northern Blot Analysis

RNA samples were denatured and electrophoresed on a 1% (wt/vol) agarose-formaldehyde gel. The gel was stained with ethidium bromide to assess the integrity and migration of the RNA species, and then transferred onto

a nylon membrane (Nytran Plus, Schleicher and Schuell, Inc., Keene, NH) by capillary transfer in 5 X sodium citrate salt solution (SSC, 0.75 M NaCl, 0.75 M Na citrate). The ribonucleic acids were fixed to the membrane by exposure to UV light in a Stratalinker UV Crosslinker (Stratagene, LaJolla, CA). Blots were prehybridized and hybridized in 5% dextran sulfate, 40% deionized formamide, 4 X SSC (0.6 M NaCl, 0.6 M Na citrate), 7 mM Tris (pH 7.4), Denhardt's (200 ug/ml each of polyvinyl pyrrolidine, bovine serum albumin, and Ficoll), 100 ug/ml herring sperm DNA, and 1% sodium dodecyl sulfate. Prehybridization was performed at 42°C overnight, and the blots were hybridized with $1x10^6$ cpm/ml of ^{32}P-labeled probe at 42°C overnight. The membranes were then washed and exposed to imaging film (X-OMAT AR, Kodak, Rochester, NY). To control for loading and transfer efficiency, the blots were rehybridized with a labeled cDNA for 18S rRNA.

2.2.6 Pancreatic Insulin Content

The frozen head of the pancreas was homogenized twice in 5 ml extraction medium (1 N HCl containing 5% (v/v) formic acid, 1% (v/v) trifluoroacetic acid (TFA), and 1% (w/v) NaCl) with a Polytron homogenizer (Kinematica, Switzerland). Peptides and small proteins were adsorbed from the extracts by passage twice through a cartridge of C18 silica (Waters Associates, Milford MA). Absorbed peptides were then eluted with 4 ml of 80% (v/v) isopropanol containing 0.1% (v/v) TFA. The eluant was stored at -70°C prior to being assayed for insulin content in duplicate using an insulin radioimmunoassay kit (Linco) with rat insulin as a standard. Total protein in the eluant was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Pancreatic insulin content is represented as ng insulin/ug total extracted protein.

2.2.7 Statistics

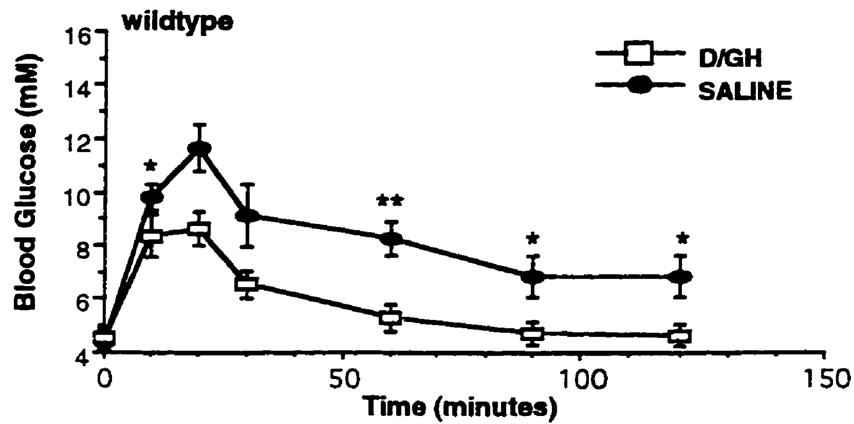
All data values are expressed as the mean ± S.E.M. Statistical significance was assessed by an unpaired t test between experimental groups and controls. All statistics were analyzed using an InStat 1.12 program for MacIntosh computers (GraphPad Software).

2.3 Results

As treatment of rodents with glucocorticoids and growth hormone induces a state of insulin resistance (86-89), we examined the capacity of the GLP-1R-/- mouse to respond to oral glucose challenge following 1 week of dexamethasone and growth hormone (D/GH) treatment. Although glucose levels were elevated in saline-treated GLP-1R-/- mice following OGTT as compared to wildtype mice, as described (80), glycemic excursion after oral glucose tolerance was actually significantly lower in both wildtype and GLP-1R-/- mice treated with D/GH (Fig. 1. A. and B.). Analysis of the levels of glucose-stimulated insulin in plasma samples obtained 20-30 minutes after glucose administration demonstrated that the improved glucose tolerance was associated with increased insulin secretion in both wildtype and GLP-1R-/- D/GH-treated mice (Fig. 1. C.).

As GLP-1 has also been shown to increase the levels of proinsulin RNA (60) via a cAMP-dependent induction of insulin gene transcription (59), we examined the levels of proinsulin mRNA and insulin content in the pancreas of saline- and D/GH-treated mice (Fig. 2). A small increase in the levels of proinsulin mRNA transcripts (Fig. 2. B. and C.) and no significant difference in pancreatic insulin content (compared to levels in saline-injected controls; Fig. 2. A.) was observed in wildtype and GLP-1R-/- mice treated with D/GH. These

A



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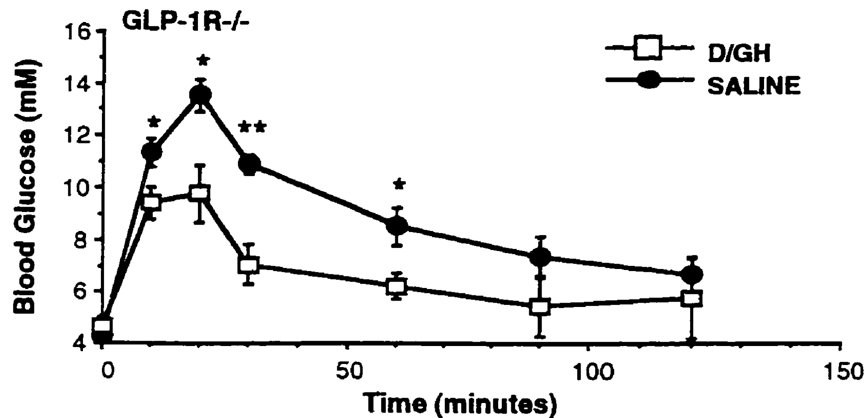

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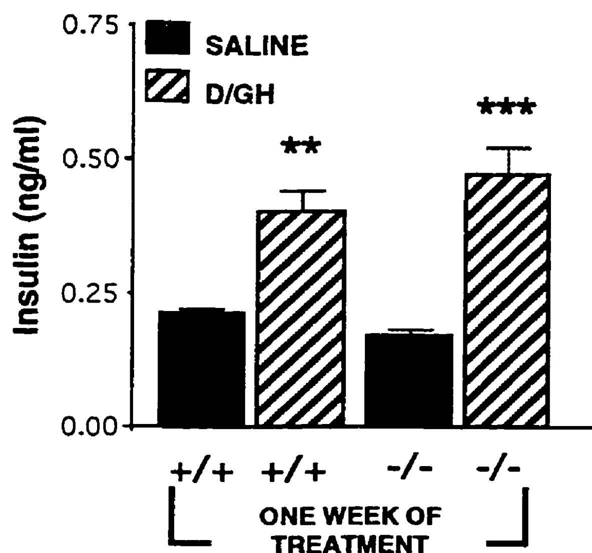


Fig. 1. Oral glucose tolerance in wildtype (**A**) and GLP-1R-/- mice (**B**) following one week of dexamethasone and growth hormone (D/GH) treatment. **C.** Insulin levels during the OGTT shown in 1.A., in D/GH-treated wildtype (+/+) and GLP-1R-/- (-/-) mice. Values are expressed as means ± S.E.M. *P<0.05, **P<0.01, and ***P<0.001, D/GH vs. saline-treated wildtype and GLP-1R-/- mice.

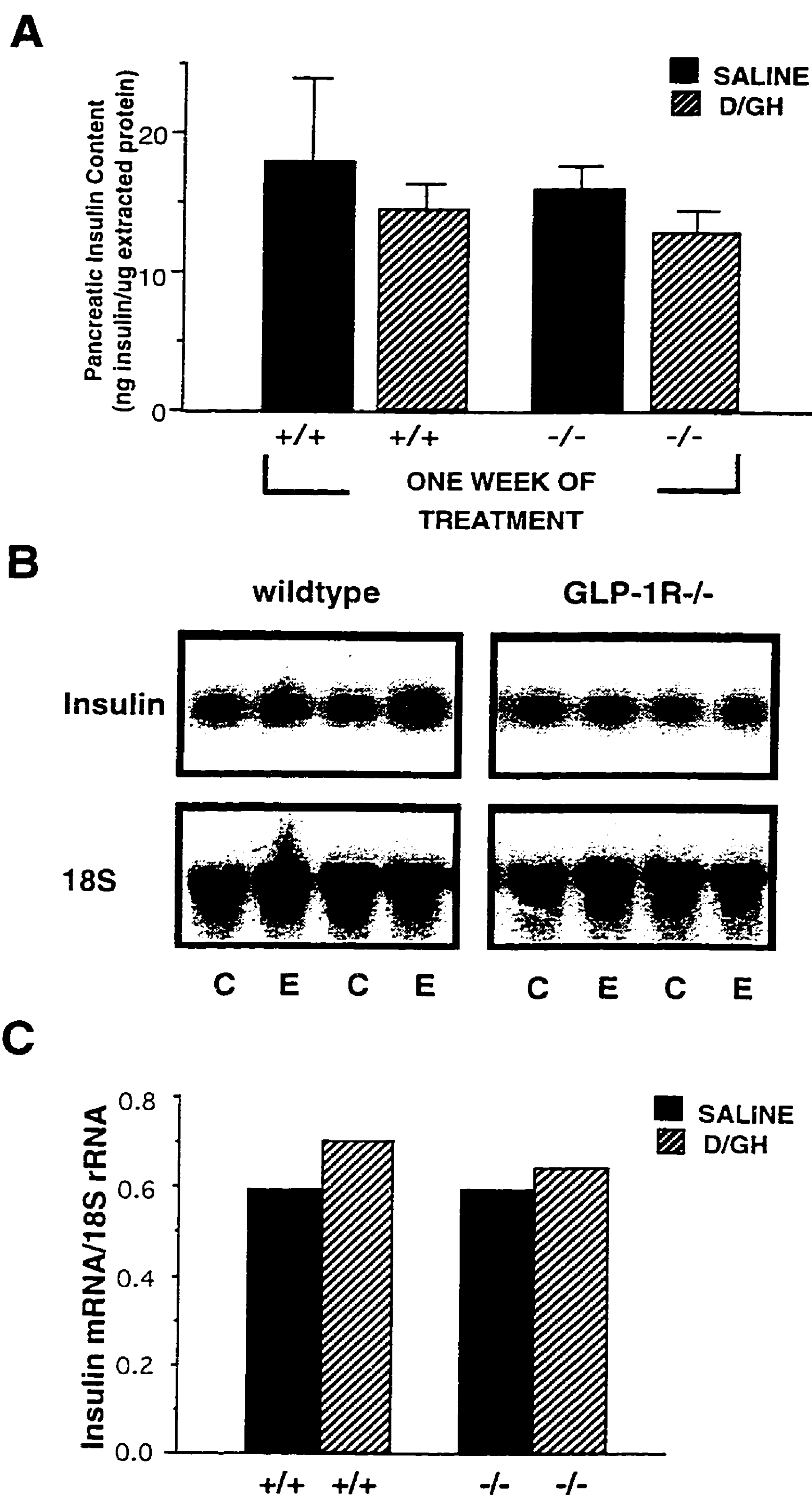


Fig. 2. A. Insulin content, expressed as ng insulin per ug extracted protein, of pancreatic tissue from wildtype (+/+) and GLP-1R-/- (-/-) mice treated with saline or dexamethasone and growth hormone (D/GH) for one week. **B.** Northern blot analysis of total pancreatic RNA from wildtype (+/+) and GLP-1R-/- (-/-) mice treated with saline or D/GH for one week. C=control group treated with saline, and E= experimental group treated with Dex/GH. The ratios of proinsulin mRNAs to 18S rRNAs, expressed as relative densitometric units, are shown in **C** (n=2).

observations demonstrate that disruption of GLP-1 signaling is not associated with an impaired ß cell response, either at the level of insulin biosynthesis or glucose-stimulated insulin secretion, to short-term administration of agents that induce insulin resistance *in vivo.*

To examine whether GLP-1R-/- mice can maintain normal insulin biosynthesis and secretion in the face of more chronic insulin resistance, and to determine the independent effects of growth hormone and dexamethasone administration on glucose homeostasis, additional groups of wildtype and GLP-1R-/- mice were treated with dexamethasone, or growth hormone, or combined dexamethasone and growth hormone for 4 weeks. Although wildtype mice treated with combined D/GH again exhibited lower blood sugars than mice treated with saline injections following challenge with oral glucose (Fig. 3. A.), a significant impairment in glycemic excursion was observed in D/GH-treated GLP-1R-/- mice (Fig. 3. B.). The improved glycemic response to oral glucose in D/GH-treated wildtype mice was associated with a significant 4-fold increase in the levels of glucose-stimulated insulin (Fig. 3. C.). In contrast, a much smaller increment in glucose-stimulated insulin levels was detected in D/GH-treated GLP-1R-/- mice (Fig. 3. C.). To determine whether 4 weeks of D/GH was also associated with reduced capacity for insulin biosynthesis in GLP-1R-/- mice, we examined the levels of pancreatic insulin content and proinsulin mRNA transcripts. No significant differences in pancreatic insulin content were detected in saline- versus D/GH-treated mice (Fig. 4. A.). In contrast, a marked induction of proinsulin mRNA transcripts was detected in wildtype mice treated with D/GH (Fig. 4. B. and C.). Although the levels of proinsulin mRNA transcripts were also increased in GLP-1R-/- mice following 4 weeks of D/GH treatment (Fig. 4. B. and C.), the relative magnitude of the increase was smaller in GLP-1R-/- mice.

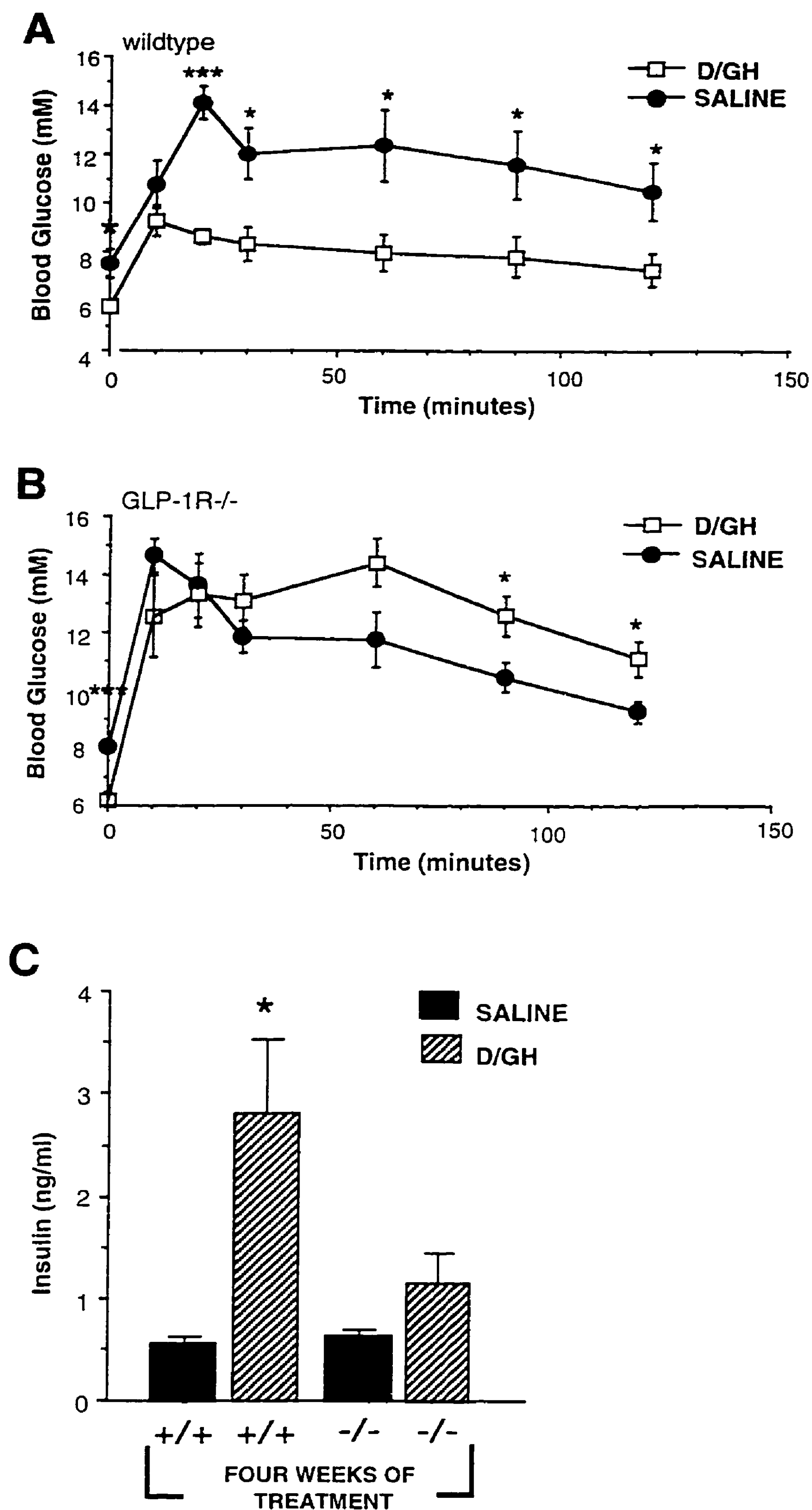


Fig. 3. Oral glucose tolerance in wildtype (**A**) and GLP-1R-/- (**B**) mice following four weeks of dexamethasone and growth hormone (D/GH) treatment. **C.** Insulin levels during the OGTT shown in 3. A., in D/GH-treated wildtype (+/+) and GLP-1R-/- (-/-) mice. Values are expressed as means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.

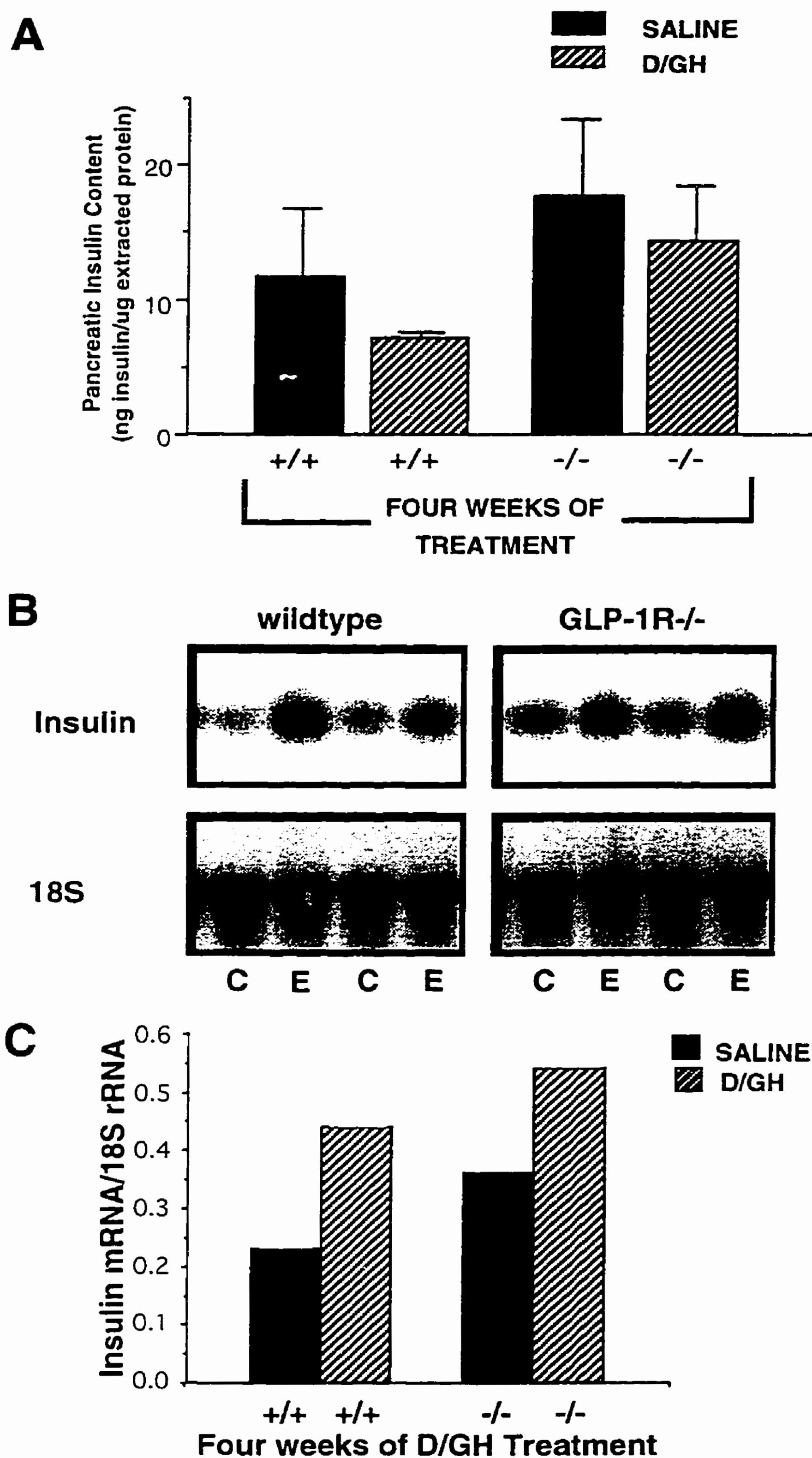
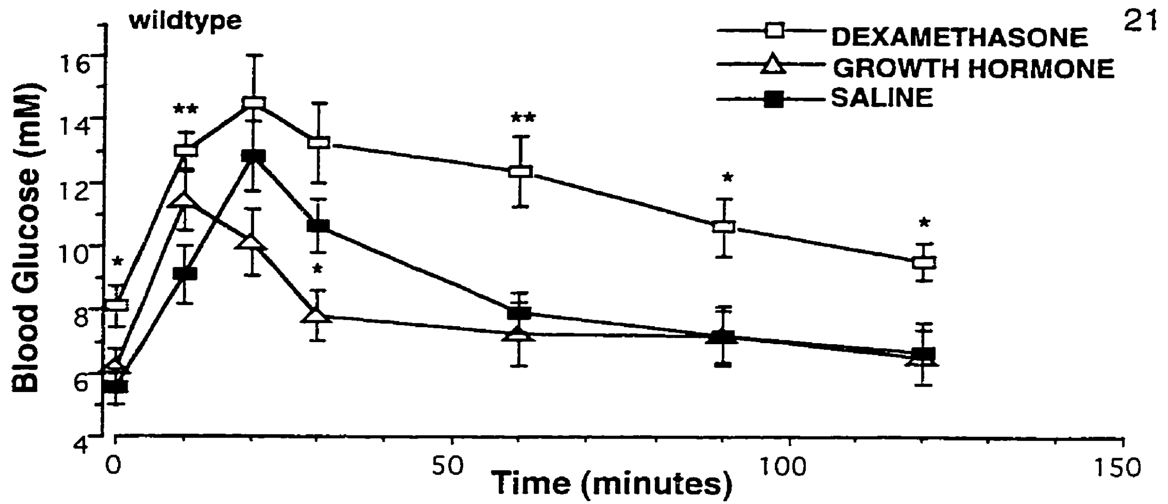
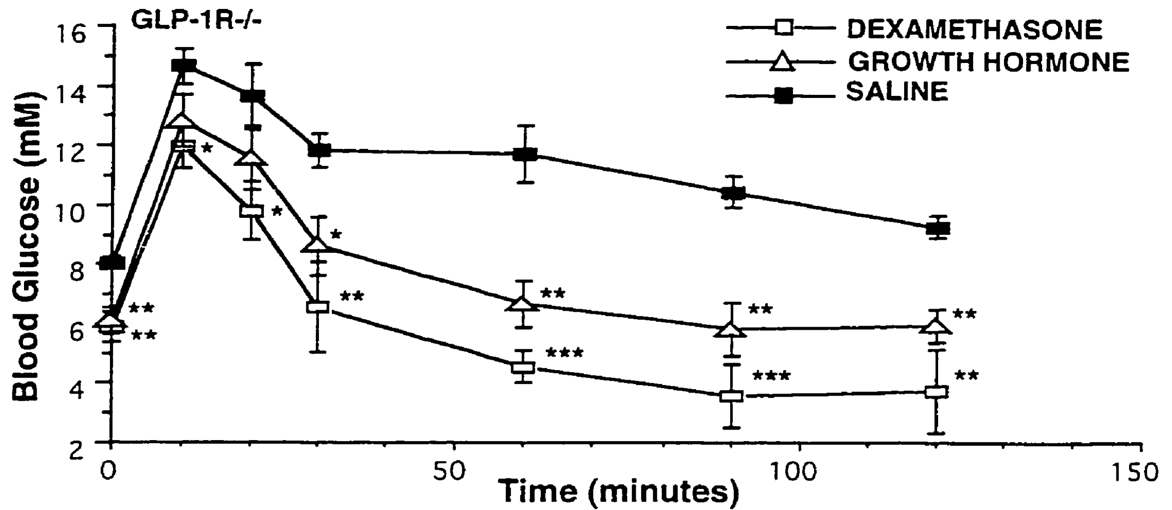


Fig. 4. A. Insulin content, expressed as ng insulin per ug total extracted protein, of pancreatic tissue from wildtype (+/+) and GLP-1R-/- (-/-) mice treated with saline or dexamethasone and growth hormone (D/GH) for four weeks. **B.** Northern blot analysis of total pancreatic RNA from wildtype (+/+) and GLP-1R-/- (-/-) mice treated with saline or dexamethasone and growth hormone (D/GH) for four weeks. C=control group treated with saline, and E= experimental group treated with D/GH. The ratios of proinsulin mRNAs to 18S rRNAs, expressed as relative densitometric units, are shown in **C.**





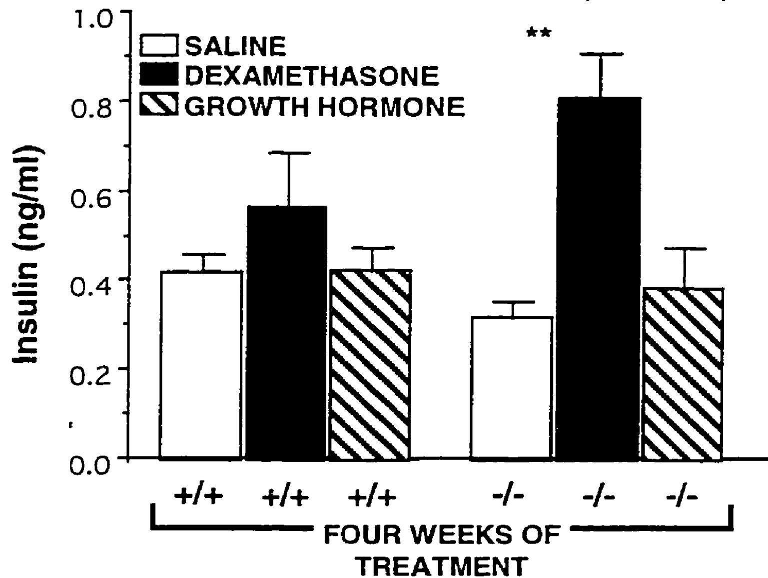
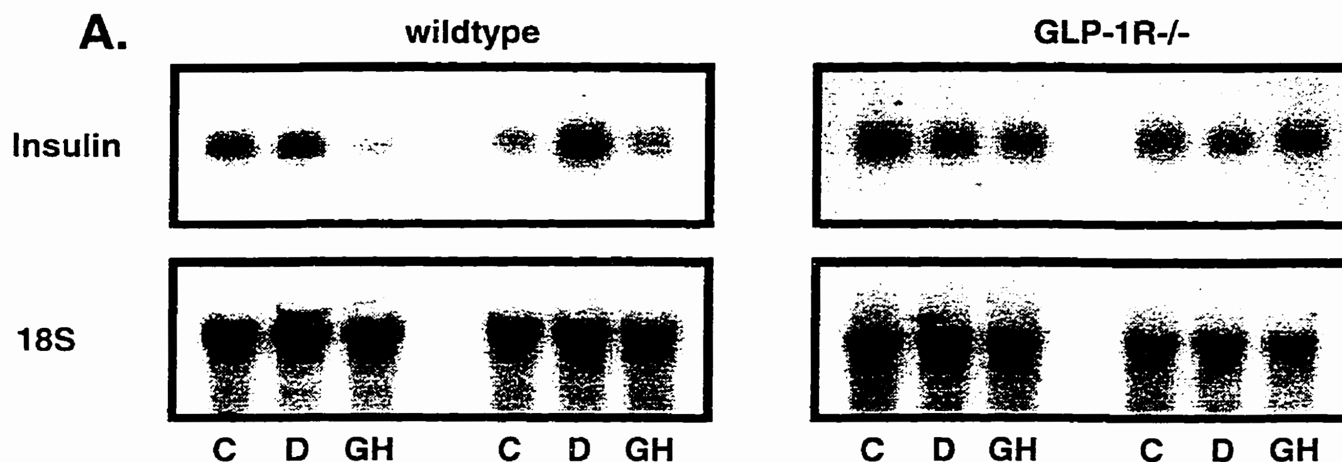


Fig. 5. Oral glucose tolerance in wildtype (**A**) and GLP-1R-/- (**B**) mice following four weeks of dexamethasone, growth hormone, or saline injections. **C.** Insulin levels during the OGTT shown in 5.A., in dexamethasone-, growth hormone-, and saline-treated wildtype (+/+) and GLP-1R-/- (-/-) mice. Values are expressed as means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.



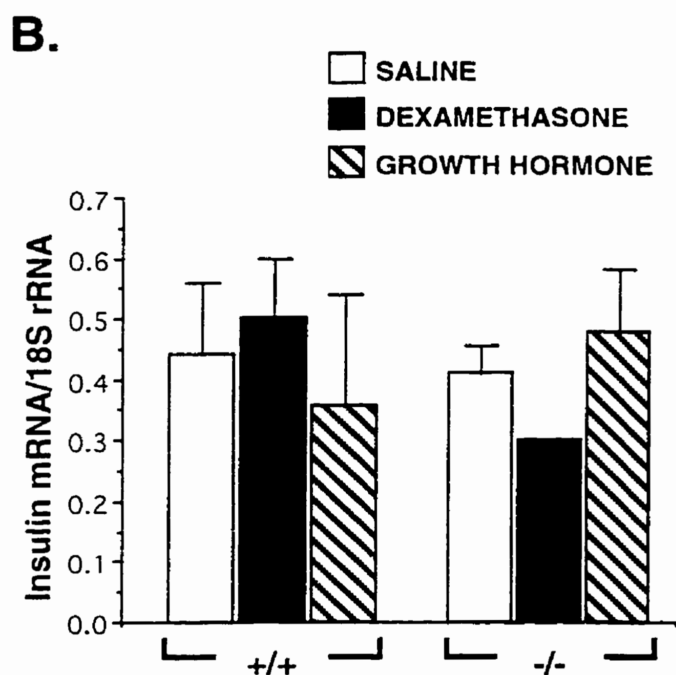


Fig. 6. Northern blot analysis of total pancreatic RNA from wildtype (+/+) and GLP-1R-/- (-/-) mice treated with dexamethasone, growth hormone, or saline for four weeks. C=control group treated with saline, D=dexamethasone-treated group, GH=growth hormone-treated group. The ratios of proinsulin mRNAs to 18S rRNAs, expressed as relative densitometric units, are shown in **B.**

Assessment of oral glucose tolerance in GLP-1R-/- mice following 4 weeks of treatment with dexamethasone or growth hormone revealed significant improvements in glycemic excursion in both dexamethasone- and growth hormone-treated GLP-1R-/- mice compared to saline-treated controls (Fig. 5. B.). The improved glucose tolerance observed in dexamethasone-treated GLP-1R-/- mice was associated with significant increases in glucose-stimulated insulin levels (Fig. 5. C.). Although there was also an increase in plasma insulin levels in mice injected with growth hormone compared to controls, the difference did not reach statistical significance (Fig. 5. C.). In contrast, while wildtype mice treated with growth hormone demonstrated slight improvements in glycemic excursion following an oral glucose load, those given injections of dexamethasone exhibited deteriorated glucose tolerance (Fig. 5. A.). While there were no significant increases in glucose-stimulated insulin secretion in wildtype mice receiving dexamethasone or growth hormone treatment compared to controls, both the dexamethasone- and growth hormone-treated wildtype mice exhibited slightly increased insulin levels (Fig. 5. C.). Neither wildtype nor GLP-1R-/- mice treated with dexamethasone or growth hormone demonstrated a pronounced increase in proinsulin mRNA transcripts compared to controls (Fig. 6).

2.4 Discussion

The essential importance of GLP-1 action for control of glucose-stimulated insulin secretion is illustrated by defective glucose-stimulated insulin secretion in GLP-1R-/- mice (80). In contrast, despite the potent inhibitory effects of GLP-1 on central control of food intake (72), GLP-1R-/- mice are not obese and do not exhibit perturbed control of feeding behaviour (80). The most likely explanation for the lack of obesity relates to the existence of functionally

redundant mechanisms for control of satiety that compensate for lack of GLP-1 action in the brain. In contrast, although GIP and other incretins may be important for control of glucose-dependent insulin secretion, disruption of GLP-1 signaling is not adequately compensated for at the level of the islet, resulting in glucose intolerance.

Administration of growth hormone or glucocorticoids, alone or in combination, has been shown to induce insulin resistance in both rodent and human studies and may also decrease glucose clearance (88). Accordingly, the normal ß cell response to insulin resistance involves increased insulin biosynthesis and secretion (88). Remarkably, glucose tolerance was actually improved after one week of D/GH treatment in both wildtype and GLP-1R-/- mice, in association with a significant increment in glucose-induced insulin secretion. These findings clearly demonstrate that the insulin secretory response following D/GH is enhanced independent of GLP-1 signaling, and hence lack of GLP-1 action does not compromise the ß cell response to short term treatment with glucocorticoids and growth hormone.

In contrast, more prolonged treatment with combined D/GH for 4 weeks was associated with improved oral glucose tolerance in wildtype, but impaired oral glucose tolerance in GLP-1R-/- mice. Furthermore, GLP-1R-/- mice failed to increase either glucose-stimulated insulin secretion or proinsulin mRNA to levels comparable to that observed in wildtype mice. These observations emphasize the importance of GLP-1 signaling for the chronic response to agents such as D/GH, not just at the level of insulin secretion, but also at the level of proinsulin gene expression. Accordingly, it appears that whereas the contribution of GLP-1 to insulin secretion and proinsulin gene expression is modest under basal conditions, GLP-1 signaling is in fact essential for the normal ß cell response to treatment with glucocorticoids and growth hormone.

The improvement in glucose tolerance observed in GLP-1R-/- mice following 4 weeks of singular treatment with dexamethasone or growth hormone, in contrast to the deteriorated glycemic control observed when these two hormones were administered in combination for 4 weeks, suggests that although the knockout mice are able to compensate for the biological effects of individual administration of these compounds with adequate increases in insulin secretion, administration of both hormones overwhelms the ß cell, resulting in glucose intolerance. However, in view of the improved glucose tolerance observed in GLP-1R-/- mice treated with singular doses of both dexamethasone and growth hormone, it is surprising that wildtype mice treated with dexamethasone exhibit glucose intolerance.

Although quantitative islet morphology was not assessed in D/GH-treated mice, no evidence for differences in islet morphology were observed in a similar study involving D/GH-treated mice (91). In addition to the induction of insulin resistance, both growth hormone and glucocorticoids exert direct effects on islet ß cells. In view of the decreased glycemic excursion observed in wildtype and GLP-1R-/- mice following one week of treatment with dexamethasone and growth hormone, which is suggestive of improved rather than impaired insulin sensitivity, the differences observed in the responses of wildtype and GLP-1R-/- mice to four weeks of D/GH treatment may primarily be the result of direct effects of these agents on the ß cell, as opposed to secondary effects on the ß cell due to the induction of insulin resistance. Growth hormone binding sites have been detected on rat insulinoma RIN-5AH cells, and incubation of these cells with growth hormone for 4 days leads to an increase in cellular insulin content (92). Growth hormone appears to stimulate insulin promoter activity via binding of STAT (signal transducer and activator of transcription) 5 to a specific site in the rat insulin gene promoter (93). In contrast to the positive effects of GH on insulin

biosynthesis, glucocorticoids have been shown to inhibit insulin biosynthesis in islet cells via destabilization of insulin mRNA transcripts (94). However, it must be considered that in vivo insulin gene expression is regulated by complicated feedback systems which are generally absent in vitro.

GLP-1 appears to exert its effects in islet ß cells via stimulation of its receptor coupled to activation of a cAMP-dependent pathway (37). Furthermore, insulin gene expression and insulin biosynthesis are also known to be upregulated by cAMP (95,96). The disruption of GLP-1 receptor signaling in mice may lead to a decrease in the basal levels of ß cell cAMP (97), perhaps explaining the comparatively diminished induction of insulin gene expression in D/GH-treated mice. Intriguingly, the levels of ß cell cAMP may also modify the negative effects of glucocorticoids on insulin gene expression. Taken together, our studies demonstrate that interruption of GLP-1 receptor expression interferes, at the level of both insulin secretion and insulin gene expression, with the compensatory ß cell response to combined dexamethasone and growth hormone administration, providing important evidence in support of the important role of GLP-1 in ß cell function in vivo.

Chapter 3 GLP-1R-/- Mice Possess Normal Peripheral Insulin Sensitivity

3.1 Introduction

While the potent insulinotropic actions of GLP-1 are well-defined, it remains unclear whether GLP-1 exerts direct actions in peripheral tissues to promote glucose-lowering. The effects of GLP-1 on insulin sensitivity and glucose uptake in tissues such as the liver, adipose tissue, and muscle continue to be debated.

GLP-1-binding activity has been observed on solubilized membranes of rat adipose tissue, and interestingly, analysis of the binding data suggests the presence of high and low affinity binding sites (42). GLP-1 reportedly exerts a lipolytic action on isolated rat adipocytes (68). Further, GLP-1 produced an augmentation of both the insulin-mediated increase in glucose uptake and insulin-mediated reduction in cAMP content of isolated rat adipocytes (98). GLP-1 has been shown to enhance insulin-stimulated glucose uptake in 3T3-L1 adipocytes (43) as well as decrease intracellular cAMP in this cell line (99). These data provide indirect evidence for the presence of a second GLP-1 receptor with signaling properties and binding characteristics distinct from those described for the pancreatic GLP-1 receptor. However, although GLP-1 receptor expression in rat adipose tissue has been detected by RT-PCR (43), subsequent studies utilizing RT-PCR, RNAse protection, and in situ hybridization, have failed to confirm this result (45).

The existence of a potent stimulatory effect of GLP-1 on glycogen synthesis in rat skeletal muscle (100,101) and isolated hepatocytes (69) has also been reported, although similar studies have failed to establish an effect of

GLP-1 on rat skeletal muscle (102) or hepatic (103) glucose metabolism. Similarly, while GLP-1 binding has been demonstrated in rat skeletal muscle (104) and liver membrane (105) preparations, GLP-1 receptor expression in these tissues remains controversial due to conflicting reports (44,45,106). Additional investigations are clearly warranted in order to more completely define the role of GLP-1 in peripheral glucose and lipid metabolism, as well as the mechanisms and receptors through which these effects are mediated.

Remarkably, a hypoglycemic effect of GLP-1, independent of its ability to stimulate the secretion of insulin, has been reported in humans (71). GLP-1 has been shown to increase glucose disposal in Type 1 diabetic patients (71) and glucose effectiveness in normal individuals (107), suggesting a role for GLP-1 in lowering blood glucose independent of its actions on the pancreatic β-cell. However, other studies have found that GLP-1 has no effect on insulin sensitivity in Type II diabetic patients (108) and normal individuals (109) during a hyperinsulinemic, euglycemic clamp. Further, determination of glucose disappearance rates during inhibition of endogenous insulin secretion with somatostatin suggested that GLP-1 had little to no effect on glucose elimination (70). It has also been proposed that the principle glycemic-lowering effect of GLP-1 in both Type I diabetics (110) and normal individuals (70) is mediated through suppression of pancreatic glucagon. A GLP-1-induced delay in gastric emptying also appears to be a major contributor to the decreased postprandial glycemic excursion observed following GLP-1 administration to IDDM patients (111).

The fasting hyperglycemia and glucose intolerance observed in GLP-1R-/- mice may, in part, be due to changes in peripheral insulin sensitivity as a result of disrupted GLP-1 signaling (80). To further elucidate a role for GLP-1 in

peripheral glucose uptake and insulin action, GLP-1R-/- mice were treated with a novel insulin-sensitizing agent, troglitazone.

Troglitazone is a member of a new class of orally active antidiabetic drugs, the thiazolidinediones, which were discovered during the search for novel hypolipidemic agents (113). The effects of troglitazone were initially evaluated in insulin-resistant diabetic animal models. Chronic administration of troglitazone to genetically insulin-resistant yellow *KK*, and *ob/ob* mice as well as Zucker *fa/fa* rats, markedly decreased plasma glucose, insulin, and triglyceride levels without any effect on body weight or food intake (114). Some nongenetic models of insulin resistance, such as rats fed high-fructose (115) or high-fat diets (116), also respond to troglitazone treatment with decreased plasma insulin and glucose levels. However, the thiazolidinediones have no glucose-lowering effect in insulin-deficient diabetic animal models, such as the streptozotocin diabetic rat (117). This suggests that the thiazolidinedione compounds act as insulin-sensitizing agents in hepatic and peripheral tissues, thereby improving the action of insulin without directly stimulating insulin secretion.

Thiazolidinedione treatment was found to restore the ability of insulin to suppress hepatic glucose output and increase peripheral glucose disposal in several animal models of insulin resistance evaluated using the euglycemic-hyperinsulinemic clamp (118,119). Treatment of diabetic animals with thiazolidinediones has also been shown to restore the responsiveness of desensitized islets to stimulation, an action which is likely secondary to the insulin-sparing effects of decreased insulin resistance (120). The regranulation of ß-cells observed in association with increased pancreatic insulin content when severely diabetic db/db mice were administered troglitazone is also consistent with an insulin-sparing effect (121).

Although the exact mechanism of action remains to be determined, it is known that troglitazone is a ligand of the nuclear receptor peroxisome proliferator-activator receptor gamma (PPAR gamma). Evidence to date suggests that PPAR gamma and other members of the same receptor family may be important regulators of lipid homeostasis, adipocyte differentiation, and insulin action (122). The thiazolidinediones appear to exert their insulin-sensitizing actions, at least in part, by binding with PPAR gamma to alter the expression of genes involved in glucose and lipid metabolism. Thiazolidinediones have been shown to exert stimulatory effects on the expression of the insulin-responsive glucose transporters, GLUT1 and GLUT4, correlating with enhanced basal and insulin-stimulated glucose uptake, in both 3T3-L1 adipocytes (123,124) and L6 myocytes (125,126). However, in several instances, specific thiazolidinedione-responsive elements have not been identified within known thiazolidinedione-responsive genes (120). Further, the genes which are relevant to the insulin-sensitizing actions of the thiazolidinediones are not well-defined.

Thus, it was hypothesized that if GLP-1R-/- mice demonstrate impaired insulin-stimulated glucose uptake as a consequence of disrupted GLP-1 signaling, treatment with the insulin-sensitizing agent troglitazone may compensate for peripheral defects in insulin action, producing improved glucose tolerance. Further, known troglitazone-responsive genes, including GLUT1 and GLUT4, were expected to exhibit increased expression in the mice receiving troglitazone.

3.2 Materials and Methods

3.2.1 Troglitazone Administration

Individually housed 10 week-old male wildtype CD1 +/+ and GLP-1R-/- mice received daily oral administrations of 200 mg/kg troglitazone (Parke-Davis, Ann Arbor, MI) combined with 0.5 g Philadelphia® spreadable cream cheese (n=6 per group) for three weeks. Age- and sex-matched wildtype and GLP-1R-/- control animals were given cream cheese only (n=6 per group). During this treatment, the mice were permitted *ad libitum* access to rodent chow and water (see 2.2.1).

3.2.2 Glucose Tolerance and Insulin Determinations

Following a 16 h fast, oral glucose tolerance tests (see 2.2.2) were performed on day 11 and day 22 following the initiation of treatment. Blood samples for insulin content measurements were withdrawn from the tail vein during the period between 20-30 min following oral glucose load for insulin quantification using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

3.2.3 Tissue Collection and RNA Isolation

Following the oral glucose tolerance test on day 22, the mice were euthanized with CO_2 and epididymal fat and quadricep muscle tissues were collected for RNA analysis. Total cellular RNA was isolated from these tissues by the acid-guanidium thiocyanate method (90).

3.2.4 Northern Blot Analysis

Northern blot analysis was performed as described in 2.2.5.

3.2.5 Statistics

All data are expressed as the mean ± SEM. Statistical significance of data from animals following treatment was assessed by an unpaired t test between mice receiving troglitazone and controls. All statistics were analyzed using the InStat 1.12 program for MacIntosh computers (GraphPad Software).

3.3 Results

The treatment of several genetic (114) and nongenetic (115,116) insulin-resistant models of diabetes with thiazolidinedione compounds produces improvements in plasma glucose levels. In order to assess changes in glucose homeostasis, the present study evaluated the oral glucose tolerance of wildtype and GLP-1R-/- mice following 11 and 22 days of troglitazone treatment. Oral glucose tolerance was unchanged in wildtype and GLP-1R-/- mice receiving 11 or 22 days of troglitazone treatment compared to controls, and the combined data for these two OGTTs are shown in Fig. 7 and Fig. 8 for wildtype and GLP-1R-/- mice, respectively.

Plasma glucose levels in nondiabetic, euglycemic animals are unaffected by thiazolidinedione treatment (127). However, these compounds continue to exert potent insulin-sensitizing effects in normal animals (127). As insulin sensitivity increases, pancreatic insulin secretion apparently declines to maintain normoglycemia. Analysis of glucose-stimulated insulin levels in plasma samples obtained 20-30 minutes after glucose administration in this experiment demonstrated unchanged plasma insulin levels in troglitazone-treated wildtype and GLP-1R-/- mice during an OGTT (P=0.4723 and P=0.5308 for control vs. troglitazone-treated wildtype and GLP-1R-/- mice, respectively; Fig. 9).

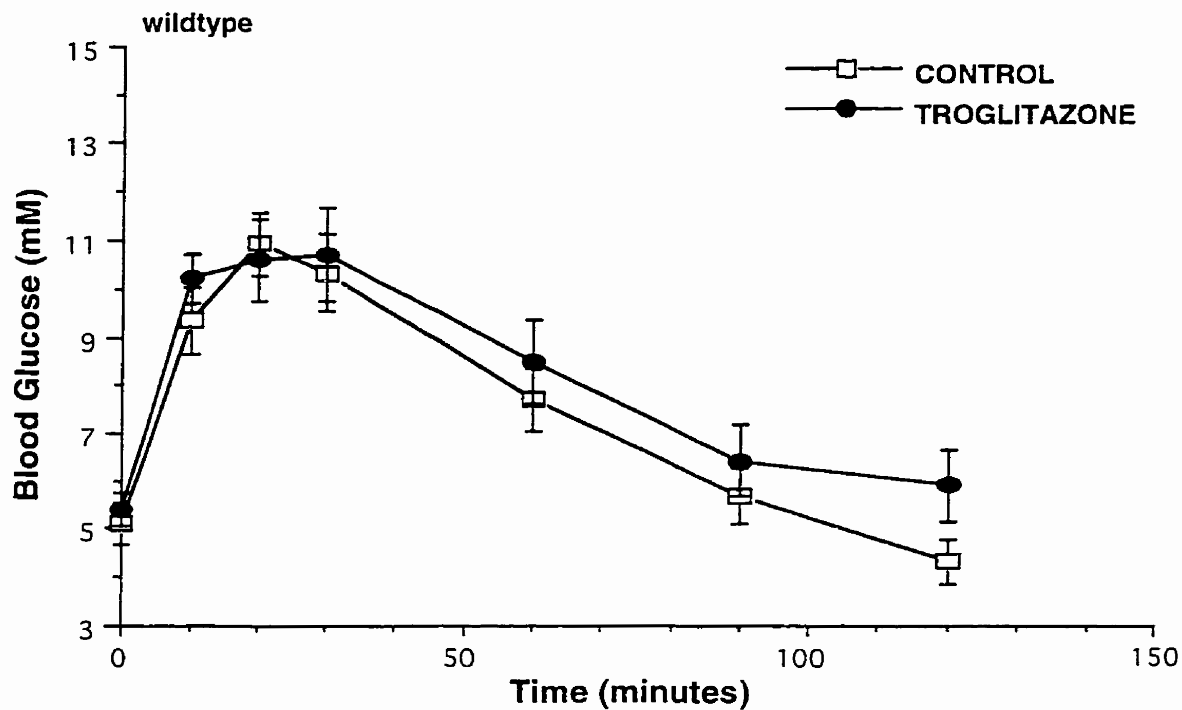


Fig. 7. Oral glucose tolerance test in wildtype mice treated with troglitazone. 10 week-old male wildtype CD1+/+ mice were treated with 200 mg/kg/day troglitazone, or vehicle, for 21 d. Oral glucose tolerance tests were performed on day 11 and day 22 following the initiation of treatment. The data presents the results of two oral glucose tolerance tests (n=12 per group). Values are expressed as means ± S.E.M.

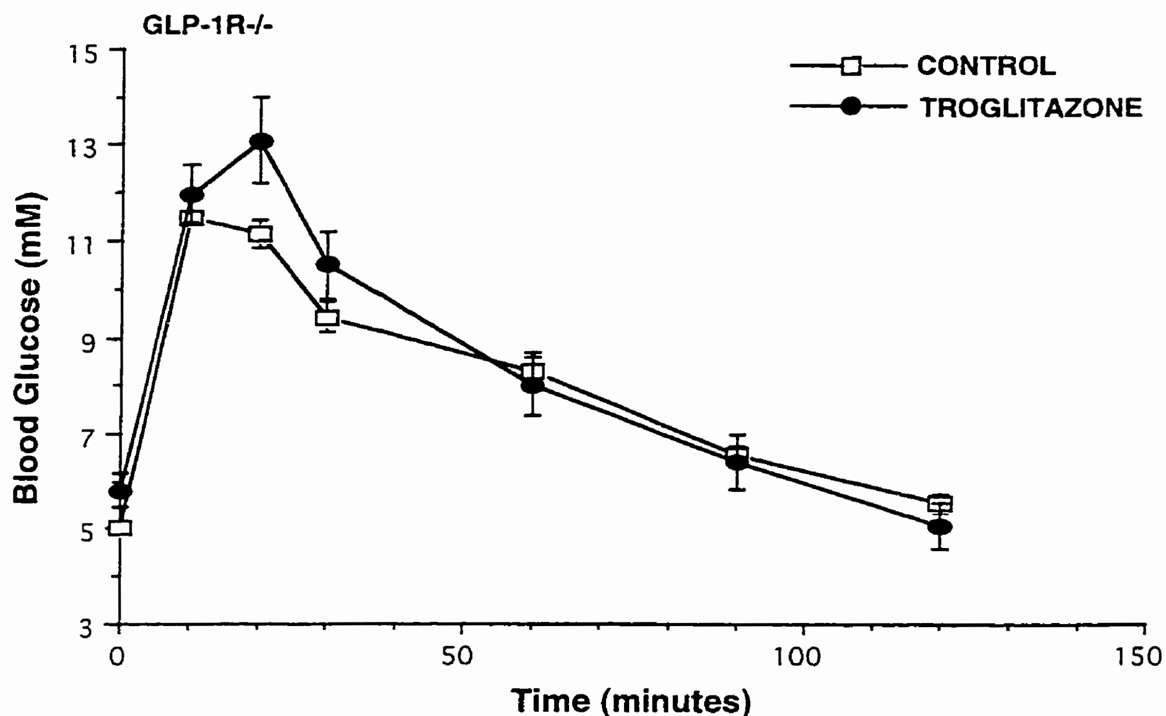


Fig. 8. Oral glucose tolerance test in GLP-1R-/- mice treated with troglitazone. 10 week-old male GLP-1R-/- mice were treated with 200 mg/kg/day troglitazone, or vehicle, for 21 d. Oral glucose tolerance tests were performed on day 11 and day 22 following the initiation of treatment. The data presents the results of two oral glucose tolerance tests (n=12 per group). Values are expressed as means ± S.E.M.

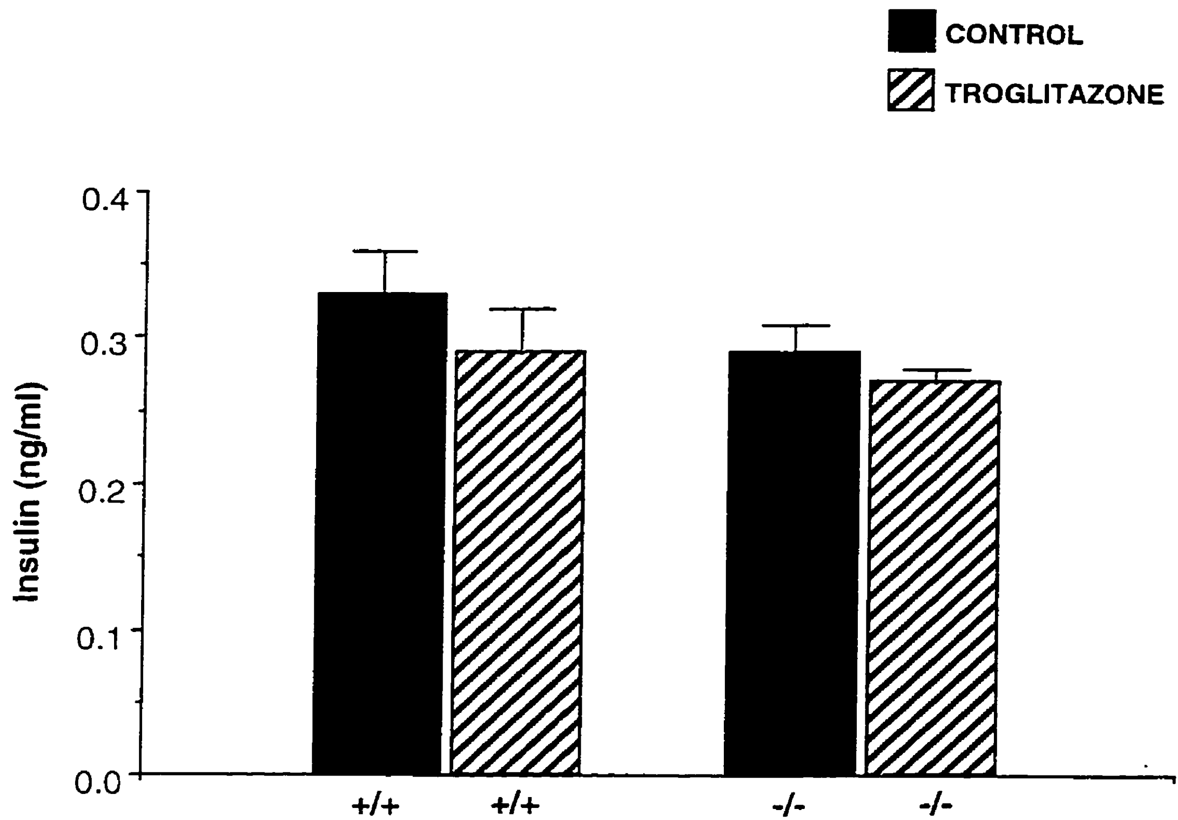


Fig. 9. Plasma concentrations of insulin during oral glucose tolerance tests in wildtype and GLP-1R-/- mice treated with troglitazone. Plasma insulin levels during the OGTTs shown in Fig. 7 and Fig. 8, (n=6 per group). Values are expressed as means ± S.E.M.

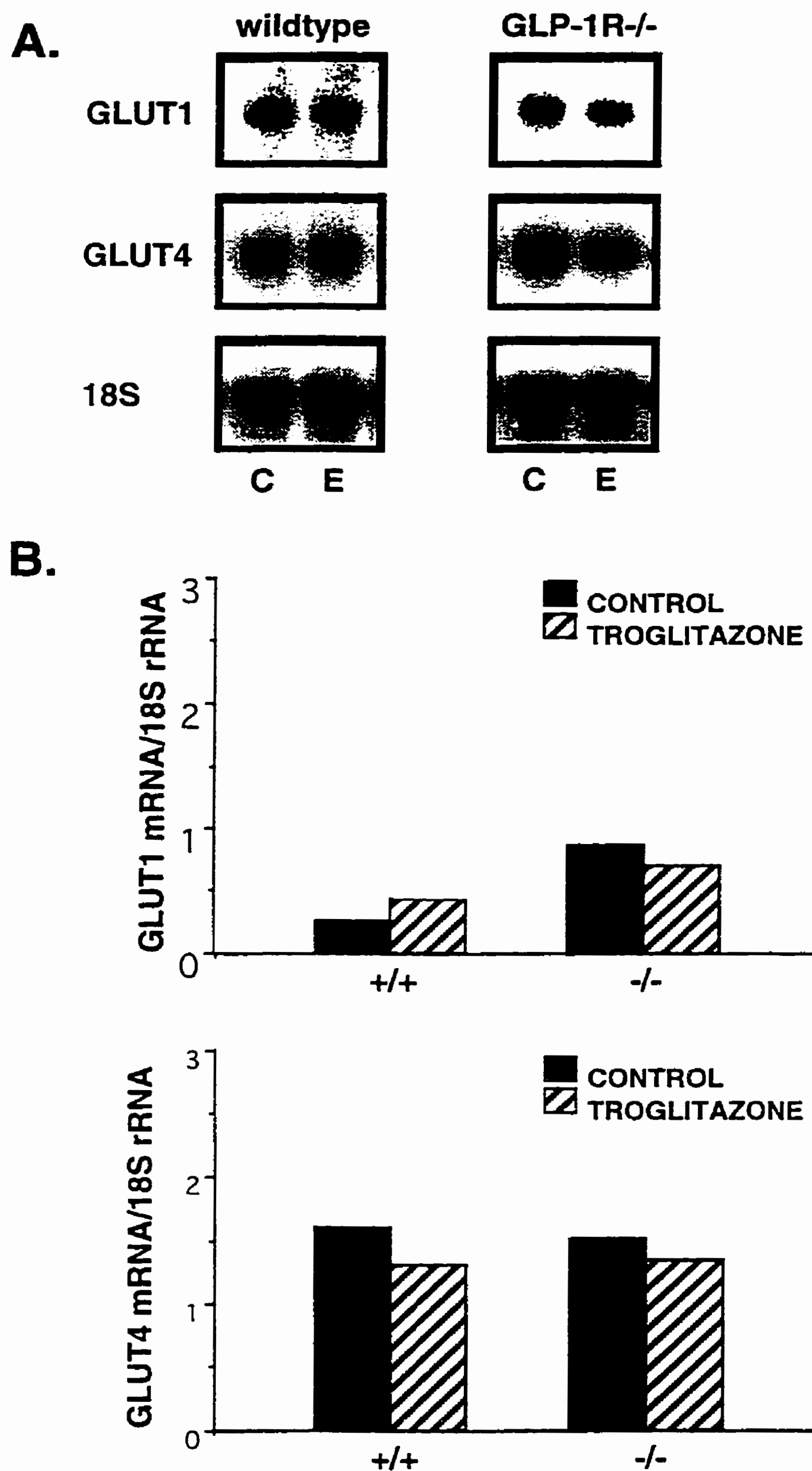


Fig. 10. A. Northern blot analysis of skeletal muscle RNA from wildtype and GLP-1R-/- mice treated with troglitazone. RNA was isolated from wildtype CD1 +/+ (+/+) and GLP-1R-/- (-/-) male mice following 21 d of 200 mg/kg/day troglitazone (E=experimental) or control (C=control) treatment. RNA was hybridized with a rat GLUT1 cDNA followed by hybridization with a rat GLUT4 cDNA and 18S rRNA. **B.** The relative densitometric data represent mean values for GLUT1 and GLUT4 mRNA transcripts from +/+ and -/- mice treated with 200 mg/kg/day troglitazone or controls (n=2 per group).

Thiazolidinedione treatment is associated with enhanced GLUT1 and GLUT4 transporter gene expression in both adipocytes (123,124) and myocytes (125,126). However, the levels of GLUT1 and GLUT4 mRNA transcripts in muscle (Fig. 10) and adipose tissue (not shown) isolated from wildtype and GLP-1R-/- mice treated with troglitazone did not differ from controls.

3.4 Discussion

A role for GLP-1 in lowering blood glucose independent of its actions on the pancreatic ß-cell has been proposed. Thus, it was hypothesized that the glucose intolerance observed in GLP-1R-/- mice may result, in part, from diminished peripheral insulin sensitivity and reduced glucose utilization in the absence of GLP-1 signaling.

Troglitazone, a novel insulin-sensitizing agent, decreases plasma glucose and insulin levels in several insulin-resistant diabetic animal models (114-116). Surprisingly, troglitazone administration failed to improve the oral glucose tolerance of GLP-1R-/- mice. As thiazolidinedione compounds have previously been shown not to affect the plasma glucose levels of normal, insulin-sensitive animals (127), these results suggest that GLP-1R-/- mice exhibit normal peripheral insulin sensitivity.

It has been speculated that although thiazolidinedione compounds increase insulin sensitivity in normoglycemic animals, the negative-feedback system between glucose and insulin remains intact, preventing hypoglycemia (120). It is proposed that as glucose levels decrease due to the heightened glucose uptake associated with increased peripheral insulin sensitivity, pancreatic insulin secretion declines. In this study, both GLP-1R-/- and wildtype mice treated with troglitazone exhibited slight, but statistically insignificant,

decreases in circulating glucose-stimulated insulin levels, suggesting that troglitazone may have modestly improved insulin sensitivity.

The observation that GLP-1R-/- mice respond to troglitazone treatment in a manner similar to that of wildtype mice suggests that the glucose intolerance observed in GLP-1R-/- mice results largely from factors independent of peripheral insulin sensitivity. In support of this hypothesis, it has recently been shown that the absence of GLP-1 signaling in GLP-1R-/- mice produces no change in whole body glucose utilization during hyperinsulinemic, euglycemic clamp studies (128). In fact, there was a tendency (N.S.) for the GLP-1R-/- mice to be more sensitive to insulin when compared with wildtype CD1 +/+ controls. This is consistent with recent human studies which employed the hyperinsulinemic, euglycemia clamp method to measure insulin sensitivity following GLP-1 administration (129), or alternatively, determined glucose disappearance rates in response to GLP-1 during inhibition of endogenous insulin secretion with somatostatin (130). Collectively, these data suggest that the effects of GLP-1 on insulin sensitivity, if any, are probably insignificant in their contribution to the glucose intolerance and fasting hyperglycemia observed in GLP-1R-/- mice.

Although in vitro studies suggest that thiazolidinediones increase expression of the GLUT1 and GLUT4 transporters in adipose and muscle cells, this study did not detect augmented expression of these genes in muscle or adipose tissue isolated from wildtype and GLP-1R-/- mice treated with troglitazone. However, glucose and lipid metabolism in vivo is regulated by complex feedback systems which are generally absent in tissue culture cells. Further, few studies have evaluated the effects of thiazolidinediones on gene expression in insulin-sensitive animal models. Although thiazolidinedione administration to Zucker fa/fa rats has been shown to increase the expression of

GLUT4 in adipose tissue (131), our study suggests that GLUT4 (as well as GLUT1) mRNA levels do not increase when thiazolidinediones such as troglitazone are administered to insulin-sensitive animals.

The failure of the insulin-sensitizing agent troglitazone to improve glycemic control in GLP-1R-/- mice suggests that these mice possess normal peripheral insulin sensitivity, and is consistent with the observation that GLP-1R-/- mice display normal glucose utilization during hyperinsulinemic clamp studies. Although subtle defects may exist in the peripheral tissues of GLP-1R-/- mice as a result of the disruption in GLP-1 signaling, a prominent effect of GLP-1 on insulin sensitivity seems unlikely.

Chapter 4 Inhibition of Dipeptidyl Peptidase-IV Improves Oral Glucose Tolerance in Wildtype and GLP-1R-/- Mice

4.1 Introduction

Interest in utilizing GLP-1 for the enhancement of insulin secretion in patients with diabetes has stimulated research into the duration of biological activity, and mechanisms of degradation, of circulating GLP-1. Immunoreactivity measurements following the cessation of GLP-1 infusion indicated that the peptide is rapidly cleared from circulation via the renal route of elimination (132), with a half-life ranging from 4-11 min (19,36). However, since peptidases in plasma may render a hormone biologically inactive while maintaining immunoreactivity, hormone measurements associated with immunoreactivity do not necessarily correspond to bioactivity. Indeed, it has been shown that GLP-1(7-37/36NH2) is hydrolyzed in human serum to the biologically inactive fragment GLP-1(9-37/36NH2) by the serine-type peptidase dipeptidyl peptidase-IV (DPP-IV) (78). The half-life of intact, biologically active GLP-1(7-37/36NH2) is estimated to be less than two minutes (77).

DPP-IV was initially identified as a T-cell-activating antigen by histochemistry and assigned to the CD26 cluster (133). It is comprised of two identical subunits with molecular masses of approximately 110 kDa, and is predominantly extracellular, with only six cytoplasmic amino acids (134). DPP-IV is ubiquitously expressed in mammalian tissues (133). The highest DPP-IV activity is found in the kidney and intestinal brush-border membrane, although it is also present in the liver and on the cell surface of many epithelial cells, almost all endothelial cells, and white blood cells (135). The major component of serum DPP-IV is believed to be shed from T cells through proteolytic digestion (136). DPP-IV is known to participate in a number of biochemical

processes, including the activation of immunocompetent cells and cell-matrix interactions (133). It is essential for the renal transport and intestinal digestion of proline-containing peptides (133).

DPP-IV is involved in the hydrolysis of several biologically relevant peptides in addition to GLP-1; including GIP, substance P, and several releasing hormones (78,137,138). The DPP-IV catalytic site cleaves dipeptides from the amino end of polypeptides consisting of at least three residues, where the penultimate amino acid is proline or, less effectively, alanine, thus liberating X-Pro or X-Ala dipeptides, where X refers to any amino acid (133). Since the N-terminus of GLP-1(7-37/36NH2) is crucial for its biological activity, DPP-IV cleavage of the N-terminal dipeptide His-Ala from GLP-1(7-37/36NH2) produces an inactive peptide, GLP-1(9-37/36NH2) (77). Evidence suggests that the C-terminal sequence of GLP-1 is required for binding of GLP-1 to its receptor, while the N-terminus is essential for biological activity (139). N-terminally truncated fragments of GLP-1, therefore, continue to exhibit receptor-binding activity but are unable to stimulate second messenger production (140). Thus, GLP-1(9-37/36NH2) may function as a competitive antagonist of the intact peptide at the GLP-1 receptor (140,141).

Further development of GLP-1 as a therapeutic agent in the treatment of diabetes is limited by its rapid degradation by DPP-IV *in vivo*. Although the bioactivity of the incretin hormone GIP is also limited *in vivo* by DPP-IV hydrolysis, NIDDM patients show a diminished response to GIP, thereby limiting its therapeutic potential (5). Efforts have focused on the clinical development of GLP-1 because its potent insulinotropic effects are preserved in patients with NIDDM (5).

The strict substrate requirements of DPP-IV have encouraged the development of GLP-1 analogues which are resistant to DPP-IV degradation

but retain bioactivity at the GLP-1 receptor. These analogues possess prolonged metabolic stability in vivo and may therefore have greater potency and duration of action than native GLP-1 (143). The development of specific DPP-IV inhibitors provides an alternative mechanism to increase GLP-1 activity in vivo. Dipeptides of the type X-boroPro, in which boroPro is an amino boronic acid analog of proline, are specific inhibitors of DPP-IV and exhibit slow-binding kinetics which produce stable enzyme-inhibitor complexes (135). Injection of 160 ug of Pro-boroPro into mice has been shown to almost completely inhibit plasma DPP-IV activity within 2 h, with no sign of toxicity (144). Recovery of the inhibited DPP-IV activity appears mainly due to dissociation and inactivation of the inhibitors (144).

In view of the role of DPP-IV in degradation of the incretin hormones GLP-1 and GIP, it was hypothesized that administration of the DPP-IV inhibitor Pro-boroPro to wildtype mice prior to an oral glucose load would increase the biological half-lives of endogenous GLP-1 and GIP, as well as exogenously administered GLP-1, facilitating enhanced insulin secretion with concomitant improvements in glucose tolerance. Further, Pro-boroPro was administered to mice with disrupted GLP-1 signaling in order to assess the role of GIP in glucose homeostasis during DPP-IV inhibition, independently of GLP-1.

4.2 Materials and Methods

4.2.1 DPP-IV Inhibitor

Pro-boroPro was generously provided by Dr. Andrew G. Plaut (Tufts University, Boston, MA). It is a potent and specific inhibitor of DPP-IV, and exhibits kinetic properties consistent with a mechanism of slow-binding inhibition and production of stable enzyme-inhibitor complexes (144). Lyophilized Pro-boroPro was dissolved at pH 2, and aliquots were stored at -

20°C. Immediately prior to injection, the aliquots were thawed and diluted in phosphate-buffered saline.

4.2.2. Pro-boroPro Administration Prior to Glucose Tolerance and Insulin Determinations

Male, 7 week-old wildtype CD1 +/+ mice (n=4) were fasted for 16 h and given an intraperitoneal injection of 150 ug Pro-boroPro in 0.25 ml phosphate-buffered saline 1 h prior to the initiation of an oral glucose test (OGTT, see 2.2.3). Age- and sex-matched controls (n=4) were injected with phosphate-buffered saline. 5 min prior to glucose loading, the mice in the experimental (Pro-boroPro) group and the control (saline) group received an intraperitoneal injection of 8 ug GLP-1. Blood samples were removed from the tail vein at the 20 minute time point during the OGTT for insulin quantification using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

In a second experiment, 8 week-old male wildtype CD1 +/+ mice were fasted for 16 h and given an intraperitoneal injection of 150 ug Pro-boroPro (n=10) or saline (n=9) one hour prior to an OGTT. Blood samples were removed from the tail vein at the 20 minute time point during the OGTT for insulin quantification using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

In a third experiment, 10 week-old male GLP-1R-/- mice were fasted for 16 h and given an intraperitoneal injection of 150 ug Pro(boro)Pro (n=6) or saline (n=6) one hour prior to an OGTT. Blood samples were removed from the tail vein at the 20 minute time point during the OGTT for insulin quantification using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

4.2.3 Statistics

All data are represented as the mean ± SEM. The experimental group was compared to the control group with a two-tailed, unpaired t test. All statistics were analyzed using the InStat 1.12 program for MacIntosh computers (GraphPad Software).

4.3 Results

Exogenous GLP-1 was initially administered in combination with Pro-boroPro (Fig. 11. A.; insert) because it was speculated that potentiation of low levels of endogenous GLP-1 and GIP during an OGTT would not produce improved glucose tolerance. Pilot studies indicated that administration of 8 ug GLP-1 prior to an oral glucose tolerance test did not result in significant changes in glycemic excursion. However, the striking improvement in glucose tolerance following exogenous GLP-1 (8ug) administration during concomitant DPP-IV inhibition ($P<0.05$ at 20 and 30 min following glucose load; Fig. 11.A.) suggested that potentiation of endogenous incretin activity alone may also improve glucose homeostasis. Indeed, oral glucose tolerance improved substantially when Pro-boroPro was administered prior to glucose loading in wildtype mice ($P<0.05$ at 20 min, and $P<0.01$ at 30 and 60 min following glucose administration; Fig. 12. A.).

Previous studies have shown that GLP-1R-/- mice demonstrate increased GIP secretion in response to an oral glucose load compared to wildtype mice (152). Additionally, pancreatic perfusion studies revealed that GLP-1R-/- mice exhibit increased pancreatic sensitivity to the insulinotropic effects of GIP (152). To assess whether further increases in GIP activity are able to improve glucose homeostasis in the absence of concomitant GLP-1 potentiation, the glucose

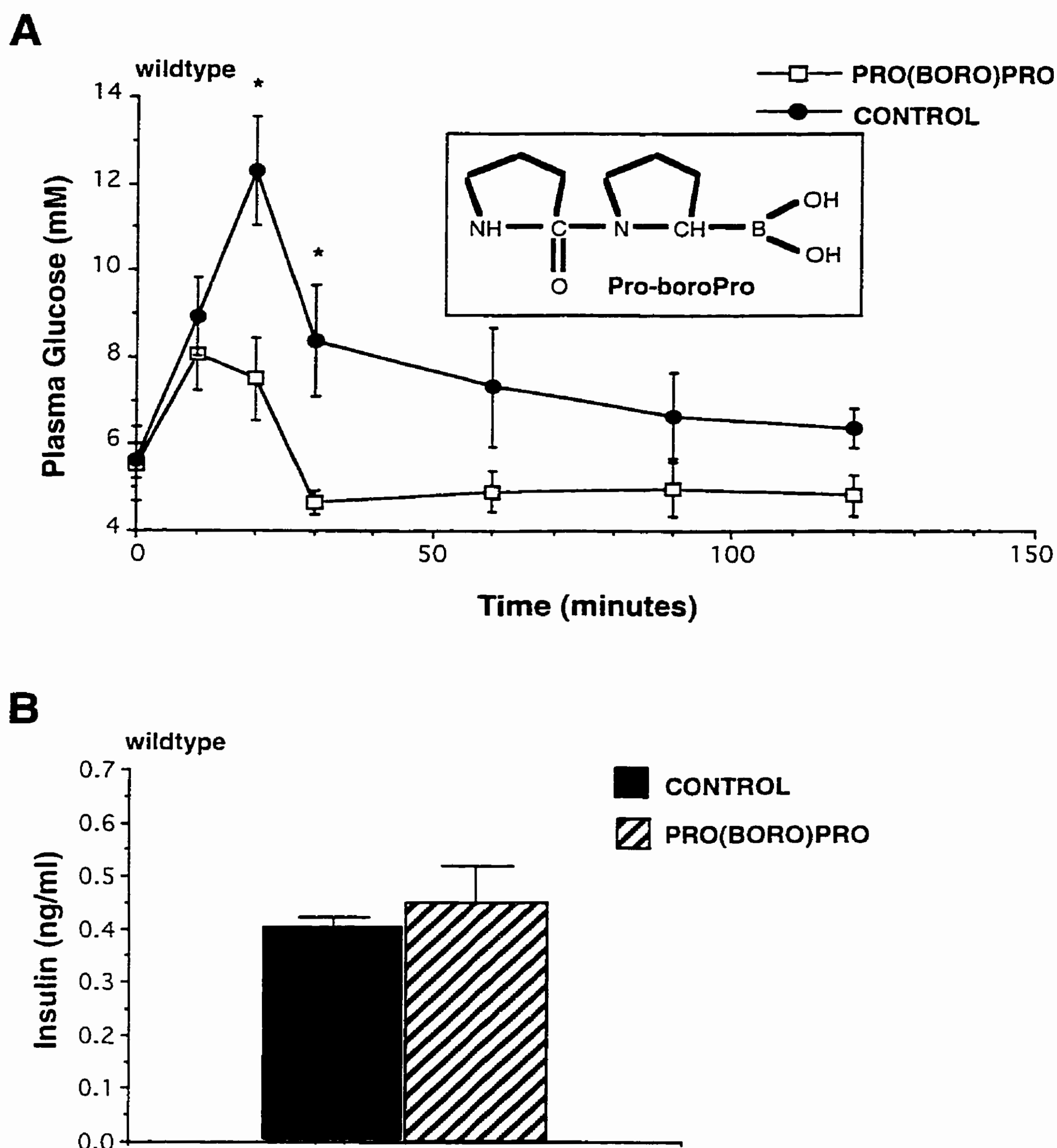
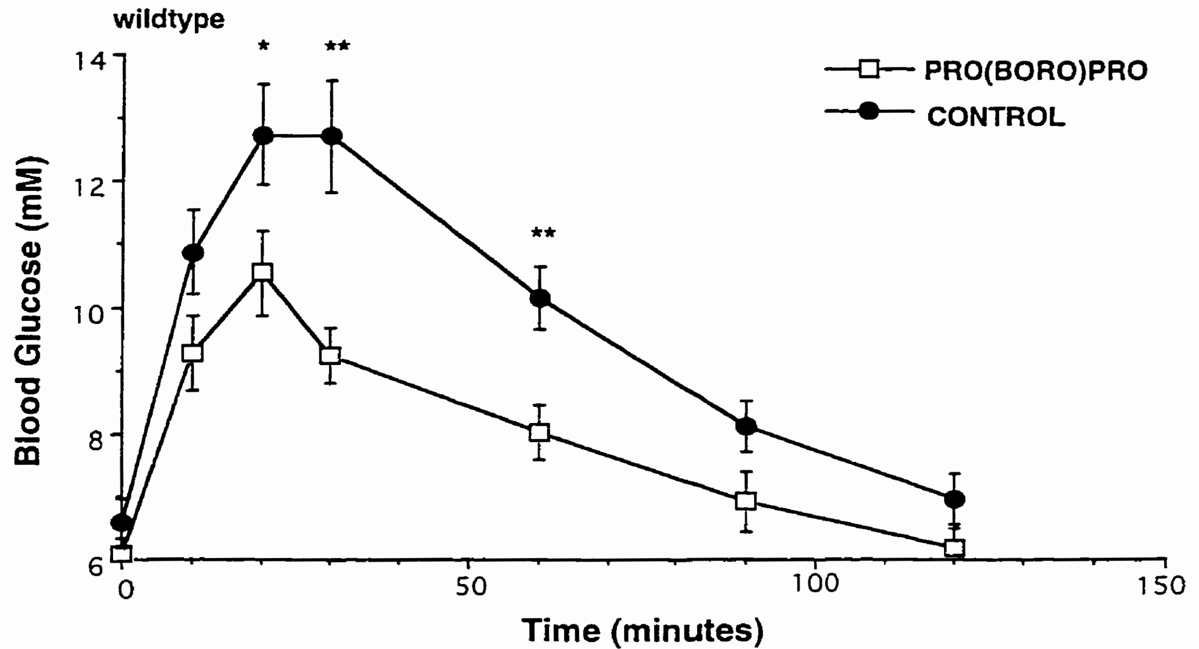


Fig. 11. Oral glucose tolerance test and glucose-stimulated insulin levels in wildtype mice injected with Pro-boroPro and exogenous GLP-1. **A.** 7 week-old male wildtype CD1 +/+ mice were injected with 150 ug Pro-boroPro or saline 1 h prior to an oral glucose tolerance test, and 8 ug GLP-1 5 min prior to OGTT (n=4 per group). *P<0.05. Inset: Structure of Pro-boroPro. **B.** Plasma insulin levels during the oral glucose tolerance test shown in A.



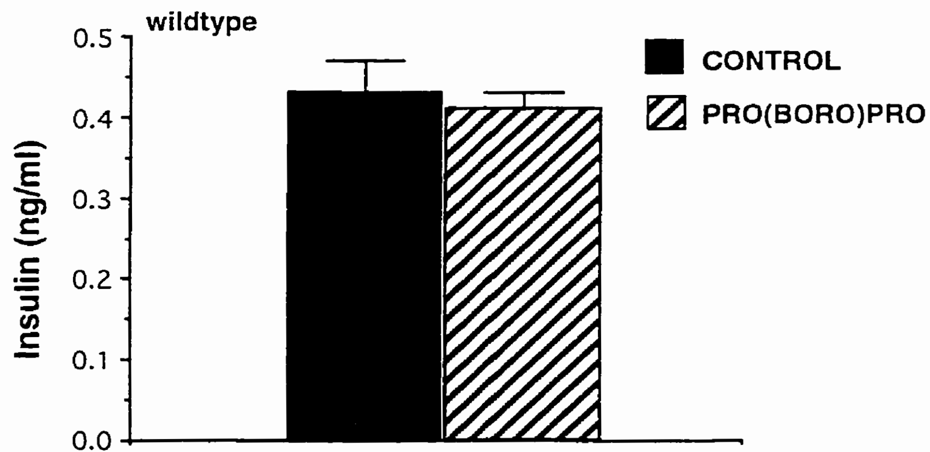
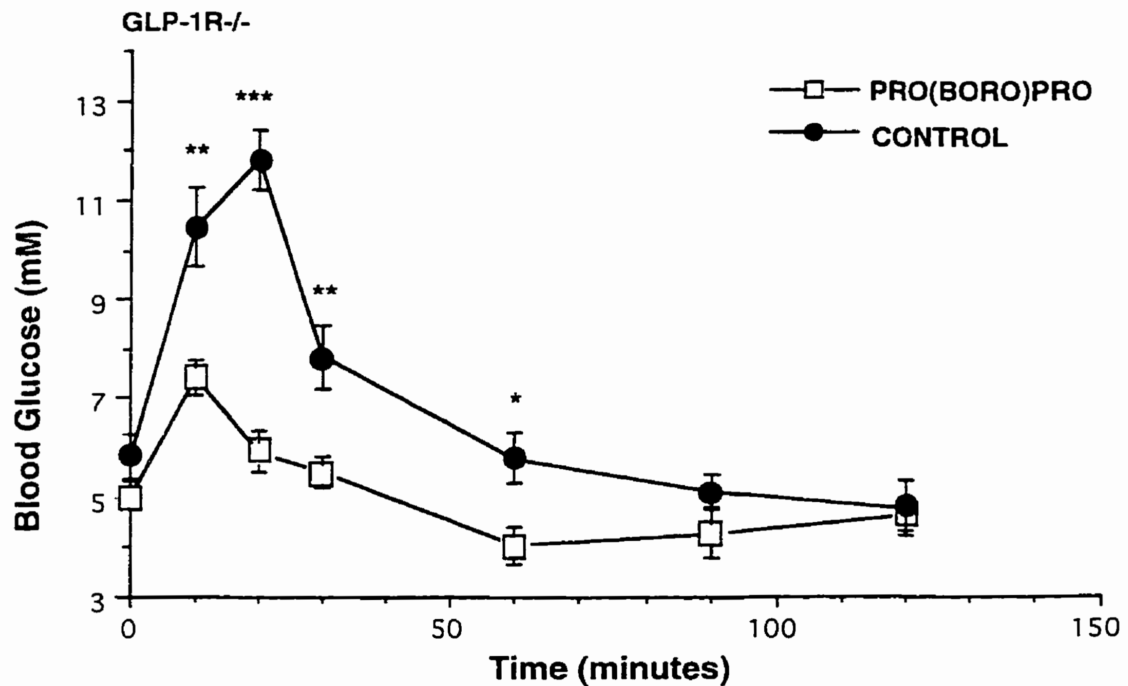


Fig. 12. Oral glucose tolerance test and glucose-stimulated insulin levels in wildtype mice injected with Pro-boroPro. **A.** 8 week-old male wildtype CD1 +/+ mice were injected with 150 ug Pro-boroPro or saline 1 h prior to an oral glucose tolerance test (n=9-10 per group). *P<0.05, **P<0.01. **B.** Plasma insulin levels during the oral glucose tolerance test shown in A (n=5 per group).

A



B

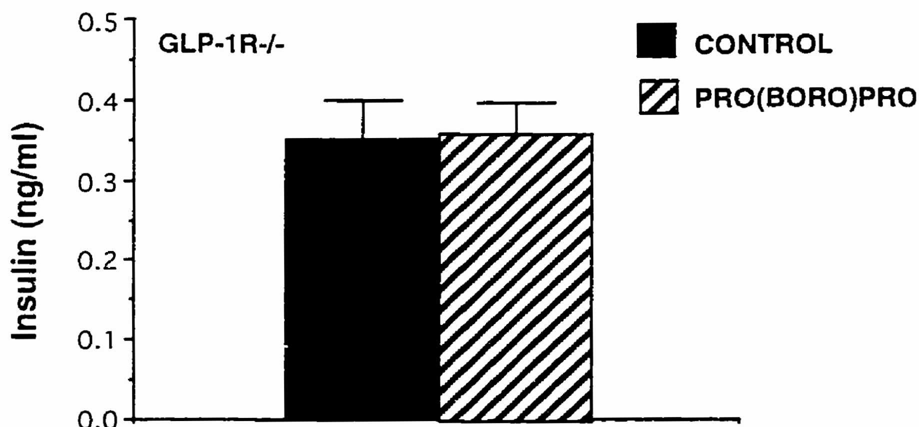


Fig. 13. Oral glucose tolerance test and glucose-stimulated insulin levels in GLP-1R-/- mice injected with Pro-boroPro. **A.** 10 week-old male GLP-1R-/- mice were injected with 150 ug Pro-boroPro or saline 1 h prior to an oral glucose tolerance test (n=6 per group). *P<0.05, **P<0.01, ***P<0.001. **B.** Plasma insulin levels during the oral glucose tolerance test shown in A (n=5 per group).

tolerance of GLP-1R-/- mice was evaluated following an oral glucose load, with or without Pro-boroPro. GLP-1R-/- mice showed very significant improvements in oral glucose tolerance during DPP-IV inhibition ($P<0.01$ at 10 and 30 min, $P<0.001$ at 20 min, and $P<0.05$ at 60 min following glucose load; Fig. 13.A.).

The speculated inhibition of endogenous GIP and both exogenous and endogenous GLP-1 degradation by DPP-IV inhibition was presumed to produce improved glucose tolerance as a consequence of increased incretin-stimulated insulin secretion. However, the insulin levels at the 20 min time point during the oral glucose tolerance test did not change significantly when GLP-1 was administered to wildtype mice with or without Pro-boroPro, or when wildtype or GLP-1R-/- mice were given the inhibitor prior to an oral glucose tolerance test ($P=0.5390$; Fig. 11. B., $P=0.6107$; Fig 12. B., $P=0.8154$; Fig. 13. B., respectively).

4.4 Discussion

Since the first report indicating GLP-1 was degraded by DPP-IV (78), studies have clearly demonstrated that DPP-IV is the main enzyme responsible for GLP-1 inactivation in vivo (77,79,145). Importantly, exogenous GLP-1 is also rapidly metabolized to the peptide GLP-1(9-37/36NH2), which likely possesses antagonistic properties in vivo (141). Thus, in order to maximize the potential of GLP-1 as a therapeutic agent, DPP-IV-resistant analogues and compounds which inhibit DPP-IV activity in vivo are under investigation as a means of increasing GLP-1 bioactivity and decreasing production of the GLP-1 (9-37/36NH2) antagonist.

This study aimed to assess the role of DPP-IV in glucose homeostasis in wildtype and GLP-1R-/- mice. The results clearly demonstrate that inhibition of DPP-IV by administration of 160 ug Pro-boroPro improves glucose homeostasis in both murine models. Although plasma DPP-IV activity was not assessed in

this study, previous reports have indicated that administration of similar doses of Pro-boroPro produces almost complete inhibition of plasma DPP-IV activity (144).

As other serum proteases appear to play only a minor role in GLP-1 and GIP degradation (146), inhibition of DPP-IV activity by Pro-boroPro in the present study was expected to reduce degradation of these incretin hormones, facilitating enhanced insulin secretion with concomitant decreases in glycemic excursion. Although the effect of DPP-IV inhibition by Pro-boroPro on GLP-1 and GIP levels were not determined in this study, a previous study has reported that administration of a DPP-IV inhibitor prolonged the half-life of the intact GLP-1, resulting in elevated levels of intact peptide (147). Surprisingly, administration of Pro-boroPro did not produce elevated insulin levels during an oral glucose tolerance test in either wildtype or GLP-1R-/- mice. It is of interest, however, that animals injected with Pro-boroPro exhibited insulin levels comparable to those of controls, despite lower glucose levels. Since glucose is the primary regulator of insulin secretion, it may be speculated that inhibition of DPP-IV by Pro-boroPro resulted in elevated levels of intact GLP-1 and GIP, which then enhanced insulin secretion to levels equivalent to those observed in controls, despite lower blood glucose levels. Alternatively, insulin sampling at the 20 min time point of the OGTT may have missed an early peak in insulin levels in mice treated with Pro-boroPro, which may account for the improved glucose tolerance observed. Further, both GLP-1 and GIP are known to exert insulinotropic actions at blood glucose levels greater than 4.5 mM, which were generally maintained throughout the oral glucose tolerance tests in this study (148,149).

Importantly, potentiation of GLP-1 and GIP bioactivity through DPP-IV inhibition did not produce hypoglycemia in this study. Although administration

of exogenous GLP-1 during DPP-IV inhibition in wildtype mice, as well as DPP-IV inhibition alone in GLP-1R-/- mice, resulted in blood glucose levels which dropped below 5 mM at the 30 and 60 min time points of an OGTT, respectively, in both cases the blood glucose levels recovered modestly at the next measurement, suggesting that the insulinotropic actions of GLP-1 and GIP continue to be glucose-dependent despite decreased metabolism of these peptides.

GLP-1 also potently inhibits glucagon secretion (61,84). Thus, increased GLP-1 activity due to DPP-IV inhibition may have additionally contributed to the improved glucose tolerance observed in this study by potentiating the glucagonostatic effect of GLP-1. Although it was impractical to obtain parallel insulin and glucagon concentrations in the present study due to the use of small animal model, a recent investigation reported that anesthetized pigs receiving infusions of glucose and GLP-1 exhibit significantly lower glucagon levels associated with increased levels of intact GLP-1 when a DPP-IV inhibitor is co-administered (147).

The GLP-1R-/- mice provide the opportunity to examine effects of DPP-IV inhibition on potentiation of endogenous GIP activity, independent of GLP-1. Disruption of GLP-1 signaling renders these mice dependent on the incretin activity of GIP. Studies have shown that GLP-1R-/- mice demonstrate increased GIP release in response to oral nutrients compared to wildtype mice (152). Additionally, enhanced GIP-stimulated insulin secretion from the isolated perfused GLP-1R-/- pancreas was observed (152). The present study suggests that DPP-IV inhibition potentiates the action of GIP, resulting in improved glucose homeostasis during an OGTT, despite the absence of potentiated GLP-1 activity. Although GLP-1R-/- mice exhibit increased GIP secretion and pancreatic sensitivity, the substantial improvement in glucose tolerance

observed following enhancement of GIP activity indicates that GIP action is submaximal in the GLP-1R-/- mice. Yet, the mice continue to exhibit glucose intolerance and mild fasting hyperglycemia. This observation supports the contention that intact GLP-1 signaling is essential for proper glucose homeostasis in vivo.

This study suggests that DPP-IV inhibition improves glucose homeostasis through increased levels of exogenous intact GLP-1, as well as endogenous intact GLP-1 and GIP. These results support the use of DPP-IV inhibitors in the treatment of diabetes. However, additional studies are necessary to determine whether the beneficial effects of GLP-1 and GIP potentiation are maintained over a long period. Interestingly, a strain of DPP-IV-deficient rats, which would be expected to have an exaggerated insulin response to oral glucose given the presumed potentiation of incretin activity, exhibit normal glucose tolerance with no enhancement of the insulin response (150). Closer investigation revealed that although the DPP-IV-deficient rats demonstrate decreased secretion of GIP, and additionally, pancreatic desensitization to the effects of GIP, GLP-1 secretion and pancreatic sensitivity appear unimpaired (150). It remains unresolved how normal glucose tolerance is maintained in the face of unaltered GLP-1 activity, combined with its prolonged half-life in DPP-IV-deficient rats.

Although additional studies are necessary to determine further in vivo effects of DPP-IV, this study and others suggests that DPP-IV inhibitors may be useful in the management of diabetes (147,151). As DPP-IV acts on substrates other than the incretin hormones in vivo, it must be ascertained that metabolism of these would not be adversely affected by DPP-IV inhibition. Investigations into both DPP-IV inhibitors and the development of GLP-1 analogues which are

resistant to degradation by this peptidase, suggest that a clinically viable means of potentiating the effects of GLP-1 will soon be available.

Chapter 5 GLP-1R+/- Mice Are Glucose Intolerant

5.1 Introduction

The generation of transgenic mice harbouring a targeted disruption of the GLP-1 receptor gene has permitted analysis of the role of GLP-1 in glucose homeostasis in vivo. GLP-1R-/- mice demonstrate mild fasting hyperglycemia and glucose intolerance following an oral glucose challenge, associated with reduced glucose-stimulated insulin secretion (80). These results are consistent with an essential role for GLP-1 signaling in the regulation of glucose-dependent insulin secretion (80).

In view of the importance of GLP-1 signaling in glucose homeostasis, we hypothesized that mice possessing one wildtype allele and one null allele at the GLP-1 receptor locus (GLP-1R+/- heterogygotes) might exhibit abnormal oral glucose tolerance and glucose-stimulated insulin secretion as a consequence of a postulated reduction in GLP-1 receptor expression.

5.2 Materials and Methods

5.2.1 Oral Glucose Tolerance and Insulin Determinations

12 week-old male wildtype CD1 +/+ (n=6) and GLP-1R+/- mice (n=9-10) were fasted for 16 h and given an OGTT (see section 2.2.3). Blood samples for insulin content measurements were withdrawn from the tail vein during the period between 20 and 30 min following oral glucose loading for insulin quantification using a rat insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

5.2.2 Tissue Collection and RNA Isolation

The mice were euthanized with CO_2, and lung tissue was collected for RNA analysis. Total cellular RNA was isolated by the acid-guanidium thiocyanate method (90).

5.2.3 Northern Blot Analysis

Northern blot analysis was performed as described in 2.2.5.

5.2.4 Statistics

All data are expressed as the mean ± SEM. Statistical significance of data from animals following treatment was assessed by an unpaired t test between wildtype and GLP-1R+/- mice. All statistics were analyzed using the InStat 1.12. program for MacIntosh computers (GraphPad Software).

5.3 Results

To determine whether disruption of one GLP-1 receptor allele was associated with impaired glucose homeostasis, oral glucose tolerance and glucose-stimulated insulin levels were analyzed in GLP-1R+/- heterozygotes. Surprisingly, GLP-1R+/- mice demonstrated significant increases in glycemic excursion following oral glucose challenge ($P<0.05$, +/+ vs. +/- mice at 20 min following glucose administration; Fig. 14.A.). Additionally, the increased glucose levels observed in GLP-1R+/- mice following oral glucose loading were associated with significantly decreased levels of glucose-stimulated insulin secretion ($P<0.05$, +/+ vs. +/- mice; Fig. 14.B.).

The abnormal glucose tolerance and diminished insulin-stimulated insulin secretion observed in GLP-1R+/- mice was suggestive of decreased receptor expression in these mice, with a consequent reduction in receptor

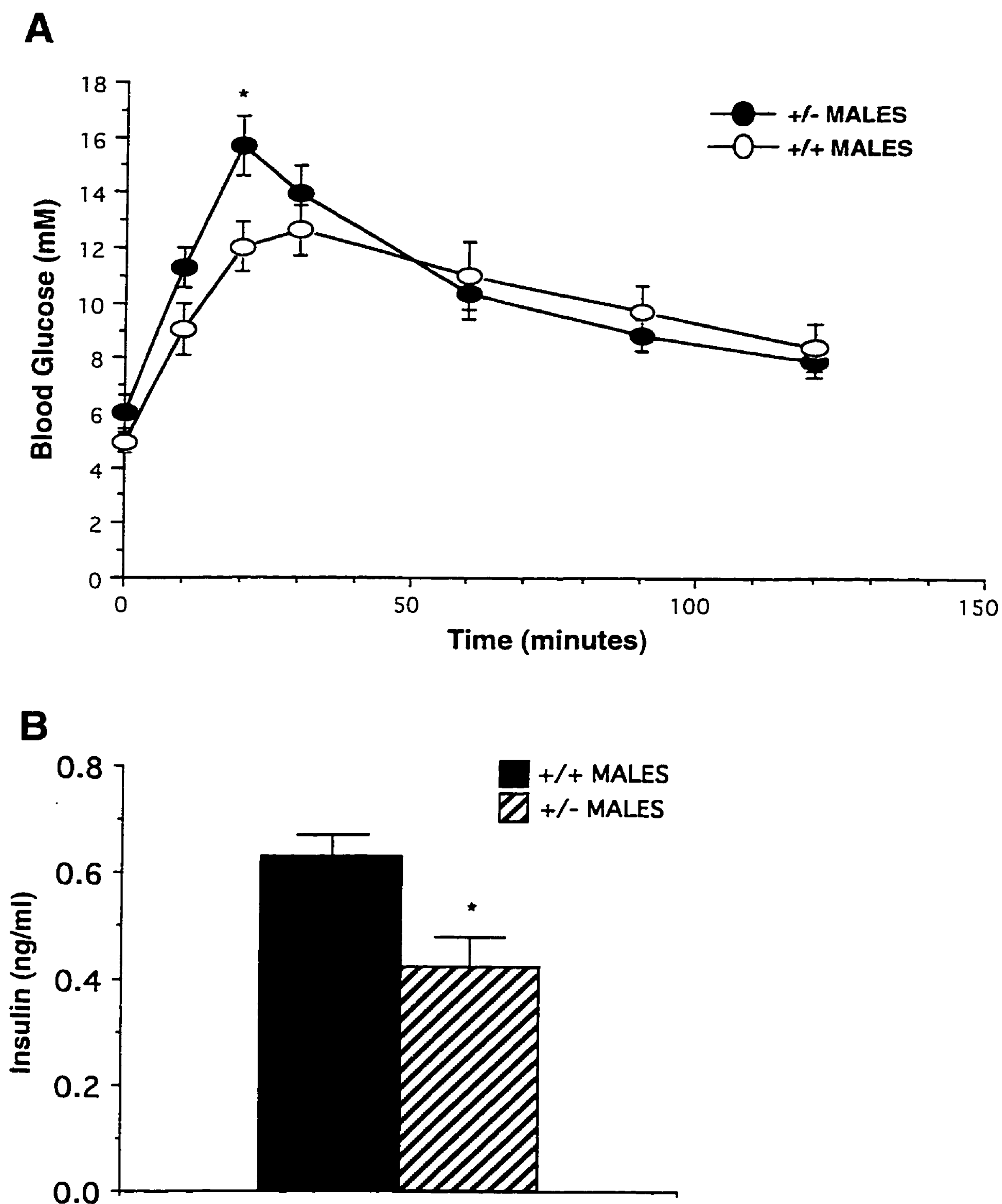


Fig. 14. A. Oral glucose tolerance test in 3 month-old male wildtype CD1 control (n=6) and GLP-1R+/- mice (n=9-10). Values are expressed as means ± S.E.M. *p<0.05, +/+ vs. +/- mice. **B.** Plasma insulin levels of GLP-1R+/- and wildtype mice during the 20-30 minute time period in the oral glucose tolerance test shown in A. *p<0.05, +/+ vs. +/- mice.

Note: The data presented in this figure was previously published as Fig. 1. B. and C. in reference 128.

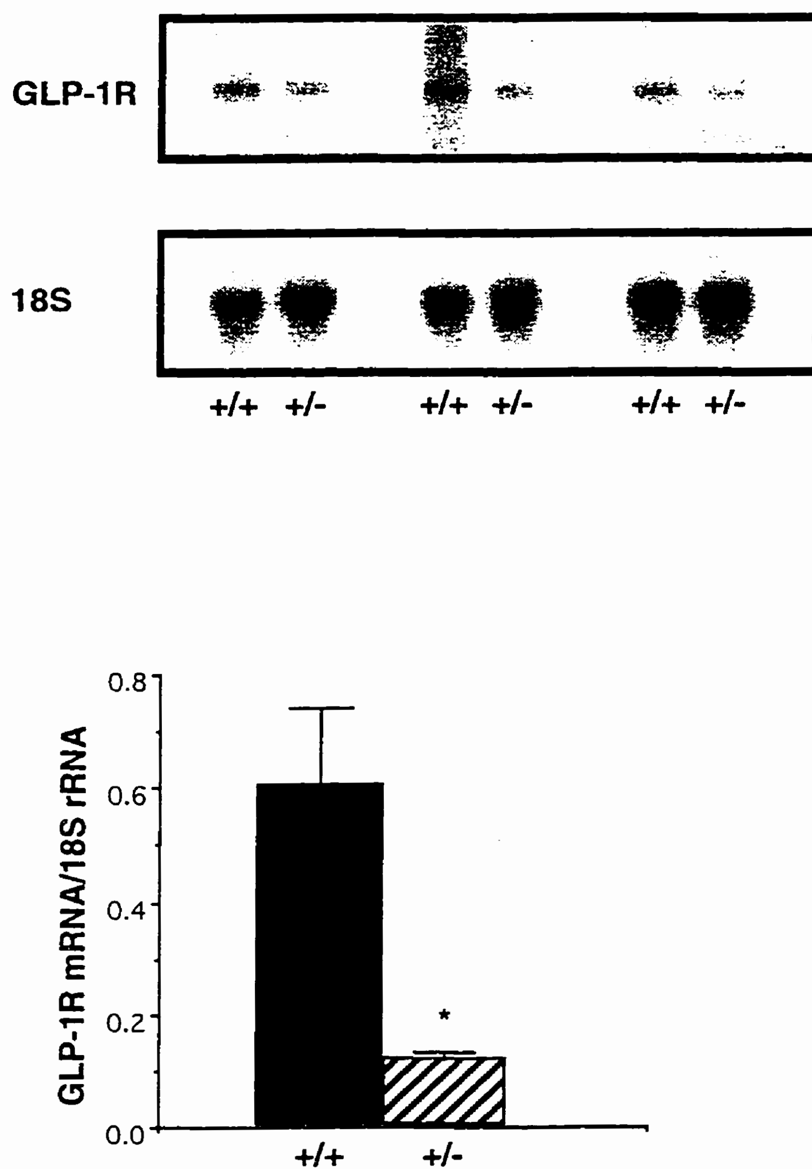


Fig. 15. A. Northern blot analysis of lung tissue RNA from wildtype (+/+) or GLP-1R+/- (+/-) male mice. RNA was hybridized with a rat GLP-1R cDNA followed by hybridization with an 18S rRNA cDNA. **B.** The relative densitometric data represent mean values for GLP-1R mRNA transcripts from +/+ and GLP-1R+/- mice (n=3 per group). *$P<0.05$.

number. Since the GLP-1 receptor is expressed most abundantly in the lung (37), receptor expression was evaluated in this tissue. Northern blot analysis of lung RNA isolated from GLP-1R+/- and wildtype +/+ mice demonstrated that GLP-1R+/- heterozygotes display significant decreases in GLP-1 receptor expression ($P<0.05$, +/+ vs. +/- mice; Fig. 15).

5.4 Discussion

In addition to its role in glucose-stimulated insulin secretion, GLP-1 exerts several actions which contribute to the reduction of blood glucose levels in vivo. These actions include the inhibition of glucagon secretion, delayed gastric emptying, inhibition of food intake, and a postulated stimulatory effect on peripheral glucose uptake and utilization (26). Analysis of the physiological consequences of complete GLP-1 signal disruption in GLP-1R-/- mice supports an essential role for GLP-1 in the regulation of glucose-stimulated insulin secretion (80). Indeed, GLP-1R-/- mice demonstrate deficient insulin secretion in response to an oral glucose challenge despite the demonstration of compensatory increases in GIP secretion and sensitivity (152). GLP-1 signaling also appears to play a central role in proinsulin gene expression and biosynthesis in the ß cell. Northern analysis of pancreatic RNA isolated from fed GLP-1R-/- and wildtype mice revealed a significant decrease in the levels of proinsulin mRNA transcripts in GLP-1R-/- mice (152). In addition to abnormal glycemic excursion and decreased glucose-stimulated insulin following oral glucose loading, GLP-1R-/- mice display fasting hyperglycemia and increased blood glucose levels following intraperitoneal glucose administration (80). The presence of aberrant glucose tolerance in GLP-1R-/- mice regardless of the site of glucose entry implicates a key role for GLP-1 signaling in glucose homeostasis.

While the insulinotropic function of GLP-1 appears crucial for normal glucose homeostasis, additional contributions of GLP-1 to glucose control appear to be well compensated for in the event of disrupted GLP-1 signaling. Although GLP-1 is known to suppress glucagon secretion, fasting and postprandial glucagon levels are not significantly altered in GLP-1R-/- mice, suggesting a redundant role for GLP-1 in control of glucagon secretion (128). Additionally, despite a postulated role for GLP-1 in peripheral glucose disposal, GLP-1R-/- mice exhibit normal whole body glucose utilization under both basal and hyperinsulinemic conditions (128).

Although a role for GLP-1 in determining the glucose-responsiveness of pancreatic ß cells has been suggested, pancreatic ß cells isolated from GLP-1R-/- mice are glucose competent and respond normally to stimulatory levels of glucose (153). Additionally, GLP-1R-/- mice exhibit normal body weight and food intake when compared with age- and sex-matched controls, implying that GLP-1 signaling is not essential for the regulation of satiety and body mass in vivo (26). These observations demonstrate that while GLP-1 is necessary for appropriate levels of pancreatic insulin secretion in vivo, many of the additional physiological roles of GLP-1 are redundant, and thus adequately compensated for in the absence of GLP-1 signaling.

The observation that GLP-1R+/- heterozygous mice exhibit abnormal oral glucose tolerance concomitant with significant reductions in circulating insulin further emphasizes the essential role of GLP-1 signaling in glucose-dependent insulin secretion. The finding of decreased GLP-1 receptor mRNA levels in the lung tissue of GLP-1R+/- mice is consistent with the hypothesis that these mice also exhibit diminished pancreatic GLP-1 receptor expression. These results suggest that even a reduction in GLP-1 signaling renders the ß-cell incapable of normal insulin secretion in response to an oral glucose challenge. It is notable

that other insulin secretagogues, such as glucose and glucose-dependent insulinotropic peptide (GIP), are not entirely able to compensate for even a partial reduction in GLP-1 signaling. Additionally, GLP-1R+/- mice have been reported to show a trend (N.S.) towards fasting hyperglycemia (128), further supporting the existence of a gene dosage effect for GLP-1 signaling in glucose homeostasis in vivo.

Chapter 6 Short-term Leptin Treatment Improves Fasting Insulin Levels in Wildtype and GLP-1R-/- Mice

6.1 Introduction

The lipostatic theory of body weight regulation, introduced more than four decades ago, proposed that adipocytes produce a circulating hormone in proportion to the mass of adipose tissue (154). This factor was postulated to regulate body size via interaction with specific brain areas, inducing subsequent modifications in energy intake and expenditure to maintain energy balance (154). Parabiosis (crosscirculation) experiments, in which the circulatory systems of genetically obese *ob/ob* or *db/db* mice were joined with wildtype mice, thereby permitting exchange of circulating hormones, further supported the hypothesis that a circulating factor plays an important role in the regulation of body weight (155). It was proposed that this substance was absent in *ob/ob* mice, for when an *ob/ob* mouse was joined with a wildtype mouse, the *ob/ob* mouse would lose weight (156). Further, it was speculated that *db/db* mice produce large quantities of the factor but are resistant to its actions, since *db/db* mice failed to lose weight when crosscirculated with wildtype mice, while conversely, wildtype mice in parabiosis with *db/db* mice died of starvation (156).

Attempts to isolate the circulating "satiety" factor were unsuccessful, prompting a molecular search for genetic mutations in *ob/ob* and *db/db* mice. The *ob* gene was identified by positional cloning in 1994 (157) and its encoded protein was named leptin, derived from the Greek *leptos*, meaning thin. Indeed, administration of leptin to *ob/ob* mice reverses the diabetes-obesity syndrome of these mice through decreased food intake and increased energy expenditure,

as well as improvements in glycemia and insulinemia, which occur prior to weight loss (158-160).

Leptin is synthesized and secreted from white adipose tissue (157), and in the fed steady-state, leptin expression and secretion reflect the percentage of body fat in both rodents and humans (161-163), supporting a role for leptin as a measure of energy storage in a feedback loop for body weight regulation. The mechanism by which adipocyte tissue mass is coupled to leptin expression is unknown (164), but circulating leptin concentrations appear to be directly proportional to the amount of leptin mRNA in adipose tissue (164), and no intracellular storage pool for leptin has been found (165). Surprisingly, the correlation of leptin levels with fat mass is drastically altered with changes in energy balance. During food deprivation, leptin gene expression and circulating levels fall rapidly and disproportionately to the loss in adipocyte stores (166-168). Additionally, leptin expression and serum levels increase upon overfeeding in both humans and rodents (169,170), but unlike rodents (170,171), leptin levels in humans do not appear to change acutely following normal meal consumption (162). Thus it appears that leptin expression and serum levels reflect both whole body adiposity and energy balance.

Leptin expression and serum levels are also responsive to hormonal influences. Insulin injection has been shown to increase *ob* mRNA expression in rodents (171), and streptozotocin-induced diabetes produces a drastic reduction in leptin mRNA (172). Furthermore, insulin has been shown to stimulate leptin mRNA expression in isolated rat adipocytes (173). It is not clear whether insulin exerts direct effects on leptin gene expression in rodents, or whether the changes are secondary to altered lipid metabolism (164). In contrast to rodents, insulin appears to have chronic rather than acute effects on leptin expression and secretion in human tissue, both *in vivo* and *in vitro*.

Hyperglycemic/hyperinsulinemic clamp studies in humans showed a significant increase in circulating leptin concentrations only after the clamp had been applied for 3 days (174). Similarly, cultured human adipocytes required 72 h of incubation with insulin before increased *ob* mRNA expression was evident (174).

Subsequent to expression cloning of the leptin receptor (175), it was determined that the *db* gene encodes the leptin receptor, and furthermore, a mutation was identified in the leptin receptor gene of *db/db* mice (176). At least five isoforms of the leptin receptor are generated from multiple splice sites in a single leptin receptor gene (177). The longest leptin receptor isoform possesses an intracellular domain with homology to the signaling domain of the type I cytokine receptor family (177). This long receptor isoform transmits the leptin signal via janus kinase 2 (178) to signal transducers and activators of transcription (STAT) proteins, which then translocate to the nucleus and induce changes in gene transcription (179).

The mRNA encoding the long leptin receptor isoform is most strongly expressed in the hypothalamus (arcuate, lateral, ventromedial, and dorsomedial nuclei), and has also been detected by RNA blot hybridization in liver, kidney, pancreatic islets, spleen and heart (180). Intracerebroventricular administration of leptin produces a more potent response than peripheral leptin administration, suggesting that the central nervous system is an important site of action of leptin (181). Leptin has been shown to induce *c-fos* activity in specific hypothalamic and brain stem regions (182). Neuropeptide Y (NPY) has also emerged as a target of leptin action. NPY is a potent stimulator of food intake and has been shown to increase plasma insulin levels (183). Importantly, *ob/ob* mice exhibit elevated hypothalamic NPY expression, which decreases following leptin administration, prior to any change in body weight (184,185). However,

studies in which NPY was knocked out in *ob/ob* mice demonstrated that while the obesity phenotype of the *ob/ob* mice is improved in the absence of NPY, it is not completely normalized (186). These results suggest that leptin possesses additional neuroendocrine targets.

Notably, *db/db* mice possess a missense mutation which results in defective mRNA splicing, preventing expression of only the long form of the receptor (176,177). Although the shorter isoforms are present wherever the long form is normally expressed, the massive obesity and leptin resistance of db/db mice indicate that the long receptor isoform is essential for appropriate leptin signaling in vivo (176,177). In agreement with this hypothesis, the short forms of the leptin receptor have not been shown to activate any of the STAT proteins (187,188). The short form splice variants of the leptin receptor are abundant in the choroid plexus of the brain, where they are postulated to facilitate uptake of leptin across the blood-brain barrier (189). Further, the short leptin receptor isoforms are more widely expressed in peripheral tissues than the long isoform, and may have a role in mediating leptin clearance in lung and renal tissue (190).

There is mounting evidence that leptin may also exert direct peripheral actions. Several studies support a role for leptin in the regulation of pancreatic insulin secretion. When leptin is administered to *ob/ob* mice, both hyperinsulinemia and hyperglycemia improve prior to changes in body weight (158-160). Further, pair-feeding studies have demonstrated that leptin administration improves insulin and blood glucose levels to a greater extent than can be explained by weight loss alone (191), suggesting leptin directly inhibits insulin secretion. Recent studies have shown that the long form of leptin receptor mRNA is expressed in pancreatic ß cells (180,192), and leptin has been shown to decrease glucose-stimulated insulin secretion from isolated

ob/ob islets (193). In view of the proposed effects of insulin on leptin expression and secretion, these data suggest a complex linkage between the endocrine pancreas and adipose tissue.

In contrast to in vivo studies demonstrating that leptin improves glucose homeostasis and insulin resistance in *ob/ob* mice (158,160), in vitro data favour a role for leptin in peripheral insulin resistance. Leptin treatment of cultured hepatoma cells, rat fibroblasts, and primary adipocytes interfered with the insulin signaling pathway (194-196). Whether leptin exerts direct effects on glucose homeostasis in normal mice is unknown because treatment of wildtype mice with leptin produced no major changes in glucose or insulin levels (160). In view of the finding that leptin inhibits insulin secretion (180), these observations suggest that leptin may adversely affect glucose homeostasis in vivo.

To further define the role of leptin at the level of the pancreatic ß cell and in glucose homeostasis, this study investigated the effects of short-term administration of recombinant mouse leptin on insulin gene expression, plasma insulin levels, and glucose homeostasis in vivo, in normal and GLP-1R-/- mice. Further, the effects of leptin on insulin gene expression in the INS-1 insulin-secreting cell line were examined.

6.2 Materials and Methods

6.2.1 Short-term Leptin Treatment

6.2.1.1 Oral Glucose Tolerance Tests and Insulin Measurements

12 week-old male wildtype CD1 +/+ (n=11 per treatment group) and GLP-1R-/- mice (n=9-11 per group) were administered 3 intraperitoneal injections of 2 ug leptin (Amgen, Thousand Oaks, CA) or saline per g body weight every 12 h for a period of 24 h. The first injection was administered at

10:00 a.m., and the mice were fasted overnight subsequent to the second injection at 10:00 p.m. The final injection was given at 10:00 a.m. the following morning, and an oral glucose tolerance test was initiated one hour later. Blood samples for insulin measurements were withdrawn from the tail vein during the period between 20-30 min following oral glucose load for insulin quantification using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

6.2.1.2 Fasting Glucose and Insulin Measurements

16 week-old male wildtype (n=5 per treatment group) and GLP-1R-/- mice (n=5 per treatment group) received 3 intraperitoneal injections of 2 ug leptin/g body weight or saline every 12 h for a period of 24 h, as above. The mice were fasted overnight following the second injection, and 1 h subsequent to the final injection, the fasting blood glucose levels were measured using the One Touch Basic Glucometer (Lifescan Ltd., Canada). The mice were then anesthetized with CO_2 and exsanguinated by cardiac puncture. Venous blood (800 ul) was collected into 80 ul of Trasylol:EDTA:Diprotin A (5000 KIU/ml:32mM:0.1nM), and the plasma was separated by centrifugation and stored at -20°C until assayed for insulin content using an insulin radioimmunoassay kit (Linco Research Laboratories, St. Charles, Missouri) with rat insulin as a standard.

6.2.2 Tissue Collection and RNA Isolation

Subsequent to the OGTT or fasting blood glucose determinations, mice were euthanized with CO_2 and the pancreas was collected for RNA analysis. Total pancreatic RNA was isolated using the acid guanidium thiocyanate method (90).

6.2.3 In Vitro Analysis of the Effects of Leptin on Insulin Gene Expression

6.2.3.1 INS-1 Cells

INS-1 cells, derived from a rat insulinoma, were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, 10 mM Hepes, 2 mM glutamine, 1mM sodium pyruvate, 50 uM ß-mercaptoethanol, 100 U/ml penicillin G sodium, and 100 ug/ml streptomycin sulfate (Gibco BRL, NY). The cells were grown to approximately 60-70% confluency and then washed with phosphate-buffered saline and incubated in low glucose DMEM, supplemented as above, for 24 h. 100 ng leptin/ml (n=3) or 1000 ng leptin/ml (n=3), or buffer alone (n=3), was then added to the culture medium for an incubation period of 24 h. RNA was subsequently prepared by the acid-guanidium thiocyanate method (90).

6.2.4 Northern Blot Analysis

Northern blot analysis was performed as described in 2.2.5.

6.2.5 Statistics

All data are expressed as the mean ± SEM. Statistical significance of data from animals following treatment was assessed by an unpaired t test between mice given leptin injections and controls. All statistics were analyzed using the InStat 1.12 program for MacIntosh computers (GraphPad Software).

6.3 Results

Previous studies demonstrated that leptin treatment of ob/ob mice is associated with reduced serum insulin and glucose levels, suggesting improved insulin sensitivity (158-160). However, in vitro studies with hepatoma

cell lines suggested that leptin attentuates insulin action (194), and further, leptin has been shown to exert direct inhibitory actions on insulin secretion (193). Chronic leptin treatment of GLP-1R-/-, but not wildtype mice, produces improved oral glucose tolerance compared to pair-fed controls, despite significant reductions in glucose-stimulated insulin secretion (197). The absence of GLP-1-mediated stimulation of insulin secretion and insulin gene expression may render GLP-1R-/- mice more sensitive to the inhibitory actions of leptin on insulin secretion, and potentially, insulin gene expression. Therefore, this study assessed the effects of short-term leptin treatment on oral glucose tolerance, glucose-stimulated insulin secretion, and insulin gene expression in age- and sex-matched wildtype and GLP-1R-/- mice. Leptin-treated GLP-1R-/- mice demonstrated significantly decreased glucose levels at the 10 min time point of an oral glucose tolerance test ($P<0.05$, Fig.16.A.), whereas leptin-treated wildtype mice did not exhibit any significant changes in glycemia compared to saline-treated controls during an OGTT (Fig. 17.A.). No changes in body weight occurred following 24 h of leptin administration in wildtype or GLP-1R-/- mice (data not shown).

To ascertain whether short-term leptin treatment altered the insulin response to oral glucose challenge, glucose-stimulated insulin levels were analyzed by radioimmunoassay. Neither leptin-treated GLP-1R-/- ($P=0.8208$, leptin-treated vs. controls; Fig. 16.B.) nor wildtype mice ($P=0.2938$, leptin-treated vs. control; Fig.17.B.) exhibited changes in glucose-stimulated insulin secretion during an oral glucose tolerance test. Further, since insulin has been shown to enhance leptin gene expression, this study assessed whether leptin inhibits ß cell function at the level of insulin gene expression. Northern blot analysis of total pancreatic RNA isolated from GLP-1R-/- and wildtype mice following short-term leptin treatment and oral glucose challenge did not

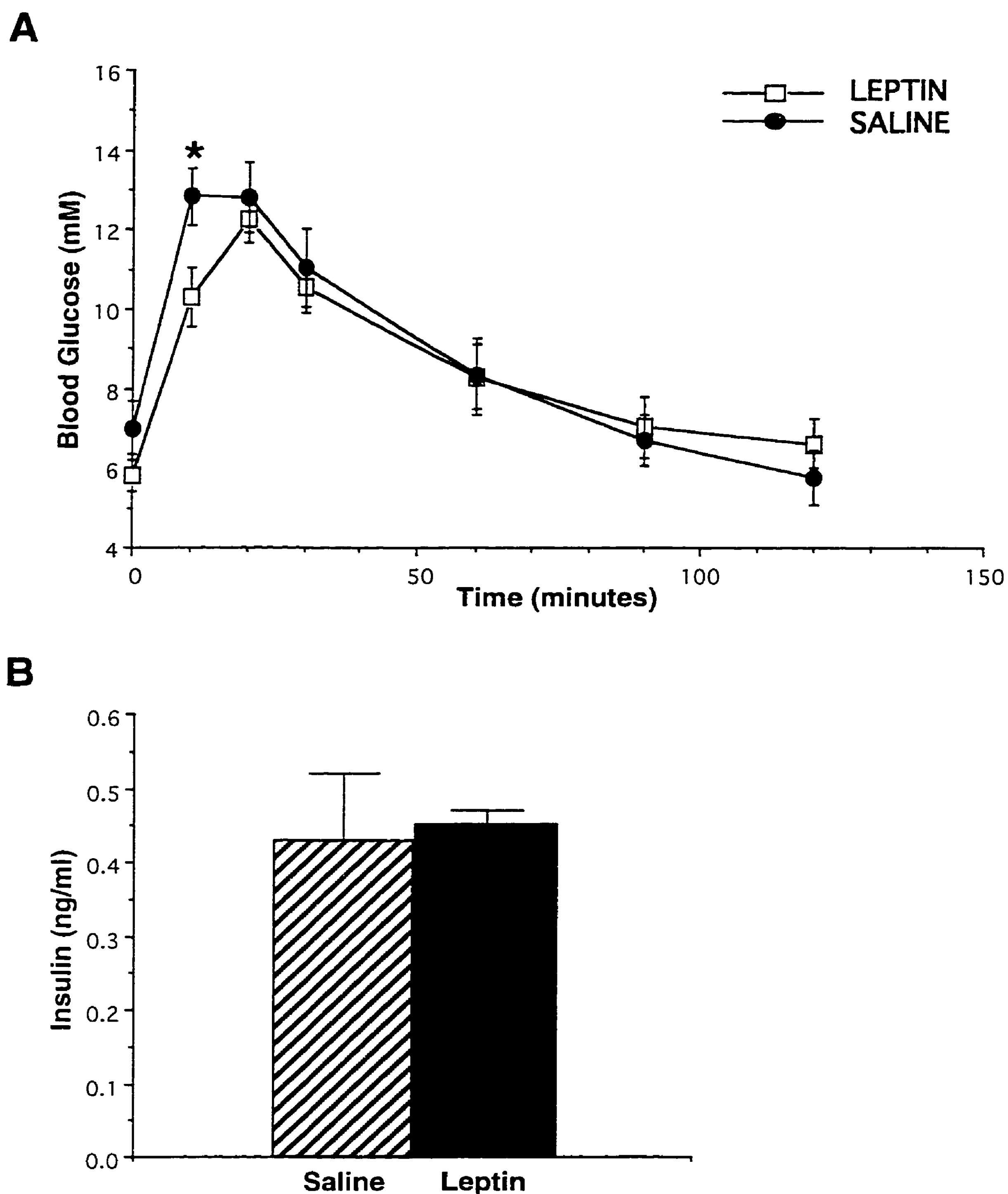


Fig. 16. A. Oral glucose tolerance test in GLP-1R-/- mice following short-term leptin treatment. 12 week-old male GLP-1R-/- mice were administered 3 injections of 2ug leptin per g body weight, or saline, in 24 h (n=9-11 per group) prior to an OGTT. *P<0.05. **B.** Plasma insulin concentrations during the 20-30 min time period of the oral glucose tolerance test shown in 16. A.

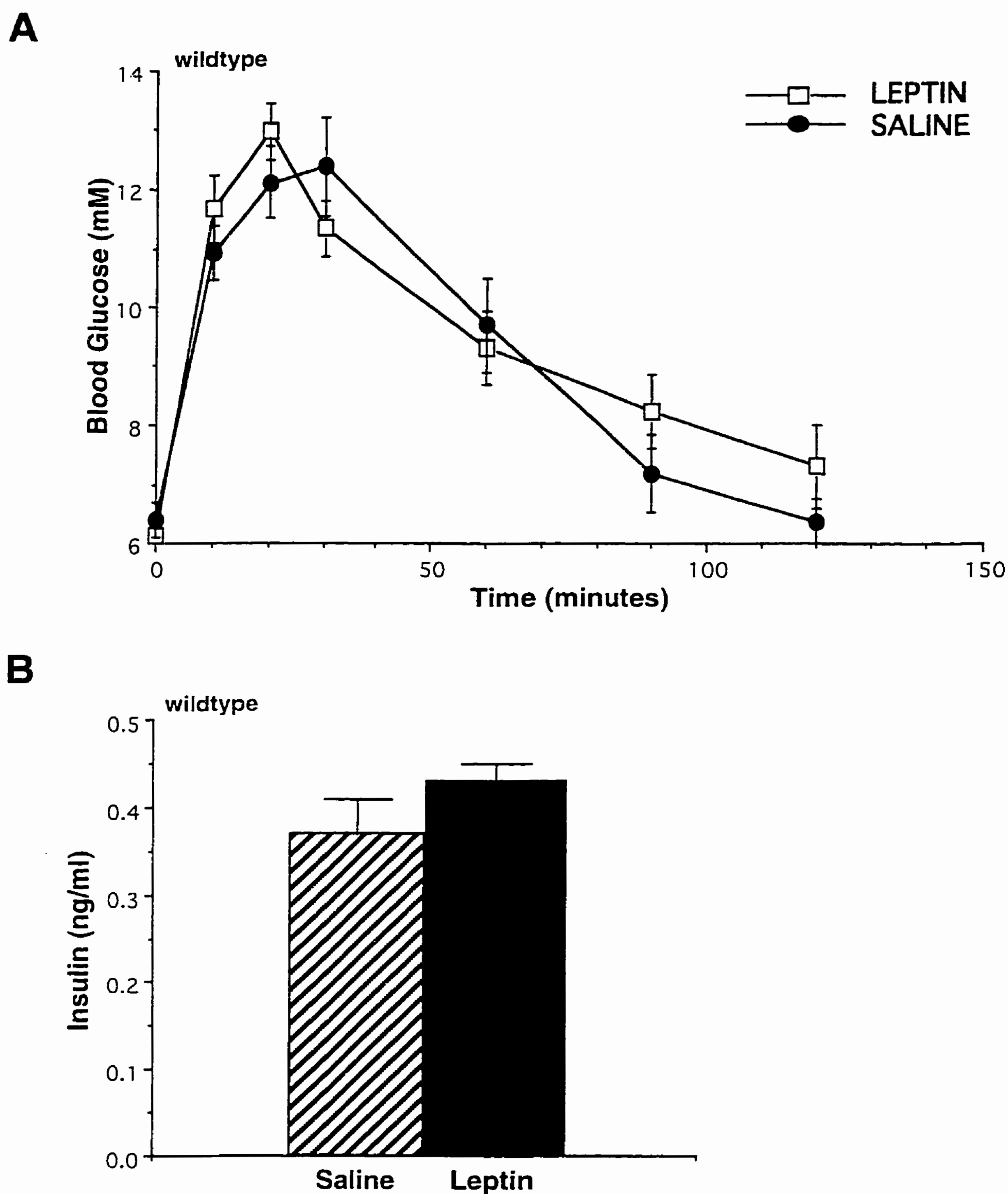
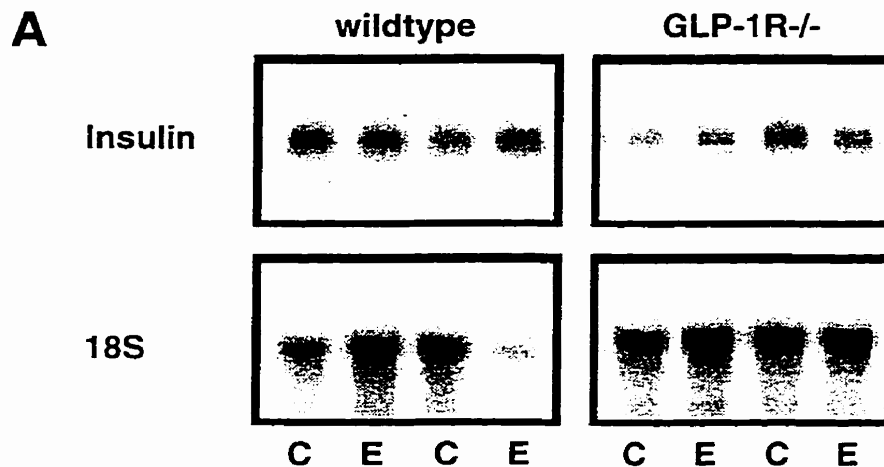


Fig. 17. A. Oral glucose tolerance test in wildtype CD1 +/+ mice following short-term leptin treatment. 12 week-old male wildtype mice were administered 3 injections of 2ug leptin per g body weight, or saline, in 24 h (n=11 per group) prior to an OGTT. **B.** Plasma insulin concentrations during the 20-30 min time period of the oral glucose tolerance test shown in 17. A.

demonstrate any alterations in insulin gene expression in leptin-treated wildtype or GLP-1R-/- mice compared to saline-treated controls (P=0.8480 and P=0.6701, respectively; Fig.18).

In view of a recent report that leptin exerts more potent inhibitory effects on insulin secretion at low (5.5 mM) compared to high glucose concentrations (11 mM) (193), this study evaluated whether short-term leptin treatment alters fasting blood glucose and insulin levels in age- and sex-matched GLP-1R-/- and wildtype mice. Short-term leptin administration produced no significant changes in fasting blood glucose levels of leptin-treated GLP-1R-/- or wildtype mice compared to controls (P=0.3281 and P=0.7151, GLP-1R-/- and wildtype mice, respectively; Fig. 19.A.). However, both GLP-1R-/- and wildtype mice exhibited significant reductions in fasting insulin levels following short-term leptin treatment (P<0.01 for leptin-treated GLP-1R-/- and wildtype mice compared to saline-treated controls; Fig. 19.B.). Northern blot analysis of total pancreatic RNA did not reveal any significant changes in fasting insulin mRNA levels in wildtype or GLP-1R-/- mice following short-term leptin administration (P=0.4652 and P=0.9616, for wildtype and GLP-1R-/- mice, respectively; Fig. 20).

To investigate whether leptin has a direct effect on insulin expression, cultured INS-1 insulinoma cells were studied. Incubation of cells with 100 or 1000 ng recombinant mouse leptin per ml of culture medium for 24 h resulted in a small but significant increase in the levels of insulin mRNA transcripts compared with untreated controls, as assessed by Northern blot hybridization (P<0.05, 100 or 1000 ng/ml leptin vs. controls; Fig. 21).



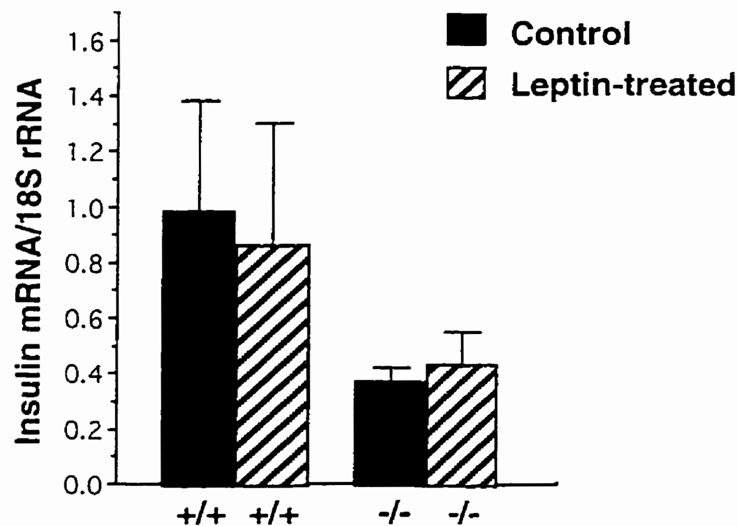


Fig. 18. A. Northern blot analysis of total pancreatic RNA from wildtype (+/+) and GLP-1R-/- (-/-) mice following short-term (24 h) leptin treatment and oral glucose challenge. RNA was hybridized with a rat insulin cDNA followed by hybridization with a rat 18S rRNA cDNA. C=control (saline) injection. E=experimental (leptin) injection. **B.** The relative densitometric data represent mean values for insulin mRNA transcripts from +/+ and -/- mice treated with leptin or controls (n=3 per group).

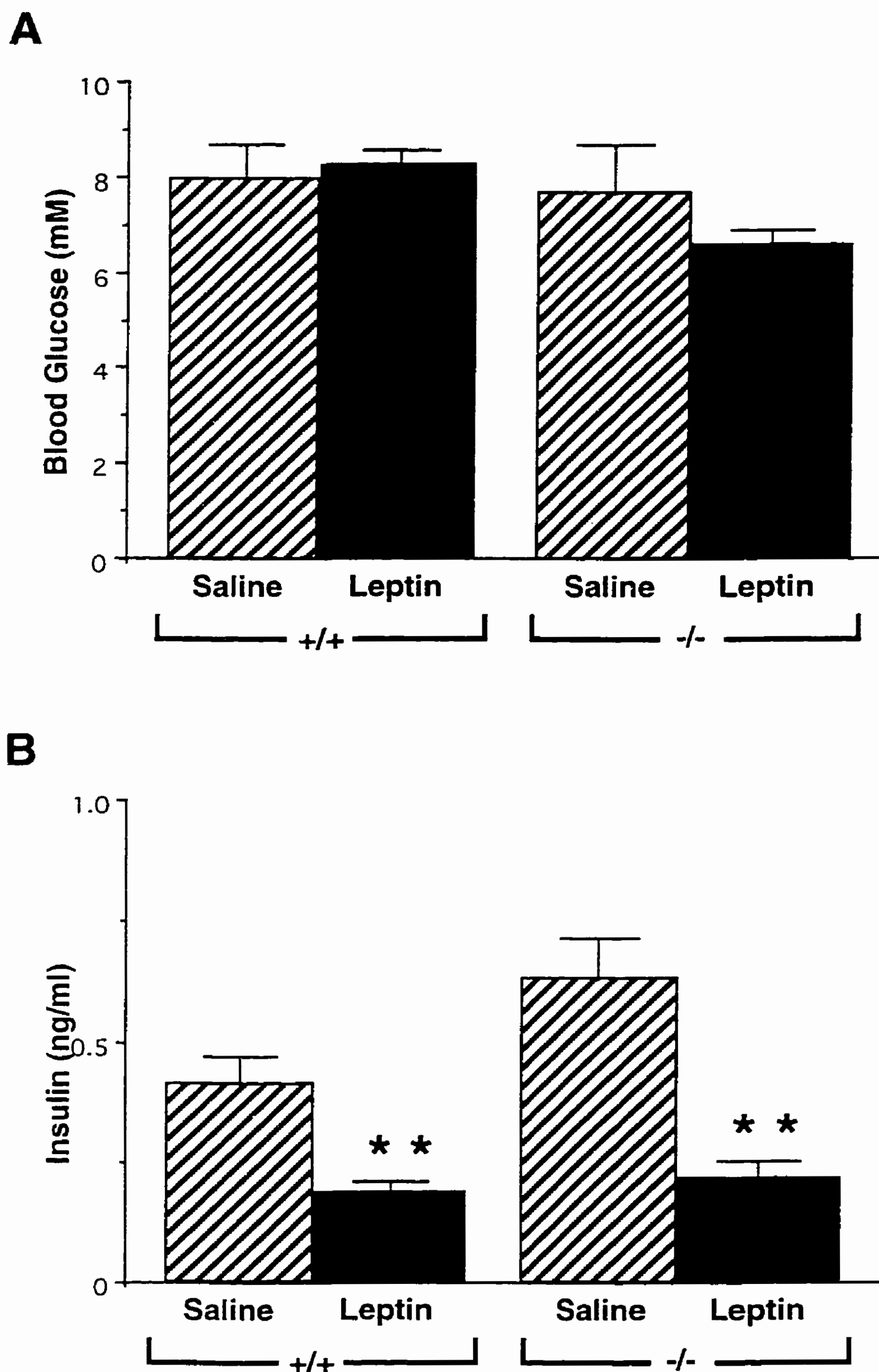


Fig. 19. A. Fasting glucose levels in wildtype and GLP-1R-/- mice following short-term leptin treatment. 16 week-old male wildtype (+/+) and GLP-1R-/- (-/-) mice were given 3 injections of 2 ug leptin per g body weight, or saline, in 24 h (n=4-5 per treatment group) prior to measurement of fasting glucose levels. **B.** Fasting plasma insulin concentrations concomitant with the fasting glucose concentrations shown in 19. A. **P<0.01.

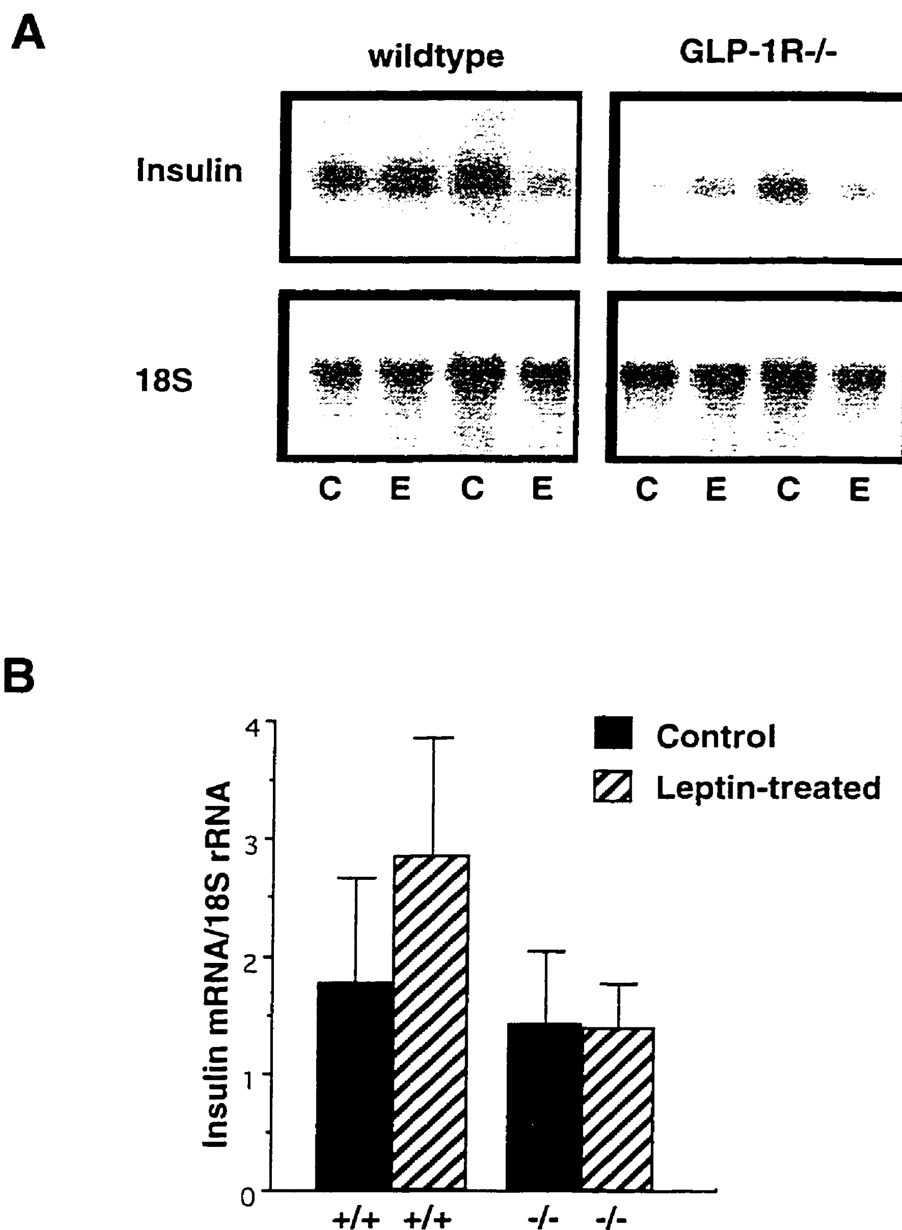
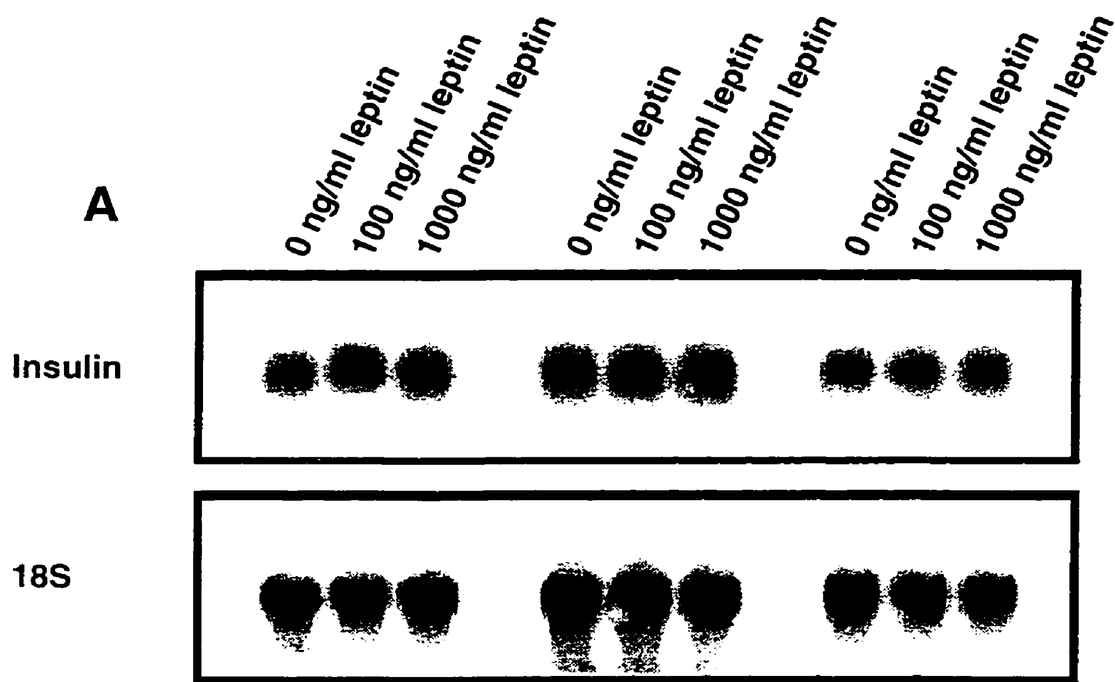


Fig. 20. A. Northern blot analysis of total pancreatic RNA from fasted wildtype (+/+) and GLP-1R-/- (-/-) mice following short-term (24 h) leptin treatment. RNA was hybridized with a rat insulin cDNA followed by hybridization with a rat 18S rRNA cDNA. C=control (saline) injection. E=experimental (leptin) injection. **B.** The relative densitometric data represent mean values for insulin mRNA transcripts from +/+ and -/- mice treated with leptin or controls (n=3 per group).



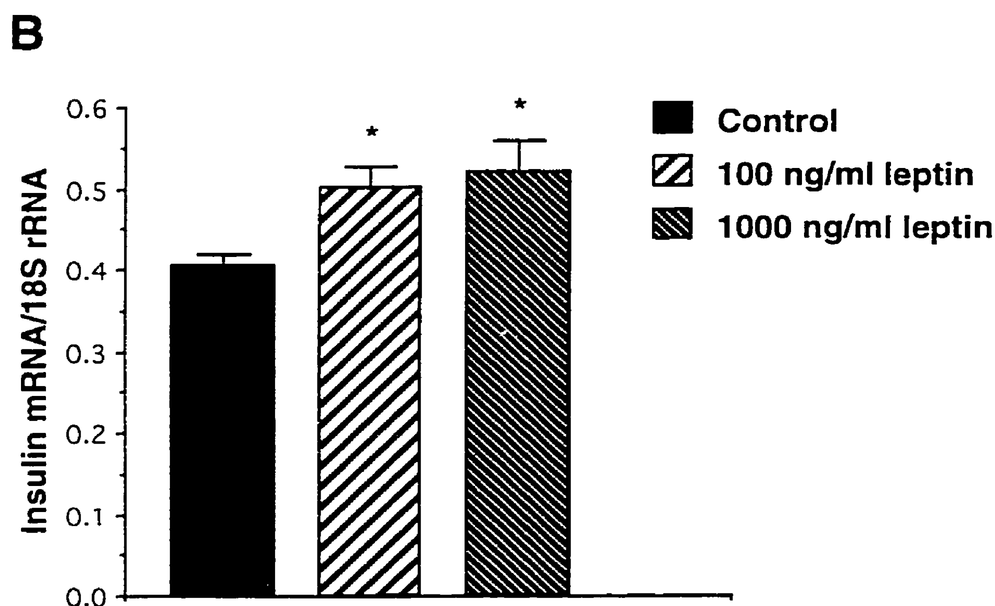


Fig. 21. A. Northern blot analysis of RNA isolated from INS-1 insulinoma cells following incubation with 0, 100, or 1000 ng recombinant murine leptin per ml culture medium for 24 h. RNA was hybridized with a rat insulin cDNA followed by hybridization with a rat 18S rRNA cDNA. **B.** The relative densitometric data represent mean values for insulin mRNA transcripts (n=3). *$P<0.05$.

6.4 Discussion

Several lines of evidence support a role for leptin in the regulation of pancreatic insulin secretion. It was reported more than two decades ago that islets isolated from *db/db* mice are partially depolarized in the absence of glucose stimulation, resulting in elevated basal insulin release (198). Further, one of the earliest detectable abnormalities in *ob/ob* and *db/db* mice is hyperinsulinemia (199). Initial studies demonstrated that daily intraperitoneal injections of recombinant leptin produced significant reductions in blood glucose and serum insulin levels in ob/ob but not lean mice (160), however, it was not clear whether insulin levels decreased secondary to reduced food intake and adiposity or via a direct effect of leptin on the pancreas. Subsequent experiments using pair-fed mice demonstrated that leptin administration produces striking reductions in plasma insulin concentrations which cannot entirely be accounted for by reductions in food intake and body weight (160,184,200), suggesting that leptin directly regulates insulin secretion from pancreatic islets.

Expression of the full-length leptin receptor has been detected in the pancreatic islets of *ob/ob* and lean mice (180), and perfused pancreas studies have shown that leptin inhibits basal insulin secretion in hyperinsulinemic *ob/ob* mice (180). Further, at doses greater than 10 nM, leptin reduces glucose-stimulated (16.7 mM glucose) insulin secretion from isolated *ob/ob* and normal islets, but not from islets isolated from *db/db* mice, which lack functional leptin receptors (180). Taken together, these results suggest that leptin influences both basal and glucose-stimulated insulin secretion via interaction with functional leptin receptors expressed in pancreatic islets.

The long form of the leptin receptor mRNA is reportedly expressed in tumour-derived cell lines representative of pancreatic ß cells (193,201).

Further, fluorescence studies indicated that leptin receptors were expressed on cells which were immunoreactive for insulin (ß cells) (193). Experiments in the isolated normal rat pancreas support earlier reports in the isolated *ob/ob* pancreas that leptin inhibits basal (2.8 mM) and glucose-stimulated insulin secretion (10 mM) (201). However, there is disagreement in the literature regarding the minimum dose of leptin necessary to inhibit insulin secretion in the perfused rat pancreas (201,202).

Interestingly, a recent report found an inhibitory effect of leptin (6 nM) on insulin secretion from isolated *ob/ob* islets which was decreased by increasing the glucose concentration from 5.5 mM to 11.0 mM glucose (193). Conversely, other studies have failed to show a reduction in the inhibitory action of leptin on insulin secretion at high glucose levels in the isolated perfused normal rat pancreas (201). It has also been reported that leptin (10 and 100 nM) inhibits insulin secretion from isolated human islets at 20 mM glucose (203), but not at lower glucose concentrations. Similarly, leptin (1 and 10 nM) inhibited insulin secretion from isolated rat islets at 16 mM, but not lower, glucose concentrations (204). The present study in mice demonstrated that short-term leptin administration in vivo is associated with significant reductions in serum insulin levels in the fasting (low glucose) but not the fed (high glucose) state, supporting previous in vitro experiments in which leptin inhibited insulin secretion from isolated *ob/ob* islets at low but not high glucose concentrations (193).

The results of a previous study demonstrated that chronic leptin treatment of wildtype and GLP-1R-/- mice for 13-20 days reduced the glucose excursion following an oral glucose challenge compared to saline-treated controls (197). However, GLP-1R-/- mice exhibited significant improvements in oral glucose tolerance and reductions in serum insulin levels compared to pair-fed controls,

whereas wildtype mice did not (197). In the present investigation, improved glycemic excursions following an oral glucose challenge was observed in short-term leptin-treated GLP-1R-/-, but not wildtype, mice. These results support the previous study which suggested that GLP-1R-/- mice may display altered sensitivity to leptin actions in vivo (197).

The effect of leptin on insulin action is not clear, and many in vivo and in vitro experiments have reported conflicting results. Despite the beneficial effects of leptin administration on glucose and insulin levels in *ob/ob* mice (158,160) which imply improved insulin sensitivity, recent studies have implicated a role for leptin in peripheral insulin resistance. High serum leptin levels have been reported to be associated with insulin resistance independent of body fat (205), and obese individuals demonstrate elevated serum leptin levels in association with increased blood glucose levels (161,162,206). Reports that short-term exposure of liver cell lines to leptin attenuates insulin action (194) further suggest that leptin may directly contribute to the deteriorated glucose homeostasis observed in obese individuals with NIDDM.

In the present study, the significant reduction in blood glucose levels observed at the 10 min time point of an OGTT in leptin-treated GLP-1R-/- mice, despite unchanged serum insulin levels at 20 min, is suggestive of enhanced insulin sensitivity. The absence of a similar effect of leptin on oral glucose tolerance in wildtype mice suggests that GLP-1R-/- mice may display increased sensitivity to potential insulin-sensitizing peripheral effects of leptin. Furthermore, the maintenance of normal fasting blood glucose levels in short-term leptin-treated GLP-1R-/- and wildtype mice, in the face of decreased fasting serum insulin levels, also suggests that leptin may improve insulin actions in vivo, independent of its effects on weight loss. In support of these observations, leptin has been shown to enhance insulin sensitivity in rats during a

hyperglycemic clamp (207) and during hyperleptinemia resulting from gene therapy (208).

It is becoming increasingly apparent that acute (hours) versus chronic (days) leptin administration produces very different results *in vivo* as assessed by serum glucose and insulin concentrations. In a recent report, acute leptin treatment of lean mice was found to inhibit whole body glucose uptake, while chronic subcutaneous leptin infusion resulted in normal glucose clearance despite the inhibition of glucose-stimulated insulin secretion (209). Further, within minutes following administration of leptin to *ob/ob* mice, insulin secretion is suppressed and plasma glucose levels double (210). However, chronic leptin treatment of *ob/ob* mice decreased food intake, body weight, and peripheral insulin resistance (158,160). Thus, leptin appears to acutely inhibit insulin secretion, resulting in deteriorated glycemic control due to suppressed insulin levels, while chronic leptin treatment may facilitate the development of peripheral changes in insulin sensitivity, resulting in decreased or normal glucose levels in the face of diminished insulin levels. The observation of a delayed improvement in glucose clearance during a glucose clamp and concomitant leptin infusion in normal rats is also consistent with a chronic role for leptin in the improvement of insulin sensitivity (207). The maintenance of normal fasting glucose levels in the present investigation, despite decreased fasting insulin levels, in wildtype and GLP-1R-/- mice treated with exogenous leptin for a period of 24 h is also suggestive of leptin-induced improvements in insulin sensitivity.

Several studies have reported that leptin suppresses insulin gene expression in cultured insulinoma cells and isolated rat islets (204,210). In this study, INS-1 insulinoma cells incubated with leptin exhibited slightly elevated insulin mRNA levels compared to controls. However, a potential caveat of

studies utilizing cultured insulinoma cells is that the leptin receptor subtypes present in these cell models may not represent those found in primary ß cells. Furthermore, INS-1 cells were cultured in medium supplemented with fetal calf serum (FCS) in this study. It has been reported that leptin is present in FCS (211), and additionally, a large proportion of plasma leptin has been shown to be bound to serum proteins (212). Thus, the effects of exogenous leptin may be diminished in the presence of FCS due to downregulation of the receptor in culture conditions or poor activity of exogenous leptin due to binding of serum proteins (180,210). Additionally, the present study did not support a role for leptin in the inhibition of insulin expression *in vivo*. However, a subtle role for leptin in the control of insulin expression *in vivo* cannot be discounted because insulin expression *in vivo* is regulated by complex feedback systems which are generally absent *in vitro*.

Through stimulation of lipogenesis (210), insulin contributes to the regulation of fat mass, and thus to plasma leptin concentrations. Insulin is also known to enhance leptin synthesis and secretion (171,213). Experimental evidence now supports a role for leptin in the inhibition of insulin secretion and insulin gene expression (193,204,210), thus establishing an adipoinsular feedback loop. *In vitro*, and acute *in vivo*, leptin studies suggest that the elevated leptin levels observed in human obesity may precipitate diabetes by increasing peripheral insulin resistance and inhibiting insulin secretion (193,194,210). Conversely, as shown in this study, chronic *in vivo* leptin administration in rodents has been shown to decrease plasma insulin levels in association with normoglycemia or hypoglycemia, suggesting that leptin enhances insulin sensitivity (207-209). However, in view of the increased circulating leptin levels observed in obese (and often diabetic) humans

(162,206), the role of leptin in the development of human NIDDM remains to be elucidated.

Chapter 7 GLP-1R-/- Mice Exhibit Altered Behaviour and a Diminished Ability to Adapt to Water Deprivation

7.1 Introduction

The neuropeptide concept initially included only the small group of hypothalamic peptides which regulate the release of pituitary hormones. However, it is now accepted that neuropeptides constitute a large number of highly potent transmitters expressed in all regions of the central and peripheral nervous systems (214). The discovery of gastrin- or cholecystokinin-like immunoreactivity represented the first evidence for the existence of gastrointestinal peptide hormones in the brain (215). Hormones which are also synthesized in the endocrine cells of the digestive tract now constitute one of the largest groups of neuropeptides. Colocalization of GLP-1 and its receptor in the same brain regions (216), and the findings that GLP-1 is released in a calcium-dependent manner from hypothalamic tissue slices, and is present in the synaptosome fraction of the hypothalamus (217), suggests that GLP-1 may function as a neuromodulator or neurotransmitter within the central nervous system.

A central effect of GLP-1 was demonstrated when intracerebroventricular (ICV) injection of GLP-1 was shown to potently inhibit feeding in fasted rats (72). Cell bodies expressing preproglucagon mRNA, and that produce fully processed GLP-1 (73), are restricted to the nucleus of the solitary tract (73), in the caudal brainstem, and the hypothalamus (10). Conversely, GLP-1 receptor mRNA has been detected in numerous brain regions, including the telencephalon, diencephalon, brain stem, and spinal cord (74-76). While the role of GLP-1 in the regulation of neuronal physiology has not yet been clarified, the widespread distribution of GLP-1 receptor expression in the brain suggests

that GLP-1 functions not only as a satiety factor, but may possess additional actions in distinct areas of the CNS.

Ingestive behaviour is thought to be largely controlled by central hypothalamic neuroendocrine neurons. Thus, it is notable that GLP-1R mRNA is expressed in the paraventricular, supraoptic, arcuate, and dorsomedial nuclei of the hypothalamus (218). A role for the arcuate and paraventricular nuclei in the control of body weight and feeding is well-established. The arcuate nucleus contains neurons which synthesize the powerful stimulant of food intake neuropeptide Y (NPY) and project to the paraventricular nucleus (219). ICV administration of GLP-1 prior to NPY greatly reduces NPY-stimulated food intake (72). Additionally, ICV injection of the GLP-1 receptor antagonist exendin (9-39) before NPY administration has been shown to increase food intake relative to NPY administration alone (72). Notably, ICV leptin administration, which potently inhibits food intake, is associated with decreased levels of NPY mRNA (181). Although GLP-1 does not appear to decrease NPY gene expression in the arcuate nucleus (72), GLP-1 administration stimulates the expression of the immediate early gene *c-fos*, a marker of functionally activated neurons, in the arcuate nucleus (220). This effect is blocked by prior administration of exendin (9-39). These results suggest that GLP-1 may have a role in the regulation of NPY release from nerve terminals in the paraventricular nucleus (72).

Intracerebroventricular administration of GLP-1 has also been shown to induce c-fos expression in parvicellular corticotropin releasing factor (CRF)-containing neurons of the paraventricular nucleus (72,220). Furthermore, GLP-1R mRNA is reportedly expressed in parvicellular neurons of the PVN, and high densities of GLP-1 binding sites have been identified in this region of the hypothalamus (76,218). A recent study has demonstrated that central GLP-1

administration is associated with increased plasma corticosterone levels (220), supporting a role for GLP-1 in activation of the hypothalamo-pituitary-adrenocortical axis through stimulation of CRF neurons. Notably, leptin also possesses activities in the paraventricular nucleus, where it has been shown to increase CRF mRNA levels following ICV administration (181). Thus, both GLP-1 and leptin may potentially exert inhibitory effects on food intake through CRF, since parvicellular CRF-synthesizing neurons of the paraventricular nucleus are thought to mediate the inhibitory effects of CRF on ingestive behaviour (181,220).

Central and peripheral GLP-1 administration has also been shown to inhibit water intake and paradoxically increase urinary output and natriuresis (221,222). GLP-1R mRNA is reportedly expressed in magnocellular neurons of the supraoptic and paraventricular nuclei, and ICV GLP-1 administration increases circulating levels of vasopressin (220). Pilot studies in which GLP-1R-/- mice were administered hydrocortisone produced symptoms of dehydration, suggesting that the mutant mice demonstrate impaired fluid balance. Since ICV GLP-1 has been shown to increase plasma vasopressin levels (220), GLP-1R-/- mice may exhibit deficient vasopressin secretion in the absence of GLP-1 signaling. To investigate this further, GLP-1R-/- and wildtype mice were administered hydrocortisone and subsequently water restricted. It was hypothesized that hydrocortisone-mediated suppression of vasopressin in GLP-1R-/- mice with concomitant dehydration would reveal deficient vasopressin secretion in the GLP-1R-/- mice compared to the wildtype mice, as exemplified by increased plasma osmolality.

The handling of GLP-1R-/- mice during previous experiments suggested that behavioural differences, particularly with respect to their response to stress, exist between GLP-1R-/- mice and wildtype controls. GLP-1R-/- mice appear to

be more docile and exhibit less biting and exploratory behaviour. Furthermore, a study in which control GLP-1R-/- and wildtype mice were injected with saline for 13 d demonstrated that GLP-1R-/- mice failed to gain weight during the experiment, whereas wildtype mice generally showed a steady increase in body mass (197). In view of these observations, we utilized two tests which permit indirect measurement of behavioural responses to stress; the elevated plus-maze (EPM) and the acoustic startle response. These tests were employed to determine whether GLP-1R-/- mice exhibit an altered stress response compared with wildtype controls.

7.2 Materials and Methods

7.2.1 Glucocorticoid Administration and Plasma Osmolality Measurements.

Female, 8 week-old GLP-1R-/- and CD1 +/+ mice received daily 200 ul intraperitoneal injections of either 10 mg hydrocortisone sodium succinate (Abbott Laboratories, Limited, Saint-Laurent, Quebec) or phosphate-buffered saline (n=16 per group) for 7 days. Half of the mice from each group (n=8) were permitted ad libitum access to water, while the other half (n=8) were water deprived for 13 h. The mice were then anesthetized with CO_2 and exsanguinated by cardiac puncture. Venous blood (800 ul) was collected into 25 ul heparin sodium as an anticoagulant (1000 U heparin/ml, Organon Teknika, Toronto, CA) and the plasma was separated by centrifugation and stored at -80°C. The osmolality of the plasma samples was measured with a vapour pressure osmometer (Wescor, Inc., Logan, Utah). Values are represented as means ± S.E.M. Statistical significance of data from wildtype and GLP-1R-/- animals given saline or hydrocortisone injections was assessed by an unpaired t test between mice which were permitted ad libitum access to

water, or water restricted. All statistics were analyzed using the InStat 1.12 program for MacIntosh computers (GraphPad Software).

7.2.2. Elevated Plus-Maze Test

The elevated plus maze consisted of two opposite open arms (50 x 10 cm) and two arms with walls (50 x 10 x 40 cm) attached to a central platform (10 x 10 cm) to form a cross, and elevated 65 cm above the ground. The maze floor was constructed from white Plexiglas, and the walls from black Plexiglas. Black lines on the maze floor defined the centre square, and divided both the open and closed arms into two equal segments, designated close and far based on their proximity to the centre square. Testing commenced by placing each mouse on the central platform facing an open arm. The GLP-1R-/- and wildtype CD1 mice were tested in alternating order. Following each 5 min test, the maze was cleaned. Tests were carried out during the light period. The number and duration of closed-arm and open-arm entries with both forepaws were recorded. Mouse behaviour was videotaped under white light illumination. Values are expressed as means ± S.E.M. Statistical significance of data from GLP-1R-/- mice compared to wildtype controls was assessed by an unpaired t test. All statistics were analyzed using the InStat 1.12 program for MacIntosh computers (GraphPad Software).

7.2.3. Acoustic Startle Response

7.2.3.1 Startle Apparatus

Amplitudes of startle reactions were determined using an acoustic startle apparatus obtained from MED Associates (St. Albans, VT). The mouse startle chamber was housed within a sound-attenuated acoustic startle cubicle (54.6 x 50.8 x 30.5 cm interior dimensions) with a ventilation fan. The chamber was

constructed of stainless steel rods suspended between Plexiglas plates fixed to a Plexiglas base. Interior dimensions of the chamber were 5.1 x 3.8 x 3.8 cm, providing the animal some movement. Magnitude of the startle response was measured by the startle platform for a period of 500 ms from presentation of the acoustic stimuli, and subsequently transduced and collected onto a PC utilizing Startle Reflex Software for Windows (MED Associates).

Acoustic white noise stimuli were presented through a programmable audio stimulator (MED Associates). Background white noise levels were maintained at 70 dB throughout testing.

7.2.3.2 Startle Testing

16 week-old male GLP-1R-/- (n=10) and age- and sex-matched GLP-1R+/- (n=11) and wildtype CD1 (n=11) mice were tested in alternating order within the four acoustic startle chambers. Mice were placed in the startle chamber for a five minute acclimation period with a background noise level of 70 dB. After the acclimation period, mice were exposed to acoustic startle intensities of 83 dB, 85 dB, 90 dB, 100 dB, and 120 dB for a duration of 30 ms in random order. Testing consisted of 40 trials of the five, randomly presented, stimulus intensities. Each mouse was therefore exposed to a total of 200 acoustic stimuli, or 40 at each of the five intensities. The startle amplitude at each stimulus intensity was defined as the average of 40 readings. The time interval between stimuli was varied, lasting from 12 to 18 s.

Five weeks following the initial startle testing, the same experimental group of GLP-1R-/- (n=8), GLP-1R+/- (n=8) and wildtype CD1 (n=8) animals received 0.5 mg/ml hydrocortisone sodium succinate (Abbott Laboratories, Limited, Saint-Laurent, Quebec) in drinking water for a period of 6 days. The

acoustic startle response of the mice was then assessed using the startle test described in the preceding paragraph.

7.2.3.3 Statistics

All startle response data is represented as the mean startle amplitude ± S.E.M. Statistical analysis of the genotype effect on startle was carried out using analysis of variance (ANOVA). Post hoc Newman-Keuls tests were performed if ANOVAs revealed $P<0.05$.

7.3 Results

To evaluate whether GLP-1R-/- mice exhibit impaired fluid balance during water restriction, GLP-1R-/- and wildtype mice were injected with saline or hydrocortisone and subsequently water restricted or permitted ad libitum access to water. Following 13 h of dehydration, wildtype mice which received saline injections demonstrated no change in plasma osmolality compared with controls which were permitted ad libitum access to water ($P=0.9365$; Fig. 22). However, fluid-restricted GLP-1R-/- mice which received saline injections exhibited significant increases in plasma osmolality compared to non-dehydrated controls ($P<0.05$; Fig. 22). These results suggest that GLP-1R-/- mice possess a decreased capacity to conserve water during a period of water restriction.

Hydrocortisone treatment of wildtype mice and subsequent water restriction produced significant increases in plasma osmolality compared to non-dehydrated controls ($P<0.05$; Fig. 22). Similarly, GLP-1R-/- mice exhibited significant increases in plasma osmolality following hydrocortisone treatment and dehydration compared to hydrocortisone-treated GLP-1R-/- controls which were permitted ad libitum access to water ($P<0.01$; Fig. 22).

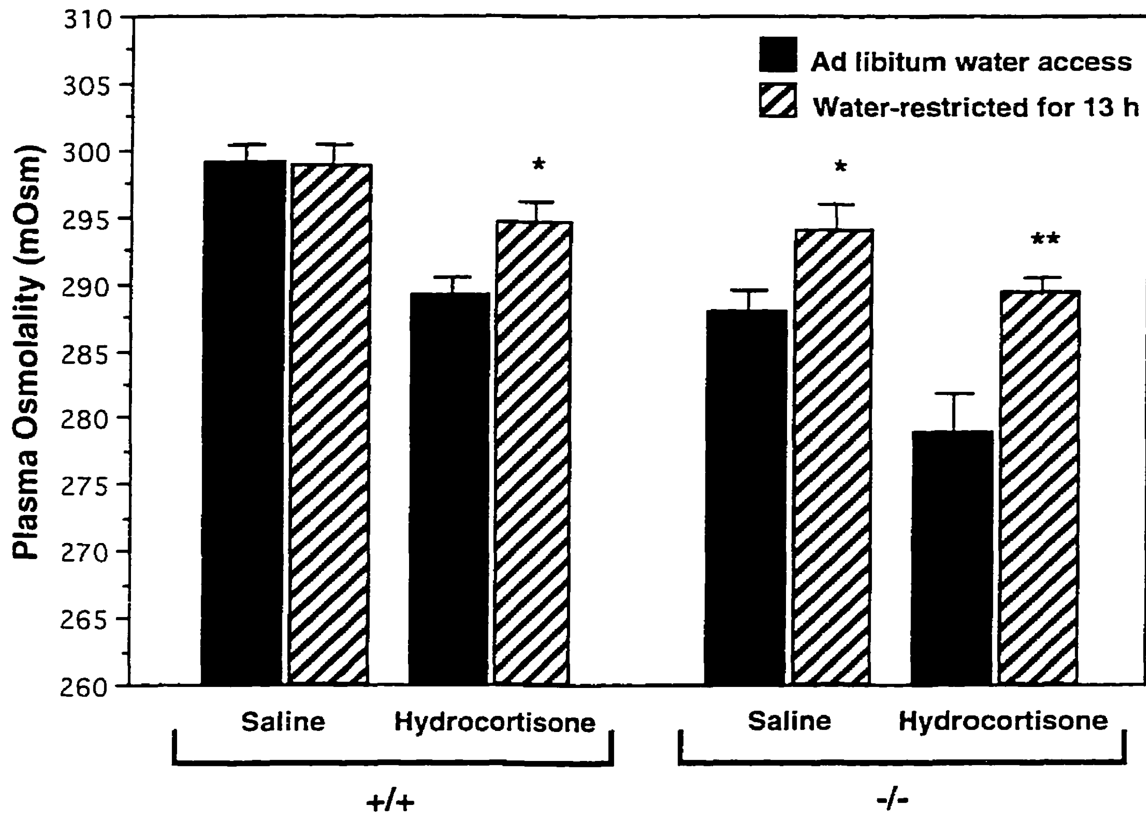


Fig. 22. Plasma osmolality of GLP-1R-/- and wildtype mice subsequent to hydrocortisone administration and water restriction. Age- and sex-matched female GLP-1R-/- (-/-) and wildtype (+/+) mice were injected with saline or 10 mg hydrocortisone for one week and subsequently permitted ad libitum access to water or water-restricted for 13 h (n=6-8 per group). *P<0.05, **P<0.01, water restricted vs. ad libitum water access.

To determine if GLP-1R-/- mice exhibit an altered behavioural response to stress, GLP-1R-/- and wildtype mice were observed in the elevated plus-maze for 5 min. The percentage of open arm entries versus closed arm entries ($P=0.1329$, wildtype vs. GLP-1R-/-; Fig. 23.) were not significantly different between GLP-1R-/- and wildtype controls. However, the GLP-1R-/- mice spent a significantly greater amount of time in the centre square of the elevated plus-maze compared to wildtype controls ($P<0.01$; Fig. 24).

The startle response of GLP-1R-/-, GLP-1R+/-, and wildtype mice to an acoustic stimulus was also assessed as a behavioural measure of stress response. A two-way ANOVA comparing genotype x stimulus intensity was performed for the startle response. There was no main effect of genotype on startle response over the range of stimulus intensities (Fig. 25; only wildtype and GLP-1R-/- results are shown for clarity), however, there was a significant interaction between genotype and stimulus intensity, $F(8,116)=2.38$, $P=0.02$. Post hoc comparisons using the Newman-Keuls test revealed a significant difference between wildtype and GLP-1R-/- mice at the highest acoustic stimulus intensity, 120 dB ($P<0.01$; Fig. 25). Thus, while there are no significant differences between the startle responses of the wildtype, GLP-1R+/- and GLP-1R-/- mice at stimulus intensities of 100 dB and lower, the GLP-1R-/- mice exhibit significantly greater startle responses than wildtype mice at the highest stimulus intensity, 120 dB. The GLP-1R+/- mice did not demonstrate startle responses significantly different form the wildtype mice at any time point. Interestingly, the GLP-1R-/- mice exhibit lower mean startle responses than wildtype mice at stimulus intensities of 83 dB, 85 dB, and 90 dB, although the differences do not reach statistical significance.

Central GLP-1 administration has been reported to induce *c-fos* expression in CRF neurons of the paraventricular nucleus of the hypothalamus

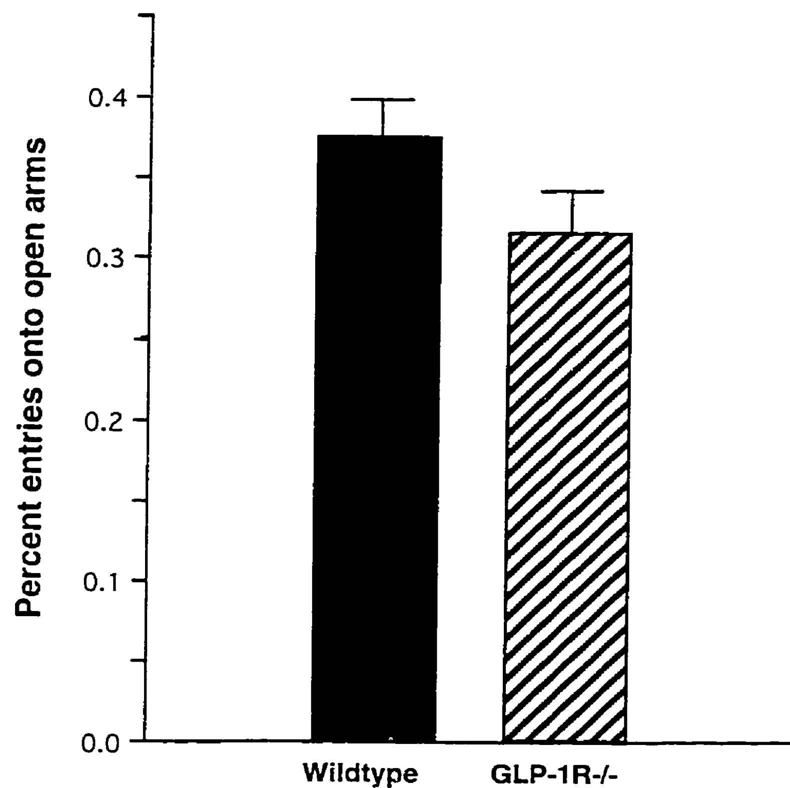


Fig. 23. Behaviour of wildtype and GLP-1R-/- mice in the elevated plus-maze. Mean (± S.E.M.) percentage of entries onto open arms of male GLP-1R-/- (n=11) or age- and sex-matched wildtype control (n=11) mice during a 5 min test on the elevated plus-maze.

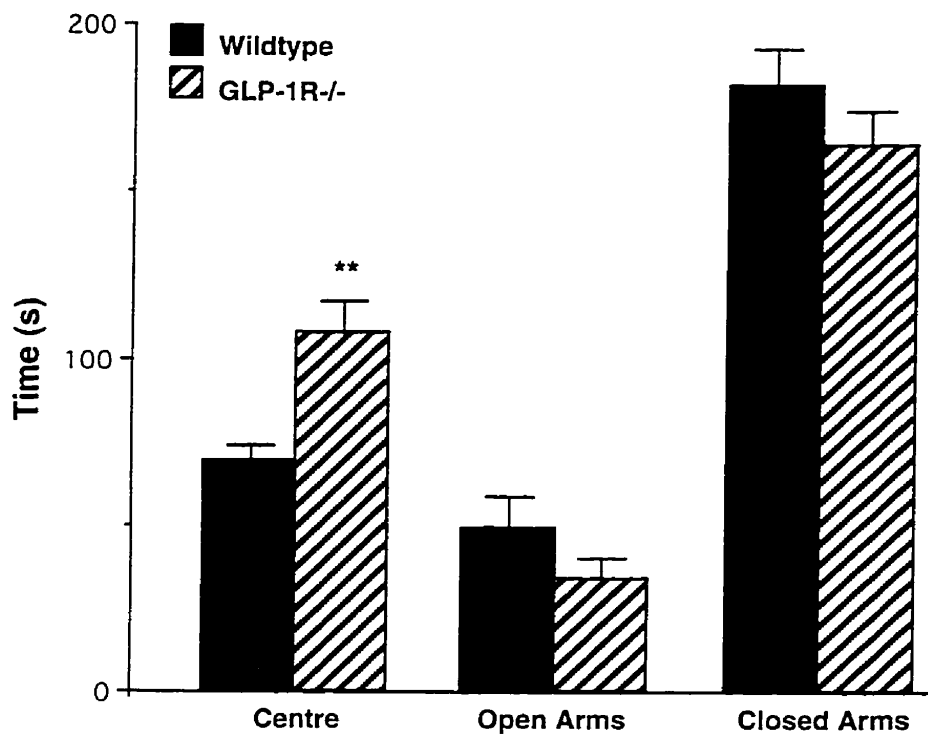


Fig. 24. Time GLP-1R-/- and wildtype mice spent in the center, open arm, and closed arm areas of the elevated plus-maze. Male GLP-1R-/- and age- and sex-matched wildtype CD1 controls were observed on the elevated plus-maze for 5 min (n=11 per group). Values are expressed as means ± S.E.M. **P<0.01, GLP-1R-/- vs. wildtype mice.

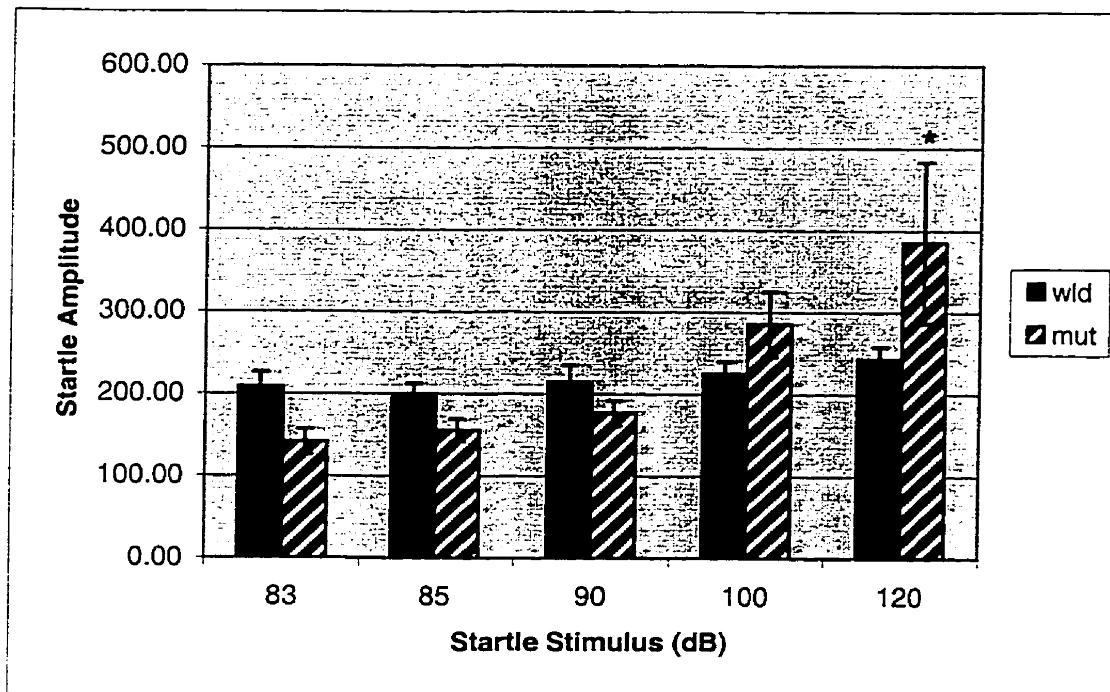


Fig. 25. Acoustic startle response of GLP-1R-/- and wildtype mice. The asterisk indicates a significant difference between the male GLP-1R-/- (mut) and age- and sex-matched wildtype (wld) controls in the Neuman-Keuls post hoc comparison, $P<0.05$.

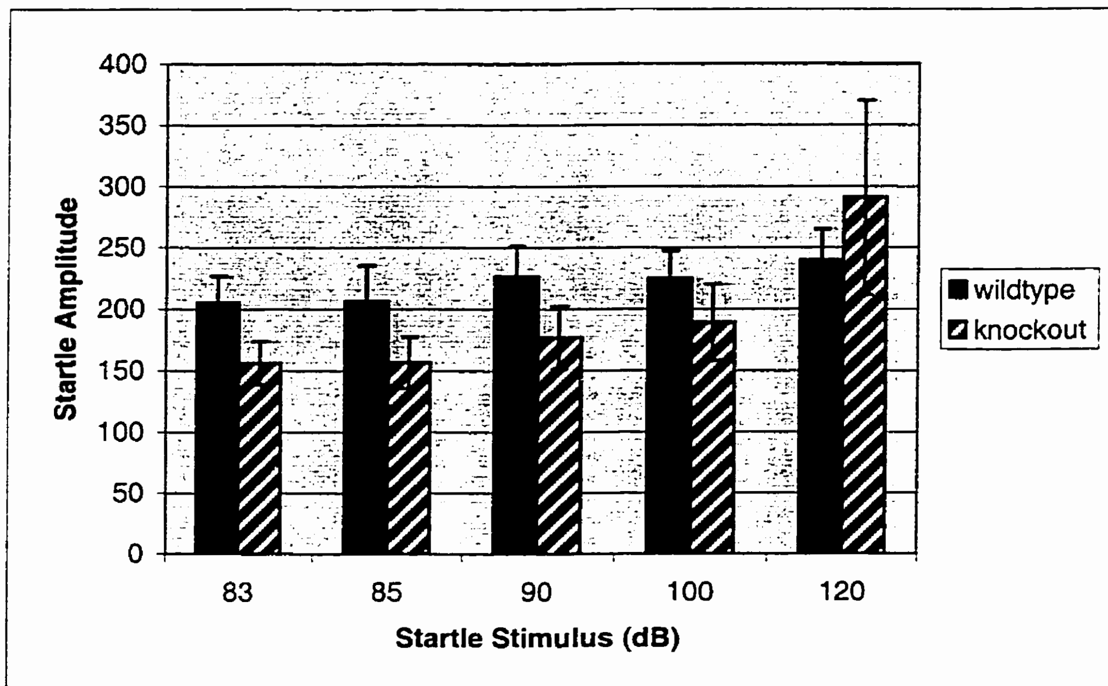


Fig. 26. Acoustic startle response of GLP-1R-/- and wildtype mice following hydrocortisone treatment. No significant differences in startle response existed between wildtype (wildtype) and GLP-1R-/- (knockout) mice (n=8 per group subsequent to hydrocortisone administration. ANOVA analysis did not reveal any significant interactions between genotype and startle amplitude at any of the stimulus intensities, F=df(8,84)=1.64, P=0.125.

(72,220), and is associated with increased plasma corticosterone levels (220). Since CRF produced in the paraventricular nucleus has been implicated in the behavioural response to stress (223), it was hypothesized that GLP-1R-/- mice may exhibit an altered startle response due to an effect of GLP-1 signal disruption on CRF. Therefore, GLP-1R-/-, GLP-1R+/-, and wildtype mice were administered exogenous hydrocortisone in an attempt to equilibrate the HPA axis in GLP-1R-/-, GLP-1R+/-, and wildtype mice through feedback inhibition of CRF. Following hydrocortisone treatment, there was no main effect of genotype on startle response, $F=(df\ 8,84)=1.643$, $P=0.125$, and no significant interactions were observed between genotype and startle amplitude at any of the stimulus intensities (Fig. 26; only GLP-1R-/- and wildtype mice are shown for clarity). Similar to what was observed in the first startle experiment, GLP-1R-/- mice exhibited decreased mean startle responses (N.S.) compared to wildtype mice at the lower stimulus intensities (83, 85, 90, and 100 dB) and greater startle responses (again N.S.) than wildtype mice at the highest stimulus intensity, 120 dB.

7.4 Discussion

The processing of proglucagon in the central nervous system produces GLP-1 as a major biologically active product (217). GLP-1 immunoreactivity is widely distributed throughout the CNS and has been colocalized with GLP-1 binding sites in regions which are known to be involved in the control of endocrine and behavioural responses (224). Intracerebroventricular injection of GLP-1 has been shown to inhibit food (72) and water intake (222), and a role for GLP-1 in the stimulation of luteinizing hormone-releasing hormone (LHRH) and CRH secretion from the hypothalamus (220,225), has been proposed. Thus, in addition to its role as a satiety signal, GLP-1 is emerging as a factor

involved in activation of the hypothalamo-pituitary-adrenocortical and hypothalamo-pituitary-gonadal axes.

Central injection of GLP-1 has been shown to increase plasma levels of vasopressin (220), suggesting that in the absence of GLP-1 signaling vasopressin secretion may be deficient. The finding that water restriction produces significant increases in plasma osmolality in GLP-1R-/- but not wildtype mice supports a role for GLP-1 in vasopressin secretion in vivo, although plasma vasopressin measurements must be performed in order to confirm this.

Vasopressin is produced in parvicellular neurons in the paraventricular nucleus, which also produce CRF, as well as magnocellular neurons of the paraventricular and supraoptic nuclei (226). Parvicellular CRF/vasopressin neurons of the paraventricular neurons and magnocellular neurons of the supraoptic nucleus express type II glucocorticoid receptors (226). The expression of glucocorticoid receptors in these neurons suggests a role for glucocorticoid-mediated suppression of vasopressin (and CRF) secretion, and in support of this, circulating glucocorticoids have been shown to regulate vasopressin levels in peripheral plasma (227). In agreement with these observations, hydrocortisone administration concomitant with water deprivation produced increased plasma osmolality in both GLP-1R-/- and wildtype mice. These results suggest that exogenous administration of hydrocortisone suppressed vasopressin secretion in both wildtype and GLP-1R-/- mice, thereby preventing secretion of adequate levels of vasopressin to maintain fluid balance in the face of the osmotic challenge.

Central injection of GLP-1 has been shown to increase plasma vasopressin levels (220). However, very few vasopressinergic neurons in magnocellular regions of the paraventricular and supraoptic hypothalamic

nuclei exhibit *c-fos* immunoreactivity, which is indicative of functionally activated neurons, following ICV GLP-1 administration (220). It is well established that vasopressin release due to an osmotic challenge is associated with *c-fos* expression within the magnocellular vasopressinergic neurons of the PVN and SON (228,229). Thus, it appears that the increased vasopressin secretion observed following central GLP-1 administration does not occur secondary to GLP-1-induced fluid depletion, which may result from the inhibitory effects of GLP-1 on water intake as well as its actions to increase urinary output and natriuresis (220). Furthermore, although GLP-1 elicits *c-fos* expression in parvicellular CRF/vasopressin neurons within the paraventricular nucleus, it is unlikely that the elevation of peripheral vasopressin levels observed following ICV GLP-1 injection are a consequence of increased parvicellular vasopressin release into the portal circulation (220). However, it is important to note that the secretory activity of the posterior pituitary is not always associated with *c-fos* expression (230). Synaptic activation at the level of the perikarya, versus the neuron terminals, appears to be necessary for the induction of *c-fos* gene expression (230). Thus, central GLP-1 administration may elicit vasopressin secretion via a stimulatory effect on vasopressinergic terminals within the posterior pituitary (220). This is supported by the finding of a high density of GLP-1 binding sites in the posterior pituitary (231). Alternatively, since central injection of GLP-1 elicits *c-fos* expression in magnocellular oxytocinergic neurons, it is possible that oxytocin, or a transmitter coexisting with oxytocin, subsequently acts within the neurohypophysis to stimulate vasopressin release at the level of the nerve terminals (220).

Intracerebroventricular administration of GLP-1 produces an elevation in plasma corticosterone levels and induces *c-fos* expression within CRF-containing neurons of the hypothalamic paraventricular nucleus (220). CRF is

the primary regulator of the endocrine, behavioural, and immune responses to stress (232). CRF stimulates the synthesis and secretion of pituitary adrenocorticotrophin (ACTH), which then results in increased adrenal glucocorticoid production (232). Additionally, central administration of CRF in rodents produces behavioural effects, many of which occur independently of HPA activation (233), that are indicative of a state of anxiety, including reduced time spent on the open arms of the elevated plus-maze and enhanced fear responses (234). In view of the proposed stimulatory effects of GLP-1 on CRF neurons, behavioural analysis of GLP-1R-/- mice may provide additional insight into the role of GLP-1 in the stress response.

A primary goal of neuroscience is the determination of how the complex mammalian brain directs behavioural changes. To approach this problem it is useful to study simple behaviours which can be elicited in mammals and experimentally manipulated. The elevated plus-maze (EPM), in which mice are allowed to freely explore two elevated open arms and two elevated closed arms, is one of the most commonly used models of anxiety (235). The EPM test is based on the natural aversion of mice for open spaces and elevation. Entry into the open arms of the EPM is associated with hormonal and behavioural changes which are indicative of increased anxiety, such as elevated plasma corticosterone levels and increased freezing, and normal behaviour therefore favours entry into the closed arms of the maze (236). Anxiolytic compounds generally increase the number and duration of open arm entries, while anxiogenics increase the aversion of the animal to the open arms. It follows that the critical factors which are considered to be correlated with anxiety are the number of entries made into the open arms as well as the total time spent on the open arms (235).

In the elevated plus-maze, GLP-1R-/- mice made a lower percentage of entries onto the open arms, compared to wildtype mice, however the difference did not reach statistical significance. As altered activity may confound the assessment of anxiety, total arm entries were used as a general index of activity (237). Although these results suggest that GLP-1R-/- mice may exhibit a slightly more anxious state than wildtype mice, consideration of the time spent in each separate area (centre, open arms, closed arms) of the plus-maze revealed that the knockouts spent significantly more time in the centre square of the EPM, which is considered to be more open and thus more anxiogenic than the closed arms. These results are difficult to interpret because, on the one hand, GLP-1R-/- mice made a lower percentage of entries onto the open arms, which suggests they are more anxious, but on the other hand, the knockout mice spent more time in the centre square than the wildtype mice, which suggests they are less anxious. However, it must be considered that the elevated plus-maze involves spontaneous behaviour in a relatively uncontrolled environment, and additional behavioural traits, such as risk-taking and exploratory behaviour, may influence the assessment of anxiety (237).

The startle response is a simple reflex behaviour which can be elicited in a highly controlled situation by a sudden, intense stimulus, and therefore relatively few variables are able to influence the results compared to the EPM. Startle can be elicited by an auditory, tactile, or visual stimulus (238). Acoustic startle is a short-latency motor response to a loud and unexpected noise, and involves a rapid and characteristic sequence of muscular responses which are easily measured by placement of the animal in a cage sensitive to movement (238). The short-latency of the startle reflex indicates that a simple neuronal circuit is involved, and although the neural circuitry that mediates acoustic startle is located entirely within the brainstem, higher neural networks appear

able to alter the startle reflex (238). Experimental manipulations which decrease or increase anxiety also decrease or increase the startle reflex, respectively.

GLP-1R-/- mice exhibit a significantly greater startle response than wildtype mice to an acoustic stimulus of 120 dB, which is indicative of an increased state of anxiety. However, this result conflicts with the observation that GLP-1R-/- mice appear less anxious than wildtype mice during most experimental manipulations (personal observation). It is interesting to note that while GLP-1R-/- mice exhibit normal basal ACTH and corticosterone levels, following exposure to a mild stress, such as movement into another room, GLP-1R-/- mice display decreased corticosterone levels compared to wildtype controls (Dr. N.J. MacLusky, personal communication). Since increased plasma corticosterone is a hormonal change which is generally indicative of an increased state of anxiety, the deficient corticosterone levels measured in GLP-1R-/- mice following exposure to a mild stress suggests that these mice exhibit a diminished ability to respond to mildly stressful stimuli, and may explain why they appear to be less anxious during experimental manipulations.

Surprisingly, when GLP-1R-/- mice are exposed to a severe stress, such as ether inhalation, they exhibit increased plasma corticosterone levels compared to wildtype controls (Dr. N.J. MacLusky, personal communication), suggesting that GLP-1R-/- mice display an exaggerated response to a highly stressful stimulus. This observation supports the finding of an increased startle response, which implies an increased state of anxiety, to an acoustic stimulus of 120 dB in the GLP-1R-/- mice. It is also interesting to note that while the GLP-1R-/- mice exhibit greater startle responses at the highest stimulus intensities (100 and 120 dB), they demonstrate consistently lower (N.S.) mean startle amplitudes than wildtype mice at sound intensities of 83 to 90 dB. This

observation further supports the hypothesis that GLP-1R-/- mice demonstrate a diminished response to mildly stressful stimuli, and an exaggerated response to highly stressful stimuli.

Recent studies have supported a role for GLP-1 in the stimulation of CRF-containing neurons (220), and therefore suggest that GLP-1 may be important in the physiological response to stress. At this time it is not possible to explain the results of the current experiments in any but speculative terms. However, it is feasible that in the absence of GLP-1 signaling CRF secretion is decreased in the GLP-1R-/- mice. The maintenance of normal basal levels of ACTH and corticosterone despite the proposed decrease in CRF secretion in GLP-1R-/- mice implies that the adrenocorticotrophs secrete normal levels of ACTH in response to diminished CRF stimulation, suggesting that the corticotrophs display an increased sensitivity to CRF. The failure of GLP-1R-/- mice to exhibit increased corticosterone levels in response to a mild stress may result from the inability of these mice to rapidly increase CRF secretion in response to mild stress in the absence of GLP-1 signaling. Additionally, since CRF exerts behavioural effects independent of activation of the HPA axis (233), diminished CRF secretion could conceivably produce the decreased stress response observed in GLP-1R-/- mice following exposure to a mild stress. However, under conditions of high stress, the CRF neurons must become activated through mechanisms other than GLP-1 which are responsive to stressful stimuli. The increased CRF levels could then result in an exaggerated ACTH response due to the increased sensitivity of the corticotrophs to CRF. It may also be postulated that increased sensitivity of brain regions involved in behaviour to CRF may produce the elevated response to highly stressful stimulus observed in the GLP-1R-/- mice. Clearly, further investigations are warranted in order to clarify the role of GLP-1 in CRF secretion.

The finding that GLP-1R-/- mice exhibit a startle response which is not significantly different from wildtype mice following hydrocortisone administration supports the existence of a perturbation in the HPA axis of the GLP-1R-/- mice. These results suggest that glucocorticoid administration may have partially normalized the HPA axis of the GLP-1R-/- mice, presumably through feedback inhibition. However, these results must be interpreted cautiously because a smaller sample was used than in the first startle experiment, and while the startle responses of GLP-1R-/- and wildtype mice were not significantly different at 120 dB, the GLP-1R-/- mice exhibited a trend towards increased startle amplitude at this stimulus intensity.

Phenotypical changes in mice with a null mutation may indicate a function of the targeted gene product. However, it must also be considered that the observed changes may be secondary to compensatory alterations during development. It will be necessary to determine whether use of GLP-1R antagonists such as exendin (9-39) produce the same changes in startle response in order to speculate whether the observed behavioural changes in the GLP-1R-/- mice are the result of compensatory mechanisms. Nonetheless, these studies in GLP-1R-/- mice implicate a key role for GLP-1 in the control of hypothalamic CRH- and vasopressin-containing neurons. In view of additional studies which propose a role for GLP-1 in the stimulation of hypothalamic LHRH secretion, current evidence suggests that GLP-1 is emerging as an important factor in the control of the hypothalamo-pituitary-adrenocortical and hypothalamo-pituitary-gonadal axes.

Chapter 8 Discussion

The generation of mice with a null mutation in the glucagon-like peptide-1 receptor gene has permitted analysis of the physiological effects of disrupted GLP-1 signaling. GLP-1R-/- mice are viable and do not demonstrate perturbations in feeding behaviour or body weight (80). However, GLP-1R-/- mice exhibit fasting hyperglycemia and deteriorated oral glucose tolerance in association with deficient glucose-stimulated insulin secretion (80). These observations are consistent with an essential role for GLP-1 in the control of glucose homeostasis *in vivo* (80).

The normal ß cell response to a state of insulin resistance involves increased production and secretion of insulin (88). In order to assess the ability of the ß cell to respond to insulin resistance in the absence of GLP-1 signaling, GLP-1R-/- and wildtype mice were treated with dexamethasone and growth hormone (D/GH), agents known to induce insulin resistance *in vivo* (86-89). Following one week of treatment, both GLP-1R-/- and wildtype mice treated with D/GH exhibited improved glucose tolerance as a consequence of increased glucose-stimulated insulin secretion. In contrast, prolonged treatment with combined dexamethasone and growth hormone for four weeks was associated with improved glucose tolerance in wildtype, but deteriorated glucose tolerance in GLP-1R-/-, mice. Furthermore, while wildtype mice demonstrated greater than four-fold increases in glucose-stimulated insulin secretion, insulin levels in the GLP-1R-/- mice increased less than two-fold. These observations emphasize the importance of intact GLP-1 signaling for increased insulin secretion in response to chronic, but not acute, insulin resistance.

Underlying the inability of GLP-1R-/- mice to increase insulin secretion in the face of insulin resistance may be a defect in insulin expression and biosynthesis. *In vitro*, GLP-1 has been shown to increase levels of proinsulin mRNA via a cAMP-dependent induction of insulin gene transcription (59,60). Initial investigations suggested that some, but not all, GLP-1R-/- mice exhibited small decreases in basal fasting proinsulin mRNA transcript levels (128). Subsequent northern analysis of pancreatic RNA isolated from fed GLP-1R-/- and wildtype mice revealed a significant decrease in the levels proinsulin mRNA transcripts in GLP-1R-/- mice (152). This is consistent with the observation that wildtype, but not GLP-1R-/-, mice exhibited a marked induction of proinsulin mRNA transcripts following four weeks of combined D/GH treatment. These findings support a role for GLP-1 signaling at the level of proinsulin gene expression and biosynthesis in the ß cell.

Physiological characterization of GLP-1R+/- heterozygotes further support a crucial role for GLP-1 in glucose homeostasis. While phenotypical changes in mice with a null mutation may indicate the functions of the targeted gene product, it is possible that the observed differences may be secondary to compensatory alterations which occurred during development. Analysis of heterozygous mice, which possess only one null allele, avoids this caveat to some extent because the targeted protein is expressed during development, although perhaps in diminished quantities. Heterozygotes often fail to exhibit a phenotype different from that of wildtype mice. However, GLP-1R+/- mice exhibit abnormal oral glucose tolerance concomitant with significant reductions in circulating insulin. Additionally, GLP-1R+/- mice show a trend (N.S.) towards fasting hyperglycemia (128). These data imply that even diminished expression of the GLP-1R impairs the ability of the ß cell to respond adequately to glucose and, furthermore, suggest that the impaired glucose tolerance and defective

insulin secretion observed in GLP-1R-/- mice are a direct consequence of GLP-1 signal disruption.

It is notable that other insulin secretagogues (such as glucose, GIP, and glucagon) are not entirely able to compensate for the partial reduction in GLP-1 signaling which is postulated to be present in GLP-1R+/- mice. Nonetheless, GLP-1R-/- and GLP-1R+/- mice exhibit only mild diabetes, suggesting that in the absence of GLP-1 other signaling factors assume a larger role in the potentiation of glucose-induced insulin secretion. Indeed, a recent study has demonstrated that GLP-1R-/- mice show compensatory increases in GIP secretion as well as in the insulin response to GIP (152). However, the substantial improvement in glucose tolerance observed in GLP-1R-/- mice following the presumed inhibition of GIP degradation by the DPP-IV inhibitor Pro-boroPro suggests that, despite compensatory increases in GIP secretion and action, GIP activity remains submaximal in the GLP-1R-/- mouse.

In view of the proposed role for GLP-1 in peripheral glucose utilization, it was hypothesized that the fasting hyperglycemia and glucose intolerance observed in GLP-1R-/- mice may, in part, be due to changes in peripheral insulin sensitivity resulting from disrupted GLP-1 signaling. A hypoglycemic effect of GLP-1, independent of its augmentation of insulin secretion, has been reported (71). GLP-1 reportedly enhances insulin-dependent glucose uptake in 3T3-L1 adipocytes (43,98), as well as glucose utilization and glycogen synthesis in liver and muscle tissue (69,100). However, administration of the insulin-sensitizing compound troglitazone to GLP-1R-/- mice produced no changes in glucose tolerance compared to controls, suggesting that GLP-1R-/- mice are not insulin resistant. Consistent with the presence of normal insulin sensitivity in GLP-1R-/- mice, a hyperinsulinemic clamp study demonstrated that

GLP-1R-/- mice exhibit normal whole body glucose utilization (128). This is consistent with recent studies employing the hyperinsulinemic, euglycemic clamp, or determination of glucose disappearance rates during inhibition of insulin secretion, which demonstrate that GLP-1 has no effect on insulin sensitivity or glucose elimination, respectively (129,130). These data suggest that the effects of GLP-1 on glucose metabolism in peripheral tissues such as adipose and muscle, if any, are probably insignificant in their contribution to the glucose intolerance and fasting hyperglycemia observed in GLP-1R-/- mice.

Evaluation of the effects of intracerebroventricular administration of leptin on food intake in GLP-1R-/- and wildtype mice revealed that leptin produced a comparatively greater inhibition of food consumption in the GLP-1R-/- mice at certain doses (197). Furthermore, chronic leptin treatment of GLP-1R-/- mice also resulted in comparatively greater improvements in glucose tolerance and glucose-induced insulin secretion than in leptin-treated wildtype mice (197). In the present investigation, improved glycemic excursion following oral glucose challenge was observed in short-term leptin-treated GLP-1R-/-, but not wildtype, mice. Taken together, these results suggest that GLP-1R-/- mice display increased sensitivity to the central and peripheral effects of leptin, and furthermore, imply a novel interaction between leptin and GLP-1 signaling pathways at the level of the ß cell and within the hypothalamus.

In addition to its peripheral effects, central administration of GLP-1 significantly inhibits food and water intake (72,221). However, GLP-1R-/- mice do not demonstrate perturbations in feeding behaviour or body weight (80), suggesting a redundant role for GLP-1 in the control of satiety. The widespread distribution of GLP-1 receptor expression in the brain suggests that GLP-1 possesses central actions in addition to its role as a satiety factor. Consistent

with this, recent reports have implicated a role for GLP-1 in the stimulation of CRF- and LHRH-containing neurons in the hypothalamus (72,220,225), and furthermore, central GLP-1 administration has been shown to produce elevations in plasma vasopressin levels (220). The results of behavioural studies undertaken in this investigation support a role for GLP-1 in physiological regulation of the stress response via complex interactions with CRF neurons. Furthermore, this study supports a role for GLP-1 in vasopressin secretion, since GLP-1R-/-, but not wildtype, mice exhibited increased plasma osmolality subsequent to water restriction, presumably as a result of their inability to secrete sufficient amounts of vasopressin. Although the mechanisms of central GLP-1 action proposed in this study are largely speculative, it is important to note that, while the effects of GLP-1 on satiety appear to be well-compensated for in the event of GLP-1 signal disruption, GLP-1 action appears to be important for both the appropriate regulation of fluid balance during water restriction, and the behavioural response to stress.

In summary, this study supports the essential role of GLP-1 in glycemic control, glucose-induced insulin secretion, and proinsulin gene expression and biosynthesis; in both insulin-sensitive and insulin-resistant states. The existence of a gene dosage effect for the incretin activity of GLP-1 in GLP-1R+/- heterozygotes further emphasizes the crucial role of GLP-1 in glucose-stimulated insulin secretion in vivo. Additionally, the failure of insulin-sensitizing agents to improve glucose tolerance in GLP-1R-/- mice in this investigation is consistent with reports of normal whole body glucose utilization in GLP-1R-/- mice (128). Finally, these studies implicate a key role for GLP-1 in the control of hypothalamic CRH- and vasopressin-containing neurons, and suggest that GLP-1 may be an important factor in the control of the hypothalamo-pituitary-adrenocortical axis.

Chapter 9 References

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