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Bioactive GLP-1 in Gut, Receptor Expression in Pancreas, and Insulin Response to GLP-1 in Diabetes-Prone Rats

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Glucagon-like peptide-1 (GLP-1) is the most insulinogenic of the glucagon-like peptides secreted mainly by L cells in the small and large intestine in response to the ingestion of nutrients. It binds to a specific GLP-1 receptor (GLP-1R) on β-cells and can increase islet neogenesis and β-cell mass. It is not clear whether the transmission of information from the gut to islet β-cells by messengers such as GLP-1 is different in individuals who develop autoimmune diabetes. In the present study the expression of bioactive GLP-1 protein in the gut and its receptor in the pancreas was examined in diabetes-prone BioBreeding (BBdp) rats in the period before overt diabetes and in age-matched control, non-diabetes-prone BB (BBc) rats. An N-terminal directed antibody specific for the bioactive forms of GLP-1 (GLP-1$^{7–37}$ and GLP-1$^{7–36amide}$) was used to measure GLP-1 by radioimmunoassay in proximal, median, and distal gut. Pancreas GLP-1R area fraction, GLP-1R gene expression, and insulin content were analyzed, as were plasma GLP-1, glucose, and insulin. The concentration of GLP-1 protein in the jejunum and ileum of BBdp rats was lower than in BBc rats. Although these animals maintained normal blood glucose, there was impaired pancreatic endocrine function, characterized by low baseline insulin concentration in plasma and pancreas. GLP-1R mRNA expression was threefold less in islets isolated from BBdp rats, and GLP-1R$^+$ islet area fraction in pancreas sections was decreased. When injected iv with GLP-1, BBdp rats displayed lower second-phase insulin response (and insulin/glucose ratios) compared with BBc rats. Thus, young BBdp rats displayed decreased concentrations of bioactive GLP-1 in jejunum and ileum, reduced GLP-1R in islets, and lower second-phase insulin response to iv GLP-1 than controls. The decrease in insulinogenic and islet β-cell mass-promoting signal from GLP-1 in BBdp rats may contribute to impaired glucoregulation and ineffective maintenance of normal islet mass that shifts islet homeostasis in favor of development of diabetes.

Key Words: Enteroglucagon; gut; BB rat; type 1 diabetes; glucagon-like peptide-1; GLP-1 receptor.

Introduction

Glucagon-like peptide-1 (GLP-1) is produced by posttranslational modification of the glucagon precursor, proglucagon, in L cells located in the small and large intestine *(1,2)*. It is secreted in response to the presence of nutrients in the gut lumen and communicates this information to the pancreas where it acts as a potent insulin secretagogue, accounting for as much as 50% of postprandial insulin secretion *(3,4)*. GLP-1 has several pleiotropic effects related to assimilation of nutrients and maintenance of normal blood glucose levels including inhibition of food intake, gastric emptying, and secretion of glucagon, as well as stimulation of glucose-dependent insulin biosynthesis and secretion. In addition to its role as an insulinotropic agent, GLP-1 through binding to its receptor, increases islet β-cell differentiation and proliferation *(5)*.

In mammals, the proglucagon gene gives rise to several proteins from the gut including two glucagon-like peptides, GLP-1 and GLP-2, both of which regulate nutrient assimilation and energy homeostasis. There are two biologically active forms of GLP-1—the glycine-extended form, GLP-1$^{7–37}$ and the amidated form, GLP-1$^{7–36\ amide}$ that account for approximately one third and two thirds, respectively, of its biological activity *(3)*. GLP-1 is rapidly cleared from the circulation both nonenzymatically and enzymatically by the action of the exopeptidase, dipeptidyl peptidase IV (DP IV), which produces the weakly active forms GLP-1$^{9–36\ amide}$ and GLP-1$^{9–37}$. Thus, proglucagon gene expression results in several glucagon-like peptides that have considerable structural overlap and different biological activities. In this investigation we used an N-terminal directed antibody (98302) specific for the bioactive forms of GLP-1.

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Table 1
Metabolic and Hormonal Data in BBc and BBdp Rats

	Rats			
	BBc		BBdp	
Sex	Male	Female	Male	Female
Age (d)	60–64	62–66	60–64	62–66
	(9)	(7)	(7)	(9)
Body weight (g)	244 ± 4	179 ± 5	251 ± 6	192 ± 3
	(9)	(7)	(7)	(9)
Plasma D-glucose (m*M*)	11.0 ± 0.2	11.3 ± 0.8	10.8 ± 0.6	10.5 ± 0.2
	(9)	(7)	(6)	(9)
Plasma insulin (μU/mL)	28.7 ± 3.4	29.0 ± 2.8	19.0 ± 2.1	10.3 ± 1.5
	(9)	(7)	(6)	(9)
Paired plasma insulin/glucose ratio (U/mol)	2.6 ± 0.3	2.5 ± 0.3	1.8 ± 0.1	1.0 ± 0.2
	(9)	(7)	(6)	(9)
Pancreas insulin content (U/g wet wt)	3.2 ± 0.2	4.8 ± 0.2	2.8 ± 0.5	3.7 ± 0.2
	(9)	(7)	(7)	(9)

In addition to its role as an incretin hormone, recent studies indicate that GLP-1 and its agonists promote β-cell proliferation and islet neogenesis *(6,7)*. Proliferation of islet β-cells in the exocrine pancreas is increased by GLP-1 in a manner that is dependent on increased binding of PDX-1 *(8)*. A single, specific GLP-1 receptor (GLP-1R) that is a member of the G-protein coupled seven transmembrane domain superfamily mediates the biological actions of GLP-1 in islets *(9)*.

The development of spontaneous diabetes in BBdp rats results from the interaction of several risk genes, is associated with decreased islet mass, and is strongly influenced by environmental factors, among which, diet accounts for at least half of the diabetes cases *(10,11)*. It has been suggested that the gastrointestinal tract may play a role in the development of autoimmune diabetes, but experimental evidence in animals that spontaneously develop type 1 diabetes is lacking. Previous studies in young BB rats reported that colon proglucagon mRNA levels were higher *(12)* or lower *(13)* in BBdp rats compared with controls. Because the proglucagon gene produces several bioactive peptides, and there is an enhancing effect of GLP-1 on islet mass, it is important to determine whether the gut of diabetes-prone rats is deficient in bioactive GLP-1 peptides, and receptor levels are normal in the pancreas, particularly in the period before classic insulitis. N-terminal-directed assays are necessary to determine accurately the concentration of biologically active GLP-1 *(1)*. The present study was undertaken to analyze (i) gut GLP-1 peptides using an N-terminal directed antibody, (ii) GLP-1R gene and protein expression in pancreas, and (iii) GLP-1-induced insulin secretion in young diabetes-prone BB rats.

Results

Experiment 1:
Analyses of Metabolic Parameters and Gut GLP-1

Male and female rats aged 60–66 d were examined (Table 1). The body weight of BBdp rats was not different from that of BBc rats, and the daily weight gain was comparable in BBc and BBdp rats of the same sex. There was no significant difference in plasma D-glucose concentration in BBc and BBdp rats. The plasma insulin concentration was lower ($p < 0.001$) in BBdp rats (13.8 ± 2.8 μU/mL; $n = 15$) compared with BBc rats (29.0 ± 2.8 μU/mL; $n = 16$). Similarly, the paired ratio between plasma insulin and D-glucose concentration (insulinogenic index) was significantly lower ($p < 0.05$) in BBdp rats than in BBc rats, in both male and female animals. This was reflected in the lower insulin content of the pancreata of BBdp rats, which represented 82 ± 6% ($n = 16$) of the mean control value found in age- and sex-matched BBc rats ($p < 0.02$).

Pancreas Inflammation

Examination of hematoxylin and eosin stained pancreas sections revealed infrequent infiltration by mononuclear cells mainly in the form of peri-insulitis. Although relatively low, the percentage of islets infiltrated was four times higher in BBdp versus BBc rats (12 ± 5 vs 3 ± 2, $n = 16$/grp, $p = 0.01$).

GLP-1 Content in the Intestinal Tract

The content of GLP-1 expressed as nanograms per milligram protein was lower ($p < 0.05$) in the jejunum compared with ileum or colon, regardless of rat type (Table 2). Few significant differences in the GLP-1 content were found between male and female rats (Table 2).

Table 2
Immunoreactive GLP-1 (ng/mg Protein) in the Gastrointestinal Tract of BB Rats

	BBc			BBdp		
	Male	Female	Both	Male	Female	Both
Jejunum	8.0 ± 0.9 (7)[a]	4.7 ± 1.3 (5)	6.6 ± 0.9[1] (12)	4.7 ± 1.6 (5)	2.5 ± 0.3 (7)	3.4 ± 0.7[1] (12)
Ileum	37.7 ± 4.5[2] (7)	18.5 ± 4.7 (5)	29.7 ± 4.2[3] (12)	24.2 ± 3.2[2] (5)	12.5 ± 3.0 (7)	17.4 ± 2.7[3] (12)
Colon	22.1 ± 5.7 (5)	16.6 ± 4.2 (5)	19.4 ± 3.5 (10)	13.5 ± 1.3 (5)	16.3 ± 2.3 (7)	15.1 ± 1.5 (12)

[a]Number of rats/group in parentheses.
[b]Groups sharing the same superscript are significantly different (*t*-test) as follows:
[1]$p < 0.01$, [2]$p < 0.05$, [3]$p < 0.03$.

The GLP-1 content of the jejunum and ileum was always lower in BBdp rats compared with BBc animals (Table 2). GLP-1 concentration in the jejunum and ileum tended to be lower in female compared with male rats, similar to findings in streptozotocin-induced diabetes *(14)*. The difference between BBdp and BBc was significant when values were pooled for male and female and when male BBdp were compared with male BBc rats (Table 2). When the results obtained in BBdp rats were expressed as a percentage of the control value in age- and sex-matched BBc animals, similar differences were observed (Fig. 1). In the colon, although the mean values were always lower in BBdp than in BBc rats, this difference was not statistically significant. The GLP-1 content of the entire gut was 1137 ± 147 ng in BBc and 826 ± 68 ng in BBdp rats ($p = 0.06$).

Northern Analysis for Proglucagon and GLP-1 mRNA

In BBc rats, the ratio of proglucagon to β-actin mRNA was significantly lower in jejunum compared with ileum or colon extracts. Relative to total proglucagon mRNA, the jejunum accounted for 28 ± 2%, compared with 36 ± 3% and 36 ± 4% for the ileum and colon, respectively. These findings agreed with the measurements of GLP-1 peptides in the intestinal tract. The proglucagon/β-actin mRNA ratios in the ileum and colon of BBdp rats did not exceed 68 ± 13% ($n = 7$; $p < 0.05$) of the mean corresponding values found in BBc rats. Similarly, the amount of proglucagon mRNA detected in BBdp rats relative to BBc rats was in agreement with the measurements of GLP-1 peptide content.

Experiment 2:
GLP-1R Localization and Gene Expression in the Pancreas

Staining for GLP-1R protein was absent in acinar cells and blood vessels, but was very intense in the cells at the periphery of islets in BBdp rats (Fig. 2). Cells in the center of the islet, mostly insulin-producing β-cells, were weakly stained for GLP-1R (Fig. 2A). Comparing adjacent sections, stained for glucagon, somatostatin, and pancreatic polypeptide, strong GLP-1R staining was coincident mainly with glucagon$^+$ cells (Fig. 2A), as well as somatostatin$^+$ and pancreatic polypeptide$^+$ cells (data not shown). The distribution of GLP-1R staining in the islets of BBc and BBdp rats was similar. However, BBdp rats had a smaller GLP-1R$^+$ area fraction than BBc rats (Fig. 3, Panel A, $p = 0.02$). GLP-1R staining was also positive in ganglion cells and some ductal epithelial cells in the pancreas (Fig. 2C and 2D). In equal numbers of isolated islets of similar size distribution, the mRNA expression for GLP-1R relative to

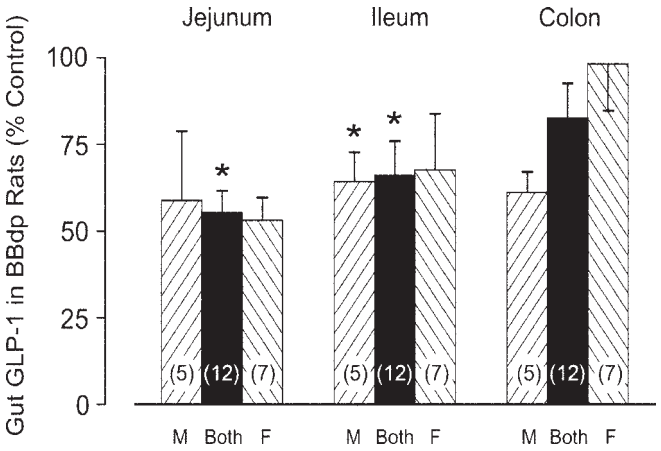

Fig. 1. GLP-1 in the gastrointestinal tract of BBdp rats as percent of control BBc values. GLP-1 content of the jejunum, ileum, and colon in male and female BBdp rats measured with N-terminal-specific antibody 98302. Results are expressed as a percentage of the mean value obtained with the same antibody in age- and sex-matched male and female BBc rats. Each bar represents the mean values (± SEM) for the number of animals shown in parentheses; * indicates $p < 0.05$ compared with BBc rats.

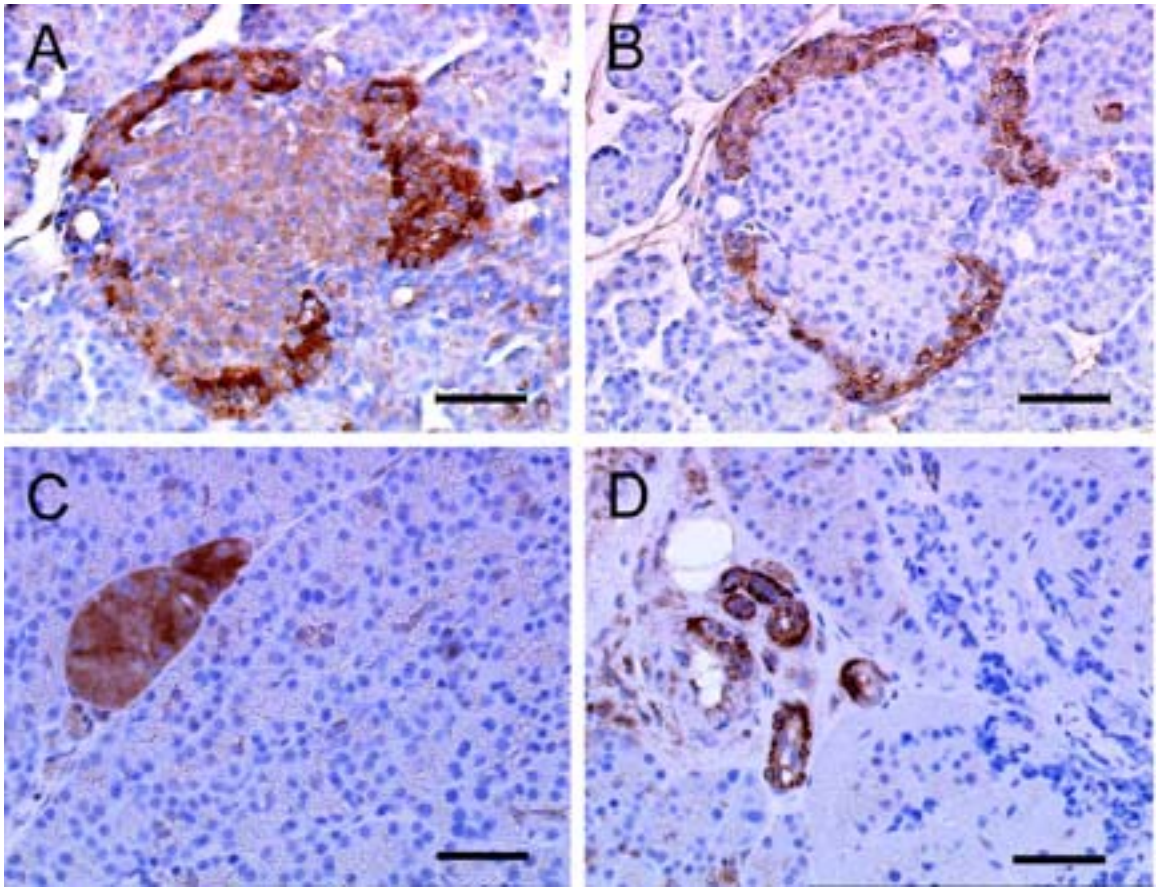

Fig. 2. GLP-1R staining in pancreas of BBdp rats. Paraffin-embedded pancreas sections stained for GLP-1R at 45 d of age. Typical staining of islets is shown (**A**). Staining was weak but uniform in the central part of the islets containing β-cells; and there was strong staining in cells at the periphery of the islet. Adjacent sections of (**A**) were stained for glucagon (**B**). GLP-1R was also localized in ganglia cells (**C**) and duct cells (**D**). Sections were counterstained with hemotoxylin. Bar = 50 μm in each image.

β-actin was threefold greater in BBc than in BBdp (Fig. 3, Panel B, $p = 0.005$), consistent with the decrease in GLP-1R staining in islets.

Experiment 3:
GLP-1 Induced Insulin Secretion

Insulin release was measured in vivo after intravenous (iv) injection of GLP-1 or saline in non-fasted male and female BBc and BBdp rats matched for age and body weight (Fig. 4). Sequential blood samples were collected at −10, 0, 2, 5, 7, and 10 min and the insulin content was measured by radioimmunoassay (RIA). Insulin response to injected saline showed similar results in BBdp and BBc rats. Plasma insulin increased during the first 7 min after injection of GLP-1 to a similar extent in BBc and BBdp rats. By 7 and 10 min, insulin concentration was less in BBdp than in BBc animals ($p < 0.01$). The total area under the insulin curve was not significantly different between BBc and BBdp rats (39.7 ± 6.3 vs 24.6 ± 4.9 ng min^{-1} mL^{-1}, $p < 0.08$). However, the area under the curve from 2 to 10 min was larger in BBc than in BBdp (35.2 ± 4.2 vs 21.2 ± 3.4 ng min^{-1} mL^{-1}, $p = 0.03$). Blood glucose concentrations were similar and hence the graph was essentially identical when insulin/glucose ratio was plotted (data not shown). Analysis of basal plasma GLP-1 by RIA in pooled samples (from five to seven rats/group) showed that GLP-1 concentration in male and female BBc rats was 0.503 and 0.439 ng/mL and in BBdp rats it was 0.353 and 0.342 ng/mL, respectively. Basal plasma insulin in BBdp (1.2 ± 0.3 ng/mL, $n = 8$) was less than in BBc rats (2.6 ± 0.5, $n = 7$, $p < 0.05$). Although the wet weight of pancreas was similar in BBc (1.33 ± 0.06 g, $n = 7$) and BBdp (1.23 ± 0.03 g, $n = 8$), the insulin content of pancreas was lower in BBdp (227 ± 77 vs 483 ± 36 ng/mg protein, $p < 0.02$). Thus, in relative terms, the decrease in plasma insulin concentration was proportional to pancreas insulin content.

Discussion

It has been suggested that the gastrointestinal tract may be involved in the pathogenesis of type 1 diabetes *(10,15–17)*. A major unanswered question is whether or not com-

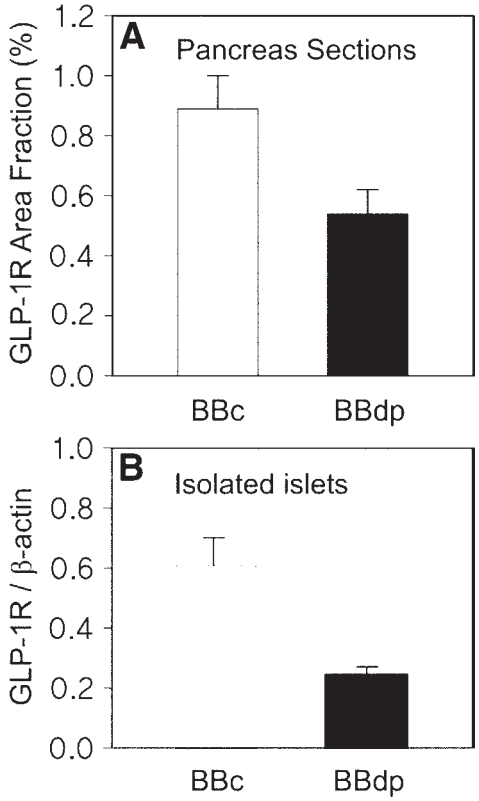

Fig. 3. GLP-1R protein and gene expression in pancreatic sections and isolated islets. Panel (**A**) GLP-1R area fraction in the pancreatic islets of 45–50 d old BBc and BBdp rats. GLP-1R$^+$ area was determined using a Zeiss AxioPlan microscope equipped with a Northern Eclipse Image Analysis System. Bouin's fixed, paraffin-embedded sections 5 μm thick were stained with rabbit anti-GLP-1R antibody, visualized with biotin, DAB and then counterstained with hematoxylin. Each bar represents the mean ± SEM of seven animals, $p = 0.02$. (**B**) GLP-1R gene expression in pancreatic islets (200 islets/rat) isolated from 30 d BBc and BBdp rats. GLP-1R expression was measured using RT-PCR and normalized to β-actin expression. Each bar represents the mean (arbitrary units) ± SEM from analyses of four BBc and four BBdp animals, $p = 0.005$.

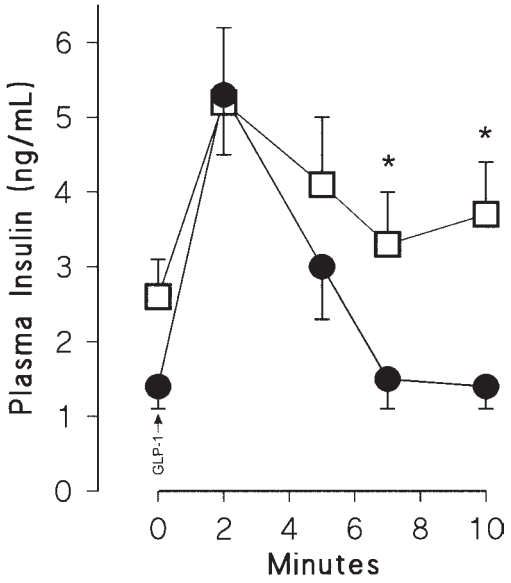

Fig. 4. Plasma insulin following iv injection of GLP-1 in BBc and BBdp rats. Plasma insulin was higher in BBc (open square, $n = 7$) than in BBdp (filled circles, $n = 8$) at 7 min and 10 min ($p < 0.05$) post GLP-1 injection; area under the curve from 2 to 10 min was larger in BBc than BBdp ($p = 0.03$).

munication between the gut and pancreas is abnormal in diabetes-prone individuals. Using an N-terminal directed antibody specific for the biologically active forms of GLP-1, GLP-1$^{7–37}$ and GLP-1$^{7–36\ amide}$, we demonstrated that the concentration of bioactive GLP-1 in the jejunum and ileum of BBdp rats is lower than in BBc rats. There was no difference in colon GLP-1 peptide concentrations. However, we did observe lower proglucagon mRNA expression in the colon of BBdp rats as reported by others *(13)*, but this difference was not borne out by the GLP-1 peptide analysis. Therefore, the final concentration of bioactive GLP-1 peptides in the gut was not always reflected by differences in proglucagon gene expression.

GLP-1 or its long-acting analog, exendin-4, can increase β-cell mass in adult rodents, induce the expression of glucagon and insulin in the acinar cell line, AR42J, and stimulate expression of PDX-1 in INS-1 cells *(8,18)*. The biological effects of GLP-1 depend on its binding to a specific receptor on the β-cell. The present results show that GLP-1R$^+$ staining, which was mainly in islets, was higher in adolescent BBc than in BBdp pancreas. By this time, some of the BBdp animals were already displaying abnormally low levels of insulin in the circulation and in the pancreas (Table 1). Our previous studies showed that as early as 41 d of age, young BBdp rats have decreased islet area fraction *(19,20)*, in keeping with the low insulin levels in plasma and pancreas *(21)*. These data are consistent with a defect in insulin homeostasis, possibly related to inadequate sensing of islet β-cell mass-enhancing GLP-1 signals from the gut. It is also possible that unresponsiveness to the other major incretin, glucose-dependent insulinotropic polypeptide (GIP), is disrupted in these animals, a point that requires further study. One preliminary report indicated that treatment of BBdp rats with a DP IV inhibitor delayed diabetes onset, by ~10 d, and showed some improvements in glucose tolerance and severity of diabetes *(22)*.

Although it is known that the pancreas responds to information relayed from the gut, the extent to which this information is essential to the maintenance of islet mass in animals that spontaneously develop diabetes is unclear. Islet mass in BBdp rats is smaller than in BBc rats in the period before classic insulitis *(20,23)* suggesting that signals which normally maintain islet mass are lacking. Signals transmitted through GLP-1R are important in glucose metabolism and islet homeostasis as demonstrated by the finding that GLP-1R$^{-/-}$ mice display fasting hyperglycemia, abnormal glucose-stimulated insulin secretion, and decreased expression of pancreatic mRNA for insulin *(24)*. Decreased GLP-1R

mRNA levels in islets have been observed in other models of diabetes *(25,26)*. Thus, the low concentration of GLP-1 in the gut of BBdp rats combined with down-regulation of islet and extra-islet GLP-1 receptors that transduce signals for growth and differentiation could contribute to impaired endocrine function and maintenance of islet mass in these animals.

The localization of GLP-1R on insulin- and somatostatin-producing cells is well established. Heller et al. found that not only insulin and somatostatin-producing cells but also glucagon-producing cells express GLP-1R in rat pancreas *(27)*. In the present study we confirmed the presence of GLP-1R on β-cells and on the cells at the periphery of islets. Based on staining of adjacent sections, we demonstrate that all the endocrine cells in the islets contain GLP-1R. In addition, we observed the localization of GLP-1R in ganglia cells, and ductal epithelial cells. The function of GLP-1R expression on duct epithelial cells is not known. Considering that duct cells are a source of β-cell progenitors and GLP-1 can induce PDX-1$^+$ duct cells to differentiate into β-cells *(28)*, expression of GLP-1R on duct cells may be necessary for the maintenance of islet β-cell mass.

In summary, this study demonstrates that the jejunum and ileum of young diabetes-prone BB rats contain less bioactive GLP-1 protein compared with controls. GLP-1R mRNA and protein was also less expressed in BBdp pancreas. This difference could be attributable to the decreased islet mass in BBdp compared with BBc *(29)*, a point we cannot exclude. However, comparison of equal numbers of islets of the same size distribution showed that GLP-1R mRNA was threefold lower in BBdp islets compared with BBc suggesting a defect mainly at the level of the islet β-cells. The smaller second phase insulinogenic response to injected GLP-1 in BBdp rats compared with controls revealed a mild but significant functional difference. This suggests a partial defect in glucose-stimulated signaling whereby the triggering pathway responsible for first-phase insulin release was functioning normally but the amplifying pathways that control second-phase insulin release were less efficient in BBdp rats compared with diabetes-resistant BBc rats. The lower concentration of bioactive GLP-1 in the gut represents a decreased reserve of a known insulinogenic and islet β-cell mass promoting signal. A deficit of this nature could contribute to impaired maintenance of normal β-cell mass and shift islet homeostasis in favor of the development of diabetes. A smaller than normal islet mass may increase the susceptibility of these animals to develop diabetes, a point that finds some support in studies of human subjects *(30)*.

Materials and Methods

Animals and Diets

All studies were performed on specific-pathogen-free male and female diabetes-prone BB rats (BBdp) and non-diabetes-prone control BB rats (BBc) maintained at the Animal Resources Division of Health Canada (Ottawa, Canada) in closed colonies since 1977. Approximately 65–70% of BBdp animals develop overt diabetes between 60 and 120 d with a mean age at onset of 90 d. Unless otherwise noted, adolescent animals aged approx 45–63 d of age were used in these studies. Three sets of experiments are reported: *(1)* analyses of metabolic parameters and gut GLP-1, *(2)* pancreas GLP-1R studies, and *(3)* measurement of plasma insulin following iv challenge with GLP-1.

Experiment 1: Analyses of Metabolic Parameters and Gut GLP-1

Body weight was monitored for approx 2 wk, during which period the rats were housed in groups of three to five per cage. The animals were given free access to tap water and NIH-07 diet *(21)* up to the time of killing at approx 63 d of age. Animals were maintained in accordance with internationally recognized standards of animal care and permission was received from the animal care committee at each institution.

Blood was collected in heparinized tubes for the measurement of plasma D-glucose *(31)* and insulin *(32)* concentrations. The pancreas was divided into two parts, the first part was weighed and immediately homogenized in acid-alcohol *(33)* for measurement of insulin content *(34)*, and the second part was fixed in Bouin's solution for histological examination. The entire small intestine from the pylorus to the ileocecal opening and the large intestine (cecum excluded) was washed with cold 0.9% saline and trimmed free of all mesenteric tissues. The first 5 cm segment from the pylorus was separated and the remainder of the small intestine was divided in two equal parts: the proximal segment was designated as the jejunum and the distal segment as the ileum. Gut segments were frozen in liquid nitrogen and stored at −80°C until used for analysis of GLP-1 or extraction of RNA.

GLP-1 Analysis in Small Intestine

GLP-1 was extracted as previously described *(35)*, with some modifications. In brief, the frozen intestinal tissue was quickly minced in boiling water (5 mL/g wet tissue), and further boiled for 5 min; then glacial acetic acid was added to reach a final concentration of 0.5 *M*, and the mixture was homogenized and centrifuged at 10,000*g* for 20 min at 4ºC. An aliquot of the supernatant (extract of intestinal segment) was taken for protein determination *(36)*, and the remainder was lyophilized and re-dissolved in the GLP-1 assay buffer. GLP-1 content was measured by RIA. The samples, or GLP-1$^{7–36\ amide}$ standard were incubated for 4 d at 4ºC in 0.4 mL of 0.2 *M* glycine, pH 8.8, containing 0.25% HSA, 500 KIU/mL of Trasylol and 1 fmol mono-^{125}I-labeled tracer *(37)*, in the absence (non-specific binding) or presence of the corresponding anti-GLP-1 serum (at the final dilution given). Separation of free from bound peptide was achieved by treatment with dextran-coated char-

coal (0.5 mL 0.25%–0.50% in 0.2 *M* glycine, pH 8.8) and centrifugation at 4ºC. The antibody used was Antibody 98302 (1:120,000), which is N-terminally directed, reacts 100% with the bioactive forms of GLP-1: GLP-$1^{7–36\ amide}$ and its glycine-extended form GLP-$1^{7–37}$ and has only minor cross-reactivity with the inactive GLP-$1^{1–36\ amide}$ (0.1%) and the major proglucagon fragment (PG 72-158, 4%) (Deacon and Holst, personal communication). The antibody was a gift from Dr. J.J. Holst (Copenhagen).

Isolation of RNA and Northern Blot Analysis

Frozen tissue was homogenized in 4 *M* guanidinium thiocyanate, and total RNA was isolated *(38)*. Northern blots were performed as described previously *(39)*, except that a full-length rat proglucagon cDNA probe (the plasmid pBSGlu generously provided by Dr. S. Mosjov, New York) was used *(40)*. In each Northern, performed in duplicate, samples from two BBc and two BBdp rats were run simultaneously. The proglucagon mRNA values were normalized with respect to β-actin.

Experiment 2: GLP-1R Localization and Gene Expression in the Pancreas

Male and female animals aged 45–50 d were anesthetized with 3% halothane in oxygen and exsanguinated. Pancreata were removed, trimmed of fat, fixed in Bouin's solution for 18 h, and then placed in 70% ethanol. Pancreatic sections 5 μm thick were attached on silanized slides for staining of GLP-1R. Antigens were retrieved by heating the sections in a microwave oven for 10 min in citric buffer (pH = 6.0). Rabbit anti-rat GLP-1R antibody (1:2000), a gift from Daniel J. Drucker (The Toronto Hospital, Ontario, Canada), rabbit anti-human glucagon (1:3200, Dako Diagnostic, Canada), rabbit anti-human somatostatin (1:3200, Dako), rabbit anti-human pancreatic polypeptide (1:9600, Dako) were used as primary antibody. Goat anti-rabbit immunoglobulin (mainly IgG, 1:300, DAKO Diagnostic, Canada) conjugated with biotin was used as secondary antibody. Avidin–biotin complex (DAKO Diagnostic, Canada) was applied after incubation of the sections with secondary antibody. A dark brown color was developed using 0.06% diaminobenzidine (DAB, Sigma Chemical Co., St Louis, MO, USA) and 0.03% H_2O_2 as substrates. Sections were counterstained with hematoxylin.

Surface area of one pancreatic section (50×) from each animal was scanned using a Zeiss Axioplan2 microscope with motorized stage and Sony color CCD video camera. Thirty images were examined for area fraction measurement. Pancreatic section area was measured using Northern Eclipse software (Empix Imaging, Mississauga, Canada). GLP-$1R^+$ staining was displayed on the computer and a blue plane was subtracted from the images. Areas of interest were manually selected and grayscale thresholding was applied so that only the positive area was included in the area measurements. GLP-$1R^+$ area fraction was calculated by dividing GLP-$1R^+$ area by pancreatic section area. The GLP-1R antibody did not react with GLP-1.

Islets were isolated from 30-d-old BBdp and BBc rats (n = 4/group). Collagenase buffer was injected via the pancreatic duct *(41)*. Two hundred islets were hand picked under a dissecting microscope using a micropipettor. Total RNA was immediately extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions.

A GeneAmp RNA PCR Kit (Roche Molecular Systems, Inc., Branchburg, NJ) was used to synthesize cDNA. PCR was performed on three threefold dilutions of the products to enable linear regression analysis. PCR amplification of GLP-1R and β-actin genes was performed simultaneously at an annealing temperature of 59ºC for 25 cycles. The following primers were used: GLP-1R sense: 5'-AGTAGTGT GCTCCAAGGGCAT-3', anti-sense: 5'-AAGAAAGTGCG TACCCCACCG-3', product size 190 bp *(42)*; β-actin sense: 5'-CCAGCCTTCCTTCCTGGGTA-3', anti-sense: 5'-CT AGAAGCATTTGCGGTGCA-3', product size 343 bp. PCR products were run on a 1.3% agarose gel and band intensity was analyzed using a Kodak 440CF image station. The PCR cycles and cDNA concentrations were optimized for each primer set. Representative samples were run at different cycle numbers and the optimal cycle number was selected in the region of linearity between cycle number and PCR product intensity. The absence of PCR product signal from genomic DNA contamination was confirmed by performing PCR without reverse transcription of mRNA.

Experiment 3: GLP-1 Induced Insulin Secretion

Groups of non-fasted male and female rats matched for age, body weight, and gender were anesthetized with pentobarbital administered intraperitoneally (60 μg per g body wt.; Abbott Laboratories). At time zero, GLP-1 (Sigma) resuspended in saline (0.2 μ*M*) containing 10 mg/mL human serum albumin was injected intravenously (right femoral vein catheter) over 30 s at a concentration of 0.5 pmol/g body wt. In control experiments, the same volume of saline containing 10 mg/mL human serum albumin was administered intravenously instead of GLP-1.

Blood samples (0.5 mL) were collected, at –10, 0, 2, 5, 7, and 10 min, from a catheter inserted in the carotid artery for the measurement of D-glucose by the glucose oxidase method *(31)*, in blood (Glucocard Memory Strips, Menarini Diagnostics, Florence, Italy) and in plasma (Glucose analyzer 2; Beckman, Galway, Ireland). Plasma insulin was determined by RIA *(43)* and quantified using a standard curve of rat insulin (Linco, St. Charles, MO) and guinea pig anti-insulin serum. Immediately after each blood sample was collected, the same volume was replaced with saline through the femoral vein. Twenty minutes after test, the animal was euthanized by cutting the diaphragm and the pancreas was removed, weighed, and frozen at –70ºC for later homogenization in acid-alcohol for the measurement of insulin.

For insulin extraction, the pancreas was homogenized in 82% ethanol adjusted to pH 8.2 with phosphoric acid (2.5 mL/g tissue) at 4ºC, and then sonicated. The mixture was kept overnight at 4ºC and then centrifuged at 1800*g* for 15 min at 4ºC. The supernatant was collected and stored at 4ºC, the pellet was extracted with 65% ethanol, pH 8.2 (1.25 mL/g tissue) during 4–6 h at 4ºC, and then centrifuged (1800*g*, 15 min, 4ºC). Both supernatants were analyzed separately. The pellet was dissolved in 0.5 *N* KOH at 60ºC for protein determination *(36)*. For insulin measurement, extracted samples were dried (Speed-Vac), dissolved in RIA buffer, and analyzed as described above.

Presentation of Results

Results are presented as means ± SEM together with the number of individual determinations (*n*). The statistical significance of differences between mean values was assessed using Student's *t*-test or ANOVA with posthoc analysis.

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