Intestinal growth is associated with elevated levels of glucagon-like peptide 2 in diabetic rats

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Glucagon-like peptide 2 (GLP-2) has recently been identified as a novel intestinal growth factor. Because experimental diabetes is associated with bowel growth, we examined the relationship between GLP-2 and intestinal growth in rats made diabetic by streptozotocin (STZ) injection and treated with or without insulin for 3 wk. Ileal concentrations of the intestinal proglucagon-derived peptides, i.e., glicentin + oxyntomodulin, and GLPs 1 and 2, were increased by 57 ± 20% above those of controls in untreated STZ diabetes (P < 0.05–0.001). Similar increases in plasma concentrations of glicentin + oxyntomodulin (77 ± 15% above controls, P < 0.01) and GLP-2 (91 ± 32% above controls, P < 0.05) were seen in untreated STZ diabetes. Both wet and dry small intestinal weight increased by 74 ± 20% above controls (P < 0.01) in STZ diabetes, and macromolecular analysis indicated parallel increases in both protein (P < 0.001) and lipid (P < 0.05) content. Villus height (P < 0.01) and crypt depth (P < 0.01) were also increased in untreated diabetic rat intestine. Insulin therapy prevented the changes in plasma GLP-2 and intestinal mass seen in untreated STZ diabetes. Thus STZ diabetes is associated with both increased production of GLP-2 and enhanced bowel weight, thereby suggesting a role for GLP-2 in diabetes-associated bowel growth.

Materials and Methods

Induction of diabetes. Adult male Wistar rats (320–380 g; Charles River Canada, St. Constant, Quebec, Canada) were made diabetic on day 0 by injection of STZ (65 mg/kg; Sigma Chemical, St. Louis, MO) into a lateral tail vein. Age-matched control animals (controls) were sham-injected with vehicle (0.9% saline) only (n = 7–8 rats/group). STZ-treated rats were given 5% glucose in their drinking water for the first 24 h to counter any initial hypoglycemia. After injection, rats were housed individually in wire-bottom metabolic cages and permitted access to standard rodent chow and water ad libitum for 3 wk. On day 1 STZ-treated animals were pairwise matched on the basis of urine glucose, urine volume, and body weight. One pair was killed on day 4 after STZ or vehicle injection. Trunk blood was collected into chilled tubes containing a 10% volume of aprotinin (Trasylole)-EDTA-Diprotin A [5,000 killikren-inhibitor units/ml (Miles Canada, Etobicoke, Canada); 1.2 mg/ml; 0.1 mM (Sigma Chemical)], and plasma was collected after centrifugation at 4°C. The entire small intestine from pylorus to ileocecal junction was removed, flushed with ice-cold saline, and weighed. It was then divided into four equal pieces representing duodenum, proximal and distal jejunum, and ileum. Sections for analysis were collected from the identical anatomic position (as measured in

In mammals a single proglucagon gene is expressed in pancreatic a cells and intestinal L cells (20). In both of these tissues an identical prohormone is synthesized, and tissue-specific posttranslational processing yields the various proglucagon-derived peptides (PGDPs) (20). In the intestine, at least four distinct PGDPs are produced: glicentin, oxyntomodulin, glucagon-like peptide 1 (GLP-1), and GLP-2. It has recently been established that all of these peptides are produced through the action of prohormone convertase 1 (7), a processing enzyme expressed in intestinal L cells (28). GLP-1 has been extensively studied, and its role as a potent stimulator of glucose-dependent insulin secretion has been firmly established (21). Recent studies have now identified GLP-2 as a trophic factor for the small intestine (8, 29). Intestinal growth induced by GLP-2 is primarily due to an increase in villus height, resulting from enhanced crypt cell mitogenesis.

A number of reports have described an increase in gut weight in rodent models of poorly controlled diabetes (13, 19, 23, 26, 31). This appears paradoxical in view of the general catabolic phenotype of diabetes; however, it may constitute a compensatory response to a perceived state of starvation. Intestinal growth is apparent as early as 9 days after induction of diabetes and persists for the period of metabolic derangement (13, 31); the changes are reversible with insulin treatment (19, 31). During the early growth phase, the predominant characteristics are increased mucosal mass and enhanced DNA synthesis in the crypts (19, 26). Independent studies have shown elevated levels of both plasma and tissue intestinal PGDPs in diabetes. In rats, streptozotocin (STZ) treatment induces elevation of plasma glicentin and oxyntomodulin (4), as well as increases in the levels of ileal GLP-1 (14). In view of the recent demonstration that GLP-2 is trophic to the intestinal epithelium, we hypothesized that GLP-2 may be increased in diabetes and may play a role in diabetes-associated intestinal growth. To further study the relationship between GLP-2 and intestinal growth, we have now examined the synthesis and secretion of GLP-2 in STZ-induced diabetic rats as a model of gut adaptation.
centimeters from the pylorus and cecum) along the length of the small intestine.

Intestinal protein and lipid content. Wet weight was determined by weighing 5-cm segments from each small intestinal region (i.e., duodenum, proximal and distal jejunum, and ileum). These same segments were then lyophilized for \(\geq 12\) h and reweighed to obtain dry weight. They were then individually reconstituted overnight with a volume of distilled water equal to the difference between wet and dry weight. The reconstituted segments were subjected to lipid extraction following the method of Folch et al. (10). Briefly, tissues were homogenized in 20 volumes of chloroform-methanol (2:1). An additional 4 volumes of methanol were added and the segments were homogenized again. After centrifugation at 4°C, supernatant and pellet were separated, dried in vacuo, and weighed. Total protein content of the resuspended pellets was determined by Lowry protein assay (16).

Intestinal morphometry. Two-centimeter segments from each of the four small bowel regions were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Cross-sectional slices 5 µm thick were stained with hematoxylin and eosin. Micrometric quantitation was achieved using a Leitz microscope connected to a Mertz square-based micrometer (Leica Q500MC Image Processing and Analysis System, Leica Cambridge, Cambridge, UK). A minimum of 10 to a maximum of 70 villus-crypt units per section in 2–4 sections per region per rat were analyzed in a blinded fashion. Villus height, crypt depth, and muscle thickness (including submucosa) were recorded in micrometers (means ± SE) and averaged for each intestinal region.

RNA analysis. Five centimeters of distal ileum were homogenized in 5 ml of guanidium isothiocyanate (ICN, Montreal, QC, Canada). After phenol-chloroform extraction and ethanol purification, RNA was stored in pyrocarnonic acid diethyl ester water at \(-70°C\) until analysis by Northern blot, as previously described (4).

Peptide extraction. Ileal peptides for PGDP radioimmunoassays (RIAs) were extracted using reversed-phase adsorption techniques, as described previously (4, 5, 7). Briefly, 5 cm of distal ileum were homogenized in 10 ml of extraction medium (1 M HCl containing 5% formic acid, 1% trifluoroacetic acid (TFA), and 1% NAOH) at 4°C. Plasma for GLP-2 RIA was acidified by addition of 2 volumes of 1% TFA (pH adjusted to 2.5 with diethyl amine). Peptides and small proteins were then collected by adsorption to octadecysylsililic silica (C18 Sep-Pak, Waters Associates, Milford, MA), followed by elution with 80% isopropanol containing 0.1% TFA. Extracts were stored at \(-70°C\) before analysis.

Assays. Aliquots of ileal and plasma extracts were dried in vacuo before assay. RIAs for glucagon-like immunoactivity used antisera K4023 (Biospecific, Emeryville, CA), which is directed toward the midsequence of glucagon and recognizes glucagon, glicentin, and oxyntomodulin [glucagon-like immunoactive (GLI) peptides]. Immunoreactive glucagon (IRG) used antisera F4A (R.H. Unger, Dallas, TX), which recognizes the free carboxy-terminal end of glucagon and therefore detects glucagon but not glicentin or oxyntomodulin (4, 5, 7). Plasma gutGLI equals GLI minus IRG and thus represents plasma glicentin plus oxyntomodulin. Ileal GLP-1 immunoactivity was assessed using anti-GLP-1-(7–36)NH2 antiserum (Affinity Research, Nottingham, UK), which recognizes the COOH-terminally amidated forms GLP-1-(1–36)NH2 and GLP-1-(7–36)NH2 (7). Total immunoreactive GLP-2 (T-GLP-2) in the ileum was measured using antisemem UTH-7, which recognizes GLP-2-(25–30) and therefore detects GLP-2, proglucagon, and the major proglucagon fragment (GLP-2 attached to GLP-1) (3). Plasma NH3-terminal immunoreactive GLP-2 (N-GLP-2) was assessed using antiserum no. 92160 (a gift from Dr. J. J. Holst, Copenhagen, Denmark), which detects GLP-2-(1–33) but does not cross-react with GLP-2 degradation products, such as GLP-2-(3–33) (3). Ileal protein content was determined by Lowry protein assay (16). An insulin RIA kit (Linco, St. Charles, MO), with rat insulin as a standard, was used to determine plasma insulin levels. Plasma glucose values were determined by the glucose oxidase method (Glucose Autoanalyzer 2, Beckman Instruments, Fullerton, CA).

Statistics. All data are expressed as means ± SE. Statistical significance of data from animals after 3 wk of treatment was assessed by analysis of variance with n-1 custom hypothesis tests. Statistical significance of data from animals after 4 days of treatment was assessed by an unpaired t-test between untreated diabetics (STZ) and controls. All statistics were analyzed using an SAS program for IBM computers (Statistical Analysis Systems, Cary, NC).

RESULTS

Three weeks after STZ injection, the body weight of rats not receiving exogenous insulin was 340 ± 10 g, significantly lower than the 463 ± 10 g observed for vehicle-injected controls (\(P < 0.001\); Fig. 1). Insulin treatment partially prevented this weight loss, maintaining body weight at 92 ± 3% of controls (\(P < 0.05\)). Untreated diabetics were profoundly hypoinsulinemic (38 ± 13 vs. 414 ± 59 pmol/ml for controls; \(P < 0.001\)), and this loss of insulin was accompanied by hyperglycemia (466 ± 16 vs. 139 ± 4 mg/dl for controls; \(P < 0.001\)). Blood glucose appeared to have been poorly controlled by daily insulin injection; however, it should be noted that the rats were not fasted at the time they were killed and had received their last insulin injection 18 h before being killed.

Plasma concentrations of gut GLI (i.e., glicentin plus oxyntomodulin) in untreated diabetics were elevated to 177 ± 15% those of controls (\(P < 0.01\); Fig. 2). A parallel increase was observed for plasma N-GLP-2 (415 ± 69 vs. 217 ± 19 pg/ml for controls; \(P < 0.05\)). IRG levels in plasma were also slightly greater in uncontrolled diabetics (\(P < 0.05\)). Insulin replacement prevented the
GLP-1 increased to 160 expressing L cells (15). Ileal concentrations of GLI and it contains the greatest population of proglucagon-

Fig. 3. Ileal concentrations of GLI, GLP-136NH2, and total immunore-

plasma (*). The correlation between the concentrations of gut GLI and N-GLP-2 in diabetic control animals (*P < 0.001) is consistent with previous results (12, 31). Stereological analysis of intestinal mucosa revealed a significant increase in villus height and crypt depth (Fig. 5). Villus height was 51 ± 6% greater in diabetic intestine than in non-diabetic control intestine. This increase was associated with a significant increase in intestinal weight, which was not prevented by insulin treatment. These findings provide further evidence for a relationship between elevation in GLP-2 levels and the bowel growth response. The significantly augmented mucosal compartment observed in the present study is consistent with previous reports that describe elongated villi and enhanced crypt depth as the primary structural changes associated with experimental diabetes (18, 31). Stereological

increments in plasma levels of all the PGDPs. Interestingly, there was a highly significant correlation between the concentrations of gut GLI and N-GLP-2 in plasma (r = 0.86, P < 0.001).

Distal ileum was sampled for PGDP content because it contains the greatest population of proglucagon-expressing L cells (15). Ileal concentrations of GLI and GLP-1 increased to 160 ± 13 and 137 ± 13% of controls, respectively, in untreated diabetic animals (P < 0.05–0.001; Fig. 3A), a change that was prevented by insulin treatment. A similar trend was detected in ileal T-GLP-2 content, rising to 144 ± 31% of controls in STZ rats and remaining at control levels with insulin treatment. Analysis of total RNA from ileum showed increased levels of proglucagon mRNA transcripts in STZ animals (1.5-fold increase by laser densitometry; data not shown). Proglucagon gene expression in STZ+I animals was not different from that of controls.

The weight of the entire small intestine was significantly increased in untreated diabetes. Wet weight of STZ-diabetic bowel was 73 ± 18% (P < 0.01) and dry weight was 76 ± 20% (P < 0.001) greater than that of control animals (Fig. 4). With insulin treatment, diabetic small intestinal wet and dry weights were not different from control values. Macromolecular analysis of the small intestine indicated that the observed weight increase was due to overall increases in both the protein (P < 0.001) and lipid (P < 0.05) content of the diabetic gut.

Morphometry of bowel sections revealed major changes in the mucosal compartment in untreated diabetes (Fig. 5). Villus height was 51 ± 6% greater in duodenum from STZ-diabetic rats compared with non-diabetic control animals (P < 0.001), and this correlated strongly with plasma N-GLP-2 concentrations (r = 0.78, P < 0.01). Duodenal crypt depth was also augmented slightly in untreated diabetic animals (117 ± 5% of controls; P < 0.05). Insulin treatment prevented these increases in villus height and crypt depth. No differences in thickness of the underlying submucosal and muscle layers were detected between any of the treatment groups.

In a second series of experiments, rats were killed 4 days after STZ or vehicle treatment. In untreated diabetic rats, plasma levels of N-GLP-2 were significantly increased to 92 ± 57% above controls (P < 0.05; Fig. 2). Small intestinal wet weights of 4-day diabetic rats were not different from those of controls (data not shown).

DISCUSSION

Substantial evidence supports a role for the intestinal PGDPs in gut growth (2). Small bowel hypertrophy was described in a patient carrying a proglucagon-expressing tumor that secreted the intestinal PGDPs (11). Tumor resection resulted in normalization of the affected bowel, and subsequent injection of the tumor cells into normal mice stimulated intestinal growth in these animals (2). Recent work in our laboratory has shown that treatment of mice with exogenous GLP-2, but not with intervening peptide-2 or GLP-1, leads to a highly specific stimulation of intestinal epithelial proliferation (8, 29). The present study is the first to demonstrate increased concentrations of both plasma and tissue GLP-2 that correlate with intestinal growth and adaptation to STZ diabetes. Furthermore, both the elevation in GLP-2 levels and the bowel growth response were prevented by insulin therapy. Interestingly, the increments in plasma levels of GLP-2 preceded any changes in intestinal weight, thereby providing further evidence for a relationship between GLP-2 and the induction of intestinal growth in diabetes.
studies indicate that these changes are due to crypt and villus cell hyperplasia as well as crypt, but not villus, cell hypertrophy (31). Thus the villus-to-crypt cell ratio is unaltered in STZ diabetes (31). Increased activity of ornithine decarboxylase (ODC) and increased polyamine content have been associated with the hyperplasia of STZ diabetes (30). Treatment with difluoro-α-methylornithine, an ODC inhibitor, prevented the hyperplasia in these rats and delayed epithelial growth in control animals.

**Fig. 5.** Villus height (A), crypt depth (B), and muscle thickness (C) in intestinal sections from control (solid bars; n = 4), STZ (open bars; n = 4), and STZ+I (hatched bars; n = 4) rats after 3 wk of treatment. *P < 0.05; ***P < 0.001 vs. controls.

**Fig. 4.** Wet weight (A), dry weight (B), protein content (C), and lipid content (D) of 5-cm intestinal segments from control (solid bars; n = 4), STZ (open bars; n = 4), and STZ+I (hatched bars; n = 4) rats after 3 wk of treatment. *P < 0.05; **P < 0.01 vs. controls.
Untreated diabetic rats eat 2–3 times as much as controls, and it has been suggested that this hyperphagia accounts for the observed intestinal growth seen in these animals (13). However, diabetic rats pair-fed diets isocaloric with those of controls still exhibit significantly greater small bowel mass and enhanced crypt cell DNA synthesis (12, 19). This indicates that at least part of the growth response of intestinal adaptation in diabetes is independent of increased nutrient consumption. Nevertheless, the mechanism by which hyperphagia stimulates intestinal growth is unclear. The present study supports the idea that an increased nutrient load may stimulate release of hormones trophic to the intestine. The combination of hyperphagia and increased gastric emptying (12) observed in diabetic rats results in a greater nutrient load being delivered to the small intestine. Because luminal nutrients, including fat and carbohydrate, are physiological L cell secretagogues in vivo (6, 24), this provides a possible mechanism whereby increased luminal nutrients stimulate release of GLP-2 into the circulation, thereby exerting a trophic action on the intestinal epithelium.

Release of gastrin, a hormone trophic to the mucosa of stomach, proximal small intestine, and colon (9), is also stimulated by nutrient intake. Three weeks after induction of diabetes, plasma and antral gastrin concentrations are 1.5- to 2-fold higher in ad libitum-fed diabetic rats compared with nondiabetic controls (27). The increase in duodenal villus height and crypt depth observed in the present study could therefore be due in part to the action of gastrin. However, because the mitogenic effects of gastrin in the small intestine are specific to the duodenum (9), elevated plasma levels of this hormone cannot account for the increased growth response observed in more distal regions of the bowel. Furthermore, after massive small bowel resection, serum gastrin levels do not always correlate with the observed intestinal growth in the nonresected remnant (22).

Other humoral factors, such as growth hormone (GH) and insulin-like growth factor I (IGF-I), may also play roles in adaptation of the gut to diabetes. In STZ diabetes, plasma levels of GH are reported to increase (25), but those of IGF-I, a molecule believed to be an important mediator of GH action, are depressed to ~50% those of nondiabetic controls (1). Given the general catabolic state of diabetic animals, it is unlikely that fluctuations in plasma concentrations of general somatic growth factors like GH and IGF-I are responsible for the organ-specific changes observed in experimental diabetes. It is more likely that molecular candidates for promoting intestinal adaptation would be paracrine/autocrine regulators and/or tissue-specific trophic hormones such as gastrin and GLP-2.

In summary, these data are the first demonstration that plasma and tissue concentrations of GLP-2 are upregulated in a physiological model of intestinal adaptation. Our results provide strong correlative evidence linking the synthesis and secretion of the intestinal PGDPs, including GLP-2, to the adaptive response of the small bowel in STZ-induced diabetes in rats. Because GLP-2 is the major intestinal PGDP with significant trophic effects on the bowel (8, 29), our data strongly implicate GLP-2 as the mediator of small intestinal growth in uncontrolled diabetes.

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