ARTICLE

Hepatic insulin gene therapy prevents diabetic enteropathy in STZ-treated CD-1 mice

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Depending on the population examined, from 6 to 83% of people with diabetes mellitus exhibit symptoms of altered gut motility, manifesting as dysphagia, reflux, early satiety, nausea, abdominal pain, diarrhea, or constipation. Hyperglycemia-induced cell loss within the enteric nervous system has been demonstrated in both diabetic rodents and patients with diabetes. Glycemic control is recommended to prevent diabetic gastroenteropathy but is often difficult to achieve with current treatment modalities. We asked if hepatic insulin gene therapy (HIGT) could inhibit the development of diabetic gastroenteropathy in mice. Bowel length, bowel transit, colonic muscle relaxation, and the numbers of both stimulatory and inhibitory neurons in the colonic myenteric plexus were compared in groups of diabetic mice (DM), control nondiabetic mice (Con), and diabetic mice treated with HIGT (HIGT).

Delivery of a metabolically responsive insulin transgene to the liver of STZ-diabetic mice with an adeno-associated virus, sero-type 8 (AAV8) produced near-normal blood sugars for over 1 month and prevented anatomic, functional, and neurohistologic changes observed in diabetic mice. We conclude that in addition to normalizing oxidative metabolism in diabetic rodents, HIGT is sufficient to prevent the development of diabetic gastroenteropathy.

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INTRODUCTION

Approximately 29 million people in the United States have diabetes mellitus,1 and many exhibit some form of diabetes-associated enteropathy presenting as altered gut motility.2,3 The frequency of clinical complaints varies with the population studied, ranging from 6–27% in unselected patients with diabetes in the community setting to 76–83% in patients referred to tertiary care facilities.2,3 Symptoms include dysphagia, reflux, early satiety, nausea, abdominal pain, diarrhea, or constipation, either alone or in concert.2,3 The breadth of clinical symptoms displayed in diabetic gastroenteropathy reflects observations that all portions of the gastrointestinal tract can be affected by diabetes.2 Moreover, within a given anatomical segment of the gut, the etiology of dysfunction is likely multifactorial, ranging from dysfunction of either the autonomic nervous system (sympathetic and parasympathetic) or enteric nervous system (ENS), to altered trophic factor signaling resulting in enteric myopathy and neural apoptosis.2,4

Among the multiple factors producing bowel dysfunction in diabetes, hyperglycemia-induced cell loss within the ENS appears to play a significant role.5,6 The ENS, an arm of the peripheral nervous system with at least as many neurons as the spinal cord, is primarily responsible for autonomously mediating gut motility responses to the physical and biochemical composition of bowel contents.2,3 The ENS, with its assortment of neuronal and glial cells coordinating the action of both stimulatory and inhibitory signals, is localized within two plexuses, one between the longitudinal and circular smooth muscle layers, the myenteric plexus, and one within the submucosa, the submucosal plexus.7 Recent observations indicate that hyperglycemia-induced neuronal loss, particularly of inhibitory neurons within these plexuses, plays a significant role in altered gut function.5,8

Insulin therapy is necessary to control glycemia in all patients with type 1 diabetes mellitus and in ~30% of patients with type 2 diabetes.2,3 Treatment with exogenous insulin improves blood sugars but is often insufficient to prevent long-term complications.4,5 While intensification of insulin regimens reduces hyperglycemia, it also increases the incidence of insulin-induced hypoglycemia.8

Gene therapy may provide a means to improve and simplify treatment of diabetes mellitus. Hepatic insulin gene therapy (HIGT) has been shown to produce near-normal random blood glucose in multiple rodent models of diabetes mellitus.10–13 Significantly, HIGT-treated animals tolerate chow deprivation without lethal hypoglycemia and exhibit normal fluctuations of diurnal oxidative metabolism.14 Moreover, the glycemic control induced by HIGT was sufficient to prevent diabetes-associated macrovascular dysfunction.15
We undertook this study to determine if HIGT could inhibit the development of diabetic gastroenteropathy in mice. We compared bowel length, bowel transit, muscle relaxation, and the numbers of both stimulatory and inhibitory neurons in the myenteric plexus of diabetic mice (DM), control nondiabetic mice (Con), and diabetic mice treated with HIGT (HIGT). Delivery of a metabolically responsive insulin transgene to the liver of STZ-diabetic mice with an adeno-associated virus, sero-type 8 (AAV8) produced near-normal blood sugars for over 1 month, and this level of glycemic control was sufficient to prevent anatomic, functional, and neurohistologic changes observed in diabetic mice. We conclude that in addition to normalizing oxidative metabolism in diabetic rodents, HIGT is sufficient to prevent the development of diabetic gastroenteropathy.

**RESULTS**

**Blood glucose and body weight**

Average body weights and blood glucose of control (Con), diabetic (DM), and HIGT-treated (HIGT) mice were similar at baseline prior to induction of diabetes (Table 1). On day 0, following STZ or buffer injection, body weights of DM and HIGT declined and blood glucose values increased to consistently greater than 200 mg/dl, a diagnostic threshold for diabetes mellitus chosen a priori (Figure 1). Analysis post hoc of random blood sugar values obtained for Con (n = 1,016; average ± SD = 151 ± 22 mg/dl), and calculation of (average + (2× SD)) provided an upper limit for normal of 195 mg/dl, confirming the utility of the selected threshold value. At day 0, average blood sugars were higher among DM than HIGT (Table 1). All mice underwent laparotomy and received similar quantities of virus, AAV2/8-SC-(GIRE3) BP-1 2xfur to HIGT, and AAV2/8-SC-CMV GFP to both Con and DM, administered by hepatic portal system injection on day 0. The average time between STZ and virus administration was similar among HIGT (7.4 ± 3.7 SD days) and DM (6.63 ± 1.8 SD days) mice (P = 0.47 for difference). All mice were sacrificed 50 days after virus administration.

Treatment of Con and DM mice with AAV 2/8 SC CMV GFP failed to modify blood sugars. Blood glucose among Con mice remained below 200 mg/dl, and blood glucose among DM mice remained persistently above 500 mg/dl (Figure 1). In contrast, treatment of HIGT with AAV 2/8 SC G3 2xfur reduced blood glucose...
beginning 4 to 6 days following viral injection. HIGT mice consistently achieved normal blood glucose levels (<200 mg/dl) beginning ~11 days after receiving virus (Figure 1a). After declining to less than 200 mg/dl, blood sugars in HIGT-treated mice maintained a mean of 133 ± 3.87 mg/dl, less than the mean blood glucose of 147 ± 1.01 mg/dl for control mice (P < 0.01). However, variance among HIGT blood sugars was greater than Con (F-test, P < 0.0001).

Following abdominal surgery and viral injections, all mice lost weight. With the support of exogenous insulin injections, DM mice gained weight beginning 3 days after virus administration but weighed consistently less than Con mice. In contrast, despite receiving no insulin injections once blood glucose values began to decline, HIGT mice gained weight beginning 4 days after virus injection, achieved normal weight by 12 days postsurgery, and subsequently grew similarly to Con mice. Final body weight of Con and HIGT mice was similar (Table 1; Figure 1b; P > 0.05).

Consistent with lower body weights and prolonged hyperglycemia, mesentery weight was diminished among DM (Figure 1c). In contrast, mesentery weight in HIGT mice was similar to Con (Figure 1c). The average weight of epididymal fat pads was reduced in DM (Figure 1d). Epididymal fat pad weight was greater in HIGT than in DM (P < 0.01). However, despite similar body weights and blood glucose values, epididymal fat pad weight in HIGT was less than Con (P < 0.01).

Serum hormones and adipokines
Serum insulin levels were diminished and glucagon levels were increased in DM compared to Con mice (Table 2). Despite having also received STZ, insulin concentrations among HIGT mice measured with a mouse insulin assay were similar to Con and greater than DM mice. As expected, human insulin was undetectable in Con and DM mice but was also not detected in HIGT mice (HIGT 0.42 ± 0.24 mU/ml (n = 10), DM 0.58 ± 0.39 mU/l (n = 19), and Con 0.31 ± 0.17 mU/l (n = 11); analysis of variance, P = 0.728). Transgenic human insulin expression was confirmed in HIGT animals and excluded in Con mice by RT-PCR (Figure 2a,b). Consistent with extant insulin levels, serum glucagon concentrations in HIGT mice were similar to Con, and lower than that in DM mice (P < 0.001). While serum leptin concentrations among DM mice were diminished compared to Con mice, HIGT treatment increased serum leptin concentrations (P < 0.01 vs. DM). However, serum leptin values in HIGT remained below those found among Con mice (P < 0.05). No differences in serum concentrations of resistin, IL-6, PAI-1, or adiponectin were observed. Amounts of TNFα and MCP-1 remained below the level of detection for all groups.

Gut anatomy, gastric emptying, and insulin signaling
Percent gastric emptying was similar across groups (Figure 3a). Total bowel length was increased among DM mice compared to both Con and HIGT mice (Figure 3b). In contrast, bowel length was similar between HIGT and Con mice. Thirty-minute dye transit distance was similar across groups (Figure 3c). However, due to the increased intestinal length, percent intestinal transit among DM mice was lower than either Con or HIGT mice (Figure 3d).

We assessed insulin signaling by western blot and phosphoprotein assay of insulin receptor (IR), AKT, and GSK3 in subject mice. Tissue content of insulin receptor (IR)β was similar across groups (Figure 4a,b). However, phosphorylated IRβ was diminished in both DM and HIGT mice (Figure 4c). Despite the reduction in IRβ phosphorylation, the amount of phosphorylated (p-) AKT in ilea of DM mice assessed by western blot tended to be elevated compared to Con, but similar in HIGT and Con (Figure 4d,e). Analysis by phosphoprotein assay confirmed similar amounts of p-AKT in Con and HIGT ilea, and increased p-AKT in DM mice compared to both Con and HIGT (Figure 4f). We observed a similar pattern of GSK3 phosphorylation among the three groups (Figure 4g).

### Table 2: Hormones and adipokines determined in sera of control, diabetic, and HIGT-treated mice

|                      | Control     | DM          | HIGT        |
|----------------------|-------------|-------------|-------------|
| Insulin (pmol/l)     | 1,584.90 ± 302* | 5,975.50 ± 234** | 1,850.15 ± 226 |
| Glucagon (pmol/l)    | 53.93 ± 9.2  | 119.50 ± 19* | 36.94 ± 6.7** |
| Leptin (pmol/l)      | 3,588.52 ± 282** | 740.77 ± 347* | 2,492.73 ± 324** |
| Resistin (pg/ml)     | 3,482.78 ± 198** | 2,594.15 ± 274** | 3,478.99 ± 433** |
| Adiponectin (µg/ml)  | 8.18 ± 0.6  | 7.17 ± 0.7  | 8.36 ± 0.9  |
| PAI-1 (pg/ml)        | 2.633 ± 293  | 2.985 ± 590  | 4.654 ± 1099 |
| IL-6 (pg/ml)         | 23.22 ± 5.1  | 16.41 ± 5.0  | 28.12 ± 5.3  |

Value of insulin in HIGT-treated mice reflects cross-reactivity in murine insulin assay. Data are presented as mean ± SD; n = 8–20 for each condition. Con, control nondiabetic mice; DM, diabetic mice; HIGT, hepatic insulin gene therapy. *P < 0.05 versus control; **P < 0.01 versus DM, by t-test after one-way analysis of variance with P < 0.05.

![Figure 2: Transgenic human proinsulin expression in livers of HIGT mice.](image-url)
Colonic function, neuronal assessment, and insulin signaling

We assessed colonic function by measuring isometric contraction and relaxation of proximal colonic muscle strips in response to EFS and by measuring stool frequency and water content. Colonic smooth muscle contraction was similar among Con, DM, and HIGT mice (Figure 5a). In contrast, colonic smooth muscle relaxation was impaired in DM mice and normalized by HIGT treatment (Figure 5b). Stool frequency was similar across groups (Figure 5c). However, stool water content was diminished among DM mice and corrected by HIGT treatment.

Sustained EFS-induced colonic smooth muscle contraction combined with impaired relaxation suggested a selective impairment of inhibitory neurons with relative sparing of stimulatory neurons. We observed no difference in the number of nuclei staining for ACh, i.e., stimulatory neurons within the colon (Figure 6a). In contrast, the number of NADPH diaphorase staining nuclei, i.e., inhibitory neurons, was diminished among DM mice (Figure 6b). This deficit in inhibitory neurons was prevented by HIGT treatment.

Colonic abundance of IRβ was similar across groups (Figure 7a,b). Similar to the ileum, phosphorylation of IRβ was reduced in both DM and HIGT (Figure 7c). Western blotting indicated a profound reduction in p-AKT in the colons of DM and HIGT compared to Con (Figure 7d,e). While phosphoprotein assay revealed a tendency toward a reduction in p-AKT among HIGT, it failed to confirm any reduction in DM (Figure 7f). The pattern of phosphorylated GSK3 across groups was similar to p-AKT (Figure 7g).

**DISCUSSION**

We have demonstrated that treatment of diabetic mice with a metabolically responsive insulin transgene prevents the development of abnormal gastrointestinal anatomy, colonic loss of inhibitory neurons, and altered colonic muscle function, while normalizing blood sugars, body weights, and growth. These data demonstrate the efficacy of HIGT to inhibit the development of diabetic enteropathy.

Enteropathy associated with diabetes mellitus presents in up to 75% of patients and encompasses symptoms of nausea, bloating, abdominal pain, diarrhea, or constipation. This diversity of symptoms suggests that multiple processes are impacted by diabetes. However, accumulating evidence indicates that impairment of the ENS may be a common mediator of gastrointestinal dysfunction in diabetes. The ENS autonomously controls and coordinates the motility, blood flow, and secretion of the gastrointestinal system and displays rapid dysfunction when exposed to hyperglycemia associated with diabetes. The ENS network of sensory, motor, and interneurons, connected via the myenteric and submucosal plexuses, inervates the entire GI system, responding to luminal content and smooth muscle tension. Animal studies suggest variable susceptibility to diabetes-associated neuronal impairment and damage depending on the duration of hyperglycemia, the type of neuron, and anatomic location. However, in general, inhibitory, nitrergic neurons appear to be more susceptible to damage imposed by diabetes than stimulatory, cholinergic neurons.

In this study, insulin transgene delivery by adeno-associated virus (AAV) restored average random blood sugars to normal in STZ-diabetic mice. Similar to delivery with adenovirus, delivery by AAV induces insulin expression in the liver (Figure 2; Thulé et al. manuscript submitted) but fails to fully normalize blood glucose variance. Serum insulin concentrations appeared to normalize in HIGT-treated mice. However, the modified human insulin produced...
by our transgene exhibits a concentration-dependent enhancement of cross-reactivity with the mouse insulin assay utilized in these studies (Thulé, data not shown). In addition, the transgene product in HIGT mice is poorly assessed by human insulin-specific ELISA, as indicated by the similar concentrations observed in HIGT, DM, and Con animals. Consequently, the actual concentration of circulating human insulin in the HIGT-treated mice remains unclear. Irrespective of the measured insulin concentration, HIGT treatment successfully normalized average blood sugars and body weight, as well as circulating glucagon and adiponectin levels. In contrast to HIGT mice treated with adenovirus, HIGT mice treated with AAV failed to exhibit increased serum resistin levels, suggesting that the adenoviral vector, and not the insulin transgene, were responsible for previously documented elevations. Interestingly, circulating leptin levels in HIGT treated mice increased compared to DM, but remained less than Con, a pattern previously observed in HIGT treated diabetic rats. Circulating leptin is produced by adipose tissue. It is thus noteworthy that epididymal fat pads were smaller in HIGT-treated mice than in controls, despite similar body weights, confirming another finding in HIGT-treated rats. HIGT-treated rats produce more heat during both feeding and fasting than control animals, possibly due to greater expression of uncoupling protein-3 in muscle. This phenomenon remains to be confirmed in HIGT-treated mice.

The etiology of diabetic gastrointestinal complications involves multiple factors. However, damage to the ENS is likely involved in both human subjects with diabetes mellitus and in diabetic animal models. Anitha et al. demonstrated delayed gastric emptying, aberrant colonic muscle relaxation, and diminished inhibitory colonic neurons secondary to enteric neuronal apoptosis in STZ-diabetic mice. Our findings of intact gastric emptying among DM may reflect strain differences between B6/CBA, and C57 in-bred mice, both of which exhibit prolonged gastric emptying following exposure to hyperglycemia, and the out-bred CD-1mice used in these studies. Our DM mice exhibited impaired colonic muscle relaxation and diminished numbers of inhibitory colonic neurons in the myenteric plexus.

While our study suggests that controlling glycemia is sufficient to inhibit diabetes associated gastrointestinal dysfunction, we did not evaluate other potentially influential factors. Alterations and interactions between intestinal permeability, microbiome, and the

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**Figure 4** Insulin signaling in ilea of control, DM, and HIGT-treated mice. (a,b) Total β-subunit of the insulin receptor (IRβ) was compared across groups by controlling for β-actin content. (c) Phosphorylated IRβ was assayed in a Luminex-based multiplex bead analyzer. (d,e) The amount of phosphorylated AKT was compared to total AKT based on the presented western blot or (f) assayed using assay in a Luminex-based multiplex bead analyzer. (g) The quantities of phosphorylated GSK3α/β were measured using a Luminex-based multiplex bead analyzer. n = 3–4; *P < 0.05 versus Con; #P < 0.05 versus DM. Con, control nondiabetic mice; DM, diabetic mice; HIGT, hepatic insulin gene therapy; IR, insulin receptor.
immune system may impact gut function, either directly or indi-
rectly through effects on the ENS. An altered intestinal microbiome
clearly impacts disease incidence among rodent models of autoim-
mune diabetes, and alterations in gut bacteria appear to precede
development of type 1 diabetes in children.22–24 Moreover, intestinal
permeability to gut flora, and the intestinal immune response, are
abnormal in subjects with type 1 diabetes, raising the possibility that
subsequent intestinal inflammation may underly gastrointestinal
dysfunction.23 Similarly, it remains possible that STZ treatment may
have produced gastrointestinal dysfunction that was exacerbated
by hyperglycemia. We were unable to identify literature reporting
a direct effect of STZ on either enteric neurons or gastrointestinal
function. However, a study similar to ours in non–STZ-mediated
model of diabetes would be required to exclude an STZ effect.

The DM mice in this study also exhibited bowel elongation and a
consequent percent delay in bowel transit. Because we only mea-
sured total bowel lengths, we cannot determine if the large or small
bowel was elongated, or whether both segments were affected.
Mayhew and Carson demonstrated that 12 weeks of hyperglycemia is sufficient to induce small bowel hyperplasia, increasing circumference, crypt, and villus height, as well as length in STZ-diabetic Sprague Dawley rats. The increase in small bowel length (from 114 to 121 cm) failed to reach statistical significance, but their diabetic rats were severely catabolic, losing 1.8 gm body weight per day. In contrast, Domènech et al. observed significant elongation in both the small intestine and colon of diabetic mice. However, the specific drivers of bowel elongation remain poorly defined.

Interestingly, oral insulin reduces the diabetes-induced mucosal hypertrophy observed in STZ-treated rats, raising the possibility that hepatic insulin may be secreted into the bile of HIGT-treated mice and restrict bowel growth. However, this remains speculation. Similar to Anitha et al., we observed a decline in colonic inhibitory neurons associated with impaired relaxation of colonic muscle in DM mice, and both of these findings were prevented by HIGT treatment, consistent with normalization of blood sugars. These results stand in contrast to those of Domènech et al., who failed to detect differential effects of hyperglycemia on nNOS or ACh staining neurons in vivo but documented a reduction in total myenteric neurons. In their study, Anitha et al. demonstrated that hyperglycemia-induced apoptosis was associated with diminished phosphorylation of PI3K and AKT, an effect that was inhibited both in cell culture and in vivo by glial derived neurotrophic factor. Interestingly, insulin signaling was not universally restored in gut tissue of HIGT-treated mice. Indeed, in ilea and colons, phosphorylation of IRβ was reduced compared to Con, and in colons p-AKT was diminished, consistent with diminished signaling through PI3K. None the less, phosphorylation of AKT and GSK3 were normalized in ilea of HIGT animals, and function was preserved in the ilea and colons of HIGT mice. However, it should be noted that the current study assessed insulin signaling in full-thickness ileum and colon samples, rather than the isolated enteric neurons examined by Anitha et al. Furthermore, increased amounts of p-AKT in whole-thickness ileum DM samples suggest increased activity of non–IR-mediated AKT activation, raising the potential for a combination of insulin and non–IR-mediated events in HIGT samples. Consequently, it remains possible that HIGT differentially affects insulin signaling within myenteric neurons, or induces
local secretion of alternative neurotropic factors, such as glial derived neurotropic factor, that counter the effects of diminished insulin signaling and excessive non–insulin-mediated signaling. Alternatively, preservation of enteric neurons and bowel function may reflect the glycemic control afforded by HIGT. Whether the aberrant intestinal insulin signaling observed in HIGT animals remains sufficient to preserve function over time periods longer than the current study will have to be investigated.

The capacity of metabolically responsive HIGT to effectively control glycemia has been established in multiple rodent models and a large animal model of diabetes mellitus. However, evidence that HIGT can effectively prevent morbidities associated with diabetes mellitus is sparse. Utilizing impaired endothelium-mediated aortic ring contractility as a surrogate marker for hyperglycemia associated vasculopathy, Thulé et al. demonstrated that HIGT can prevent diabetes-induced large vessel disease. In addition, Chen et al. were able to prevent the development of diabetic nephropathy and ocular cataract formation in pigs following hepatic insulin transgene production. The current study expands the efficacy of HIGT to prevent diabetic complications to the intestinal tract.

In summary, we successfully established a model of diabetic enteric neuropathy using STZ in CD-1 mice with autonomic, functional, and molecular changes. We also demonstrated that HIGT can prevent autonomic, functional, and molecular aspects of diabetic enteric neuropathy aside from normalization of blood glucose, weight growth, and hormone level with single injection of AAV2/8 SC G3 2xfur. Compared to other therapies, HIGT still has some advantage for treating STZ-induced diabetic mice. However, questions remain for hepatic insulin gene therapy. First of all, the precise mechanism of how HIGT can prevent enteric neuropathy remains unclear. Second, whether HIGT can reverse diabetic enteric neuropathy is still unknown. Last but not least, whether HIGT can prevent diabetic enteric neuropathy in large animals or human requires further study.

**MATERIALS AND METHODS**

**Animals**

Male CD-1 virus-antigen free (VAF plus) mice (Charles River Laboratories, Wilmington, MA) were group-housed in an AAALAC-accredited animal care facility under a 12:12 light–dark cycle. Water and chow was provided *ad libitum*. Mice in the DM and HIGT groups received i.v. streptozotocin (STZ, 200 mg/kg; Sigma, St Louis, MO) dissolved in 0.05 mol/l citrate buffer (pH 4.5) via penile vein injection. Control mice received injections of citrate buffer alone. Blood sugar and body weight was monitored on tail-tip venous plexus blood every 1 to 3 days using a hand-held blood glucose monitor (Freestyle; Abbott, Abbott Park, IL). Mice were diagnosed with diabetes mellitus upon two consecutive random daily blood glucose determinations greater than 200 mg/dl. In response to two consistent findings of body weight decline, STZ-treated mice received a single s.c. injection of glargine insulin (usually 0.2 U) calculated to prevent further weight loss, but not reduce blood sugars.

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**Figure 7** Insulin signaling in proximal colon of control, DM, and HIGT-treated mice. (a,b) Total β-subunit of the insulin receptor (IRβ) was compared across groups by controlling for β-actin content. (c) Phosphorylated IRβ was assayed in a Luminex-based multiplex bead analyzer. (d,e) The amount of phosphorylated AKT was compared to total AKT based on the presented western blot or (f) measured using assay in a Luminex-based multiplex bead analyzer. (g) The quantities of phosphorylated GSK3α/β were measured using a Luminex-based multiplex bead analyzer. Con, control; DM, diabetic mice; HIGT, hepatic insulin gene therapy.
All procedures were approved by the VA/Emory University Institutional Care and Use Committee.

Virus production and administration

Pseudotyped AAV8 virus was produced by first cloning coupled promoter and expression sequences, CMV GFP or (GIRE)BP-1 2xfur, into plasmids between adenovirus-associated virus, serotype 2, inverted terminal repeats, of which the terminal resolution sequence of the 5′ inverted terminal repeats had been excised, permitting viral packaging of self-complementary, double-stranded viral genomes, to create pSC-CMV GFP and pSC-(GIRE) BP-1 2xfur.10 Triple cotransfection of 120 µg total DNA containing equimolar ratios of pSC-CMV GFP or pSC-(GIRE)BP-1 2xfur, pAAV2rep-AAV8cap (graciously provided by Dr James Wilson, University of Pennsylvania, Center for Gene Therapy), and pDF (a gift from Roland Herzog and Weidong Xiao, The Children’s Hospital of Philadelphia, Philadelphia, PA), which expresses adenoviral helper-proteins, using calcium phosphate (Promega, Madison, WI) to prevent volume depletion. All mice received a similar dose, unless otherwise indicated. All mice were euthanized 50 days after development of hyperglycemia. Control of normal saline. HIGT mice received AAV2/8-SC-(GIRE3)BP-1 2xfur (2.2 × 1010 vg) by mesenteric vein injection after developing hyperglycemia. Control and DM mice received an equal volume of PBS.

Colonic segment isometric muscle recordings

Longitudinal strips of proximal colons of Control, DM, and HIGT mice suspended on hooks linked to an isometric force transducer were placed between platinum electrodes in 15 ml chambers containing 37 °C Krebs buffer (NaCl, 118 mmol/l; KCl, 4.7 mmol/l; KH2PO4, 1.2 mmol/l; MgCl2, 1.2 mmol/l; NaHCO3, 23 mmol/l; EDTA, 0.3 mmol/l; glucose, 10 mmol/l; and CaCl2, 2.5 mmol/l; pH 7.4) continuously gassed with 95% O2 and 5% CO2. After a 1-hour equilibration at 4.9 mN, tension was continuously recorded using the PowerLab recording software (version 5.2.1; AD Instruments), as relaxation was induced by transmural electrical field stimulation (EFS) (24 V, 4 Hz, repeating 0.03 millisecond pulses for a duration of 20 seconds) in the presence of atropine (1 µmol/l) and guanethidine sulfate (1 µmol/l) to block cholinergic- and adrenergic-mediated responses, respectively. Strips were precontracted with 5-hydroxytryptamine (10 µmol/l) for 30 seconds prior to EFS. The first induced relaxation was used for quantitative determination of each sample. Relaxation was abolished by coincubation with tetrodotoxin (10–7 mol/l), indicating mediation by a neuronal pathway. Constriction was measured by applying EFS 20 minutes after incubation with N[+]-nitro-arginine methyl ester (100 µmol/l). Contraction or relaxation was expressed as a % change from baseline muscle tone. For quantitative determination of constriction or relaxation, intestinal strips from at least three mice were used, and the average of contractions or relaxations was obtained from EFS.

Stool frequency and water content

Mice placed individually in a clean cage were observed throughout a 60-minute collection period. Fecal pellets were collected upon expulsion, counted, and placed into sealed, preweighed 1.5 ml tube. Tubes were weighed to obtain the wet weight of the stool, which was then dried overnight at 65 °C and reweighed to obtain the dry weight. Stool water content was calculated from the difference between the wet and dry stool weights.

Liver real-time RT-PCR

Total RNA was extracted from tissues flash frozen in liquid nitrogen and stored at −70 °C using TRI reagent (Ambion, TX, USA) or Trizol (Gibco BRL, Gaithersburg, MD) and treated with DNA-Free (Ambion) per manufacturer’s instructions. After quantification of RNA by spectrophotometry, cDNA was reverse transcribed from 1.0 µg of total RNA in a 20-µl reaction using an M-MLV RT (all from Promega, Madison, WI). Subsequent to fixing intestinal segments 1 hour at room temperature in 4% paraformaldehyde, the mucosa and circular muscle layer were separated from the preparation, while the longitudinal muscle layer and the myenteric plexus were subjected to histologic staining. The most distal ileum was excised and mounted on a glass slide. For both NADPH diaphorase- and ACh-stained segments, the most distal ileum was excised and mounted on a glass slide. For ACh staining, fixed tissue was washed in PBS, incubated in fresh copper buffer solution (100 ml dH2O, 7.2 mg ethopropazine, 115.6 mg acetyltiocoline iodide, 75.0 mg glycine, 50.0 mg copper sulfate pentahydrate, 885.0 mg sodium acetate trihydrate; pH to 5 with glacial acetic acid) for 2 hours, washed in dH2O, incubated for 1 minute in 1.25% sodium sulfide nonahydrate solution, re-washed in dH2O, and mounted on a glass slide. For both NADPH diaphorase- and ACh-stained tissues, nuclei of myenteric neuronal fibers were manually counted within 20 randomly selected squares of a 0.1 mm2 microscope grid. Samples from 6–10 mice for each experimental condition were assessed.

Histochemical tissue staining

At sacrifice, three 4-cm serial segments of colon, beginning at the cecum, were freed of mesentery, cut longitudinally to expose the mucosa and pinned mucosa side down onto hardend silicone gel in a glass dish. Subsequent to fixing intestinal segments 1 hour at room temperature in 4% paraformaldehyde, the mucosa and circular muscle layer were separated from the preparation, while the longitudinal muscle layer and the myenteric plexus were subjected to histologic staining. The most distal ileum was stained for peripherin, the most distal segment for NADPH diaphorase, and the most proximal segment for acetycholineesterase (ACh) staining. For NADPH diaphorase staining, fixed tissue was washed in PBS, incubated in diaphorase solution (15 µg/ml nitroblue tetrazolium, 0.1 mg/ml nitroblue tetrazolium, 0.2% Triton X-100 in PBS) for 1 hour at 37 °C, washed in PBS, mounted on a glass slide. For ACH staining, fixed tissue was washed in PBS, incubated in fresh copper buffer solution (100 ml dH2O, 7.2 mg ethopropazine, 115.6 mg acetyltiocoline iodide, 75.0 mg glycine, 50.0 mg copper sulfate pentahydrate, 885.0 mg sodium acetate trihydrate; pH to 5 with glacial acetic acid) for 2 hours, washed in dH2O, incubated for 1 minute in 1.25% sodium sulfide nonahydrate solution, re-washed in dH2O, and mounted on a glass slide. For both NADPH diaphorase- and ACh-stained tissues, nuclei of myenteric neuronal fibers were manually counted within 20 randomly selected squares of a 0.1 mm2 microscope grid. Samples from 6–10 mice for each experimental condition were assessed.

Fat weight and hepatic glycogen content

Fat and hepatic glycogen content

At sacrifice and weighed immediately on a Mettler MS5100 analytical balance.
Right and left epididymal fat pads from each animal were weighed independently, and statistics performed on the entire data set. The entire bowel mesentery, excluding the spleen and pancreas, was stripped manually from the excised bowel and weighed immediately. Liver samples were obtained at sacrifice from normal (Con), diabetic (DM), and HIGT-treated mice, by flash freezing in liquid N₂ and stored at −70 °C until analysis. Tissue glycogen assays were performed with modification of a glycogen assay kit (Sigma) as previously described. Briefly, 25–50 mg of liver were boiled in 500 µl 30% KOH saturated with Na₂SO₄ for 60 minutes. After cooling, glycogen was precipitated by the addition of 1.5 ml 95% ethanol, was cleaved by the addition of O-amyloglucosidase, and the produced glucose quantified by colorimetric assay per the manufacturer's instructions (Sigma). Background values of non–glycogen-derived glucose were simultaneously determined by excluding O-amyloglucosidase in parallel samples and subtracted from glycogen-derived values prior to calculating glycogen content.

Serum analysis
At sacrifice, serum was obtained by centrifugation of whole blood collected in microcentrifuge tubes containing clot activator, and frozen at −70 °C until analysis, using Milliplex multiplex assays (MADPK-71K-ADPN, MADPK-71K, MENDO-75K; EMD Millipore, Billerica, MA) run on a Luminex 100/200 device using MasterPlex QT software (MiraBio/Hitachi Solutions America, San Bruno, CA). In addition, serum was assayed for transgenic human insulin using the Mercodia UltraSenstive Human Insulin ELISA (Catalog nr 10-1132-01; Merckodia, Winston Salem, NC).

Western blotting and phosphoprotein assay
Mouse ileum and proximal colon homogenates of control, empty virus-treated diabetic or HIGT-treated diabetic mice were isolated using T-PER tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) with Phosphatase Inhibitor Cocktail Set 1 (Calbiochem, Darmstadt, Germany). Total protein was quantitated using the Pierce BCA Protein Assay Kit (Thermo Scientific). Aliquots containing equal amounts of protein combined with prestained molecular weight markers were resolved on 12-well 1.0 mm NuPAGE Novex 4–12% Bis–Tris Gels using 1× NuPAGE MES SDS running buffer (Invitrogen, Carlsbad, CA). Proteins were transferred onto nitrocellulose membranes using the XCell blot module (Invitrogen) with 1× NuPAGE transfer buffer and probed with antibodies directed against IRβ, AKT, or phospho-AKT (Ser473) (1:1,000; Cell Signaling, Danvers, MA), or β-actin HRP (1:4,000; Genescript, Piscataway, NJ) in a solution of 5% nonfat milk, 30 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% v/v Tween-20 (TBS/T) at 4 °C during continuous, gentle rotary agitation overnight. After five 10-minute washes in TBS/T solution, membranes were incubated with 10 mM of SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minutes. Relative levels of immunoreactive proteins were quantitated using the ChemiDoc XRS imaging system and Quantity One software (Bio-Rad Laboratories).

Quantities of phospho-IRβ (Tyr 1186), phospho-AKT (Ser 473), and phospho-GSK3β (Ser 21/Ser 9) were assessed using Bio-Plex phosphoprotein assays on full-thickness ileum and proximal colon homogenates per manufacturer’s instructions (Bio-Rad Laboratories). Homogenates were processed from tissue samples flash frozen in liquid N₂ at sacrifice and stored until use at −70 °C, utilizing a Bio-Plex cell lysis kit, treated with Bio-Plex phosphoprotein assays and detection reagents, and read on a Luminex 100/200 analyzer using MasterPlex QT software (MiraBio/Hitachi Solutions America, San Bruno, CA).

Statistics
Unless otherwise specified, data are presented as mean ± SEM with the utilized n. Comparisons were made following one-way analysis of variance using the statistics program resident in GraphPad Prism 4 (GraphPad Software) and Tukey’s multiple comparison test if analysis of variance delineated a P < 0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest. No author owns any intellectual property associated with this research, sits on an advisory board, or has received travel or accommodations from entities involved with this research. No author, or family member, has financial investments with commercial entities involved with this research.

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