Glucagon-Like Peptide-2 Reduces Intestinal Permeability But Does Not Modify the Onset of Type 1 Diabetes in the Nonobese Diabetic Mouse

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The development of type 1 diabetes (T1D) has been linked to environmental factors and dietary components. Increasing evidence indicates that the integrity of the gut mucosa plays a role in the development of autoimmune diseases, and evidence from both preclinical and clinical studies demonstrates that increased leakiness of the intestinal epithelium precedes the development of type 1 diabetes. However, there is limited information on modulation of gut barrier function and its relationship to diabetes development. Here we show that the nonobese diabetic (NOD) mouse, a model of T1D, exhibits enhanced intestinal transepithelial permeability before the development of autoimmune diabetes. Treatment of NOD mice with a glucagon-like peptide 2 (GLP-2) analog, synthetic human [Gly2] glucagon-like peptide-2 (h[Gly2]GLP-2, increased the length and weight of the small bowel and significantly improved jejunal transepithelial resistance. However, chronic administration of once daily h[Gly2]GLP-2 failed to delay or reverse the onset of T1D when treatment was initiated in young, normoglycemic female NOD mice. Furthermore, h[Gly2]GLP-2 administration had no significant effect on lymphocyte subpopulations in NOD mice. These findings demonstrate that h[Gly2]GLP-2-mediated enhancement of gut barrier function in normoglycemic NOD mice disease is not sufficient to prevent or delay the development of experimental T1D.

Type 1 diabetes (T1D) is a chronic disorder resulting from the autoimmune destruction of the insulin-producing β-cells. The etiology of T1D is complex and multifactorial. Major risk factors for T1D include genetic predisposition, with putative environmental triggers activating host immune responses in predisposed individuals (1). The pathogenesis of T1D has been extensively studied using animal models, principally the nonobese diabetic (NOD) mouse and the BioBreeding (BB) rat (2, 3). Studies in these animal models have established the importance of diet in the initiation of β-cell autoimmunity. Furthermore, a strong association of celiac disease with T1D, and evidence for immune activation in the gastrointestinal tract, implicate a role for the gut in the development of T1D (4–7). Intriguingly, increased gut permeability has been reported to precede the onset of T1D in diabetes-prone animal models as well as human subjects (8–12).

Glucagon-like peptide (GLP)-2 is an intestinotrophic hormone, related in sequence to GLP-1, and cosecreted with GLP-1 from intestinal endocrine L cells in response to nutrients. GLP-2 exerts proliferative and antiapoptotic actions in the bowel, and administration of GLP-2 enhances the adaptive response to intestinal injury (reviewed in Ref. 13). GLP-2 has also been shown to enhance transcellular and paracellular gut barrier function, by increasing transepithelial resistance in the normal gut and after induction of experimental gut injury (14–16).

The observations that increased gut permeability precedes the development of T1D, together with recent observations demonstrating that GLP-2 enhances gut barrier function, provide an opportunity to prospectively determine whether modulation of intestinal permeability influences the development of T1D. We hypothesized that increased intestinal permeability may represent a modifiable trigger capable of modulating the initiation of autoimmunity and T1D. Accordingly, we have now tested whether administration of a degradation-resistant GLP-2 analog...
affects gut permeability and alters the natural course of diabetes onset in diabetes-prone NOD mice.

Materials and Methods

Reagents

Synthetic human Gly2-glucagon-like peptide-2 (hGly2-GLP-2) acetate was obtained from Pepceuticals Ltd. (Nottingham, UK). This analog has a glycine substitution in position 2, rendering it resistant to the actions of dipeptidyl peptidase 4 (17). hGly2-GLP-2 was dissolved in degassed water at a concentration of 1 mg/ml. Vacuum-dried aliquots were stored at −80°C. Fresh aliquots were thawed before each use and redissolved in PBS (pH 7.4).

For flow cytometry studies, the following monoclonal antibodies were prepared as described (18): purified anti-CD16/CD32 (for Fc blocking), biotinylated anti-CD68, fluorescein isothiocyanate (FITC) anti-CD8α, phycoerythrin (PE) anti-CD25, FITC anti-CD4, PE anti-CD4, allophycocyanin anti-H57 (T-cell receptor β-chain), and PE anti-187 (mouse α-chain). Streptavidin-SRD was purchased from Southern Biotech (Birmingham, AL). Allophycocyanin anti-mouse/rat Foxp3 staining set was purchased from eBioscience Inc. (San Diego, CA).

Mice

Three- and 6-wk-old female NOD/Ltj and 6-wk-old female nonobese diabetes-resistant (NOR)/Ltj mice were purchased from the Jackson Laboratory (Bar Harbor, ME), housed in a specific pathogen-free facility and maintained on a 12-h light, 12-h dark cycle, with free access to standard rodent chow and water. Mice were allowed to acclimatize to the animal facility for at least 1 wk before treatment. All experiments were carried out in accordance with protocols and guidelines approved by the Animal Care Committees of the Toronto General Hospital and Mt. Sinai Hospital.

Experimental protocols

To determine whether GLP-2 exerts intestinotrophic effects in the NOD mouse, 4-wk-old normoglycemic female NOD mice were given sc injections of 5 μg hGly2-GLP-2 or saline, once daily for 14 d based on previously described protocols (19). In separate studies, the acute effects of GLP-2 on gut permeability were examined using 8- and 14-wk-old normoglycemic female NOD mice. Mice were given a single sc injection of 5 μg hGly2-GLP-2 or saline and then euthanized at 4 or 8 h after injection. Small bowel permeability was assessed using an Ussing chamber as described below. To determine whether chronic treatment with GLP-2 modifies the development of diabetes, 4-wk-old normoglycemic female NOD mice were given sc injections of an equal volume (200 μl) of either saline or 5 μg hGly2-GLP-2 once daily for 16 wk and monitored for diabetes onset. At the end of the treatment period, all remaining nondiabetic mice were euthanized and tissues were collected for further analyses.

Diabetes monitoring

Diabetes onset was monitored as previously described (20). Briefly, single-cell suspensions were prepared from the spleen, lymph nodes, thymus, and bone marrow of NOD mice. After lysis and removal of red blood cells by addition of ACK lysis buffer (0.15 M NaH2Cit, 0.01 M KHCO3, 0.1 mM EDTA), cell numbers were quantified using a hemocytometer. Cells (105) were incubated at room temperature with a primary and secondary (in case of biotinylated primary) antibodies in PBS containing 2% calf serum. Cells were either fixed overnight in 1% paraformaldehyde or fixed/permeabilized and stained with the Foxp3 antibody according to the manufacturer’s protocol. Cells were acquired and analyzed on a FACS Calibur flow cytometer (BD Biosciences, Mississauga, Ontario, Canada).

GLP-2 receptor (GLP-2R) expression

RNA was extracted from the jejunum, colon, and single-cell suspensions of lymphocytes, splenocytes, thymocytes, and bone marrow cells, using TRIzol reagent (Life Technologies, Burlington, Ontario, Canada). Total RNA (2 μg) was reverse transcribed at 50°C for 60 min using SuperScript III reverse transcriptase (RT; Invitrogen, Burlington, Ontario, Canada) and random hexamer primers (Invitrogen Life Technologies). A positive (RT+ and a negative (RT−, no enzyme added) control reaction were included for each RNA sample. The resulting cDNA was tested for expression of the mouse GLP-2R by PCR using the following primer pairs and conditions: 5’TGGCCCATGATGCAGAGAG-3’ and 5’TGGGTAGGGACCCCTGCTTCTAG-3’, 94°C for 5 min; 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, 40 cycles; 72°C for 10 min. These primers result in the amplification of a 1.648-kb product spanning bases 189-1837 of the GLP-2R mRNA sequence. β-Actin cDNA was amplified using the primer pairs 5’TGACTCCTGTAAGA-3’ and 5’CAGCTCAGTACAGTCC-3’, with the following parameters: 94°C for 5 min; 94°C for 30 sec, 45°C for 30 sec, 72°C for 30 sec, 40 cycles; 72°C for 4
min. The PCR products were run in 1% agarose gels and bands were visualized with SYBR Safe DNA dye (Invitrogen). The PCR products were transferred to Nytran supercharge nylon membranes (Mandel Scientific, Guelph, Ontario, Canada) by capillary transfer, and membranes were hybridized overnight with γ-32P[ATP] (PerkinElmer, Wellesley, MA)-labeled internal primers corresponding to GLP-2R or β-actin.

Statistical analysis
Results are expressed as mean ± SE. All statistical analyses were performed with Prism version 3.03 software (GraphPAD Software Inc., San Diego, CA). Statistical significance was assessed by the Kaplan-Meier life table method and the Log rank test, one-way or two-way ANOVA, and where appropriate, a two-tailed Student’s t test. Differences were considered statistically significant at P ≤ 0.05.

Results
GLP-2 modifies intestinal mucosal thickness and permeability in the NOD mouse
To examine whether the NOD mouse responds to exogenous GLP-2, we first treated 4-wk-old normoglycemic female NOD mice with 5 µg h[Gly2]GLP-2 or saline once daily for 14 d. h[Gly2]GLP-2-treated NOD mice exhibited significantly increased small bowel length (Fig. 1A) and weight (Fig. 1B), relative to saline-treated mice. In contrast, no significant changes in bowel weight or length were observed in the colon after h[Gly2]GLP-2 administration (Fig. 1, A and B). To determine whether acute treatment with h[Gly2]GLP-2 was capable of modulating permeability in NOD mice, we analyzed intestinal permeability 4 h after a single injection of h[Gly2]GLP-2 in separate groups of NOD mice. Gut permeability, measured as ion conductance, was significantly decreased in the jejunum of both 8- and 14-wk-old normoglycemic female NOD mice after h[Gly2]GLP-2 administration (Fig. 1, D and E).

Gut barrier permeability is compromised in NOD mice
To assess whether the NOD mouse displays increased intestinal permeability when compared with a congenic diabetes-free control strain, permeability to both ions and 4-kDa fluorescent dextran molecules was determined in jejunal sections from 7- to 10-wk-old normoglycemic female NOD/Ltj mice and age- and sex-matched NOR/Ltj controls. Transepithelial ion conductance in bowel sections from NOD mice was significantly higher compared with values obtained using intestinal sections from NOR control mice (Fig. 2A), indicating that intestinal permeability to ions is relatively increased in NOD mice. The compromised gut permeability observed in normoglycemic NOD mice did not worsen with the onset of diabetes because there were no significant differences in conductance values in intestinal sections from normoglycemic vs. age- and sex-matched diabetic NOD mice (Fig. 2C). Furthermore, no significant differences were observed between the two mouse strains in the mucosal-to-serosal flux rates of the 4-kDa dextran permeability probe (Fig. 2B), and permeability to dextran was not altered after the development of diabetes in NOD mice (Fig. 2D).

**FIG. 1.** GLP-2 stimulates gut growth and reduces intestinal permeability. A–C, Normoglycemic 4-wk-old female NOD mice were injected sc with an equal volume (200 µl) of either saline or 5 µg h[Gly2]GLP-2 or saline once daily for 14 d. At the end of the treatment period, the length (A) and weight (B) of both the small and large intestine was measured. Bowel weight is expressed as a percentage of body weight. Average body weight of h[Gly2]GLP-2-treated mice was 16.80 ± 0.33 vs. 16.67 ± 0.36 g in saline-treated mice (P > 0.05). C, Histological representation of jejunum sections stained with hematoxylin and eosin from mice treated with h[Gly2]GLP-2 and saline. In a separate experiment (D and E), gut permeability was measured 4 h after a single injection of 5 µg h[Gly2]GLP-2 or saline. The effects of h[Gly2]GLP-2 on jejunal ion permeability were determined in intestinal sections from normoglycemic 8-wk-old (D) or 14-wk-old (E) female NOD mice (n = 7 mice/group for A and B and n = 8–12 tissues from three to four mice/group for D and E). *, P < 0.05 and ***, P < 0.001 [Gly2]GLP-2 vs. saline-treated mice. Results are displayed as mean ± SE.
GLP-2 administration does not modify diabetes onset in the NOD mouse

Because h[Gly²]GLP-2 reduces intestinal permeability in CD1 mice (14) and the NOD mouse (Fig. 1), we next examined whether chronic administration of h[Gly²]GLP-2 might improve gut permeability and prevent or delay the onset of diabetes. Groups of 4-wk-old nondiabetic NOD mice were treated with a single injection of saline or 5\( \mu \text{g} \) h[Gly²]GLP-2 once daily and monitored for diabetes onset. After 16 wk of treatment, no significant changes were observed in the median age of diabetes onset or the cumulative incidence of diabetes (Fig. 3A). Moreover, treatment with h[Gly²]GLP-2 did not influence ambient blood glucose levels or body weight (Fig. 3, B and C). To assess whether NOD mice retained their responsivity to the actions of h[Gly²]GLP-2 after chronic administration, we assessed bowel weight and length in NOD mice after the diagnosis of diabetes. Longer-term treatment with h[Gly²]GLP-2 significantly increased the length and weight of the small bowel and colon in both diabetic NOD mice (Fig. 3, D and E) and NOD mice that remained normoglycemic at the end of the 16-wk treatment period (Fig. 3, F and G). The trends in bowel weights after h[Gly²]GLP-2 treatment were identical when values were expressed either as percent of body weight (Fig. 3, E and G) or as absolute values (data not shown).

Analysis of lymphoid subpopulations in GLP-2-treated mice

One potential mechanism for the inability of h[Gly²]GLP-2 to modify diabetes onset in NOD mice might be due to concomitant effects of h[Gly²]GLP-2 on components of the immune system in NOD mice. Accordingly, we assessed lymphocyte subpopulations and/or total cell numbers in immune organs of NOD mice treated with h[Gly²]GLP-2 for 14 d (Fig. 4, A–G) or NOD mice treated with h[Gly²]GLP-2 for 16 wk (Fig. 4, H–K). Neither the 14-d treatment in nondiabetic NOD mice (Fig. 4, A–C) nor the 16-wk treatment of older NOD mice (Fig. 4, H–K) had any effect on total cell numbers in spleen (Fig. 4, A and H), lymph nodes (Fig. 4, B and I), thymus (Fig. 4, C and J), or bone marrow (Fig. 4K). Similarly, using flow cytometry, we did not observe any significant differences in the percentage of CD4+ or CD8+ T cells or B cells in either the spleen (Fig. 4D) or lymph nodes (Fig. 4E). Likewise, compared with saline-treated NOD mice, there were no differences in the percentage of CD4+CD25+Foxp3+ regulatory T cells in the lymph nodes (Fig. 4F) or thymus (Fig. 4G) of NOD mice after 14 d of treatment with h[Gly²]GLP-2.

Discussion

Type 1 diabetes is a multifactorial disease, with major risk factors encompassing genetic predisposition and environmental triggers. Genetic susceptibility to the disease is complex and polygenic because more than 20 genetic loci have been identified from linkage analysis and genetic association studies as contributors to increased risk (24). However, several observations indicate that environmental factors are also key contributors to the development of T1D (reviewed in Refs. 25, 26). Some examples include a lower than 40% concordance rate among monzygotic
twins, a dramatic increase in the global incidence of T1D over the last 50 yr that cannot be attributed to changes in the gene pool, a wide geographic variation in the prevalence of T1D that cannot be solely accounted for by genetic background, and spatial clustering of T1D epidemics that can be best explained by environmental exposure.

The majority of evidence linking environmental factors with increased T1D risk comes from retrospective association studies; whether specific environmental factors modify gut barrier function and/or the immune system remains incompletely understood. Here we show, for the first time, that the NOD mouse displays impaired small intestinal barrier function before the development of diabetes. Permeability was measured \textit{ex vivo} using the Ussing chamber technique, and was calculated from the electrical conductance values representing the passive movement of ions across the epithelium, mainly through the paracellular pathway. It has been postulated that enhanced intestinal permeability facilitates the abnormal entry of multiple environmental antigens and, in the setting of a compromised immune system, contributes to the initiation of an autoimmune reaction against pancreatic \( \beta \)-cells (11, 27, 28). Moreover, reduction of gut permeability through blockade of zonulin-mediated improvement in intestinal barrier function significantly reduced the development of diabetes in the BB rat (9).

Accordingly, we hypothesized that administration of a protease-resistant hGLP-2 analog, h[Gly\textsubscript{2}]GLP-2, might enhance barrier function and delay diabetes onset in the NOD mouse. Although NOD mice are responsive to GLP-2, as evidenced by a significant increase in gut length and weight and an acute reduction of intestinal permeability, we were not able to detect any difference in the onset or incidence of diabetes after repeated daily administration of h[Gly\textsubscript{2}]GLP-2. Moreover, the GLP-2 receptor is not expressed in bone marrow, spleen, thymus, or lymph nodes from NOD mice (Fig. 4L) and 2 or 14 wk of h[Gly\textsubscript{2}]GLP-2 administration produced no detectable changes in immune cell numbers or populations in nondiabetic NOD mice.
FIG. 4. Lymphocyte subpopulations in NOD mice treated with h[Gly2]GLP-2 or saline. Total cell count in lymphoid organs from 8-wk-old female NOD mice treated for 14 d with 5 μg h[Gly2]GLP-2 or saline (A–C) and 20-wk-old female NOD mice that remained normoglycemic after a 16-wk treatment with saline or 5 μg h[Gly2]GLP-2 (H–K). The percentage of CD4+ or CD8+ T cells and B cells were quantified in the spleen (D) and lymph nodes (E), and the percentage of CD4+CD25+Foxp3+ cells (Tregs) were determined in the lymph node (F) and thymus (G) of NOD mice after 14 d of treatment with either h[Gly2]GLP-2 or saline. L. The expression of GLP-2R mRNA transcripts (top panel) was assessed using total cellular RNA from single cell preparations of bone marrow (lanes 1 and 2), thymus (lanes 3 and 4), spleen (lanes 5 and 6), and lymph nodes (lane 7) or in tissue sections from jejunum (lanes 8 and 9) and colon (lanes 10 and 11). Tissues were isolated from normoglycemic female NOD mice, treated with saline (lanes 1, 3, 5, 7, 8, and 10) or 5 μg h[Gly2]GLP-2 (lanes 2, 4, 6, 9, and 11) for 14 d. RT-PCR analysis of β-actin (bottom panel) was used to confirm the integrity of the samples. For A–C and H–K, results are expressed as number of total cells, as counted by a hemocytometer. For D–G, results are expressed as percentage of total gated live cells. Values are expressed as mean ± se.
Several possibilities may account for the failure of h[Gly]2GLP-2 to modify diabetes onset in the NOD mouse. First, despite considerable evidence that a leaky gut directly contributes to the pathogenesis of diabetes in the BB rat (6, 9, 11, 28), it remains possible that reduced intestinal barrier function in the NOD mouse is not central to the pathogenesis of T1D in this rodent model. Alternatively, our intestinal permeability studies demonstrated significantly higher conductance values in intestinal sections from NOD mice but were not able to detect any statistically significant changes in the transepithelial flux of dextran. Hence, although paracellular permeability in the NOD mouse model is compromised, there is still a normal restriction to transmucosal passage of molecules such as dextran. As the identity and size of potential gut-derived diabetogenic antigens remains uncertain, it is possible that the NOD gut functions normally to restrict access to key diabetogenic enteral antigens, in contrast to the antigen permeability described in studies with the BB rat (8, 11). Furthermore, the dose and method of GLP-2 administration required for sustained enhancement of gut barrier function may require further optimization in the NOD mouse model.

Compromised intestinal barrier function, measured as changes of intestinal permeability to probes, was first reported in patients with established T1D (29–31). Moreover, there is a higher-than-normal reported prevalence of celiac disease, an immune-mediated enteropathy characterized by gut barrier dysfunction, in T1D compared with the general population (32). Of even greater interest is the observation that changes in intestinal morphology and permeability in both the BB rat and humans precede the onset of diabetes (8, 11, 12). Furthermore, enteropathy, identified as disrupted mucosal architecture, increased density of intraepithelial lymphocytes, intestinal inflammation, and reduced brush border enzymatic activity, was also reported in diabetes-prone animal models before the onset of disease (4, 33, 34). The observations that GLP-2 administration restores intestinal integrity and enhances gut barrier function in preclinical studies (14–16, 35–37), taken together with recent findings suggesting that the GLP-2 analog teduglutide exerts similar actions in human subjects with compromised intestinal function (38), suggests that additional studies delineating how GLP-2 modulates gut permeability appear warranted.

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