Enteric neural pathways mediate the anti-inflammatory actions of glucagon-like peptide 2

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Am J Physiol Gastrointest Liver Physiol 293: G211–G221, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00530.2006.—Glucagon-like peptide-2 (GLP-2) is an important regulator of nutritional absorptive capacity with anti-inflammatory actions. We hypothesized that GLP-2 reduces intestinal mucosal inflammation by activation of vasoactive intestinal polypeptide (VIP) neurons of the submucosal plexus. Ileitis or colitis was induced in rats by injection of trinitrobenzene sulfonic acid (TNBS), or colitis was induced by administration of dextran sodium sulfate (DSS) in drinking water. Subsets of animals received (1–33)-GLP-2 (50 μg/kg sc bid) either immediately or 2 days after the establishment of inflammation and were followed for 3–5 days. The involvement of VIP neurons was assessed by concomitant administration of GLP-2 and the VIP antagonist [Lys1-Pro2,5-Arg3,4-Tyr6]VIP and by immunohistochemical labeling of GLP-2-activated neurons. In all models, GLP-2 treatment, whether given immediately or delayed until inflammation was established, resulted in significant improvements in animal weights, mucosal inflammation indices (myeloperoxidase levels, histological mucosal scores), and reduced levels of inflammatory cytokines (IFN-γ, TNF-α, IL-1β) and inducible nitric oxide synthase, with increased levels of IL-10 in TNBS ileitis and DSS colitis. Reduced rates of crypt cell proliferation and of apoptosis within crypts in inflamed tissues were also noted with GLP-2 treatment. These effects were abolished with coadministration of GLP-2 and the VIP antagonist. GLP-2 was shown to activate neurons and to increase the number of cells expressing VIP in the submucosal plexus of the ileum. These findings suggest that GLP-2 acts as an anti-inflammatory agent through activation of enteric VIP neurons, independent of proliferative effects. They support further studies to examine the role of neural signaling in the regulation of intestinal inflammation.

Vasoactive intestinal peptide; trinitrobenzene sulfonic acid colitis; dextran sodium sulfate colitis; Crohn’s disease

MATERIALS AND METHODS

Animals. Male rats (Sprague-Dawley, 250–300 g; Charles River, Trois Rivieres, PQ, Canada) were acclimatized for 1 wk before the study. Animals were housed under constant temperature and humidity, with free access to food and water. Protocols were approved by the University of Calgary Animal Care Committee, following the guidelines of the Canadian Council on Animal Care. All animals were between 280 and 300 g at the start of the study, and weight gain and food intake were monitored throughout.

Models of intestinal inflammation. We used three models of intestinal inflammation to ensure that the validity of these findings was not limited to a single region of gut or to a specific model of intestinal inflammation. The TNBS model of chronic intestinal inflammation was used to model Th1 inflammation in the ileum and colon (5), whereas DSS was used to simulate aspects of ulcerative colitis (32). Ileitis was induced as previously described (29); nonfasted animals were anesthetized with halothane (1–2% by nose cone), and at laparotomy 1 ml of TNBS (40 mg/ml in 40% ethanol; Sigma, St. Louis, MO) was injected intraluminally, 10 cm proximal to the ileocecal valve. Controls received saline or 40% ethanol. The incision was closed with 4-0 absorbable suture. Animals were allowed immediate access to food and received analgesia (buprenorphine; 0.2 mg/kg

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Treatment groups included controls (ileal saline or ethanol injection treated with saline sc injection), controls + GLP-2 (ileal ethanol injection + GLP-2 sc), TNBS (ileal TNBS injection + saline sc), TNBS + GLP-2 (ileal TNBS injection + GLP-2 sc), and TNBS + delayed GLP-2 (ileal TNBS injection + GLP-2 sc treatment starting on day 2). To show the effects of GLP-2 in established colonic inflammation, animals with TNBS colitis or DSS colitis were treated with identical dosages of GLP-2 at 36 h following establishment of colitis (TNBS animals) or on day 5 of DSS treatment (n = 8 in all groups). In the TNBS colitis study, the groups were controls (rectal installation of ethanol + saline sc), controls + GLP-2 (rectal installation of ethanol carrier + GLP-2 sc), TNBS (rectal installation of TNBS + saline sc), and TNBS + delayed GLP-2 (rectal installation of TNBS + GLP-2 sc). Animals were studied on day 5. In the DSS study, the groups were controls (normal drinking water + saline sc), controls + GLP-2 (normal drinking water + GLP-2 sc), DSS (DSS in drinking water + saline sc) and DSS + delayed GLP-2 (DSS in drinking water + GLP-2 sc) All injections started on day 5, and animals were studied on day 8.

To determine the pharmacokinetics of GLP-2 with this dosing pattern and the interaction of ileitis on GLP-2 metabolism, a specific set of animals was used. Ileitis was induced by injection of TNBS or control injection of saline (n = 6), as described earlier. After 3 days, following an overnight fast, GLP-2 levels were drawn; animals were injected with GLP-2 (14 nmol/kg (50 μg/kg) sc); levels were repeated in groups of 3 animals each from the control or ileitis groups at 1, 2, 6, and 12 h; and the animals were then euthanized. Blood for GLP-2 samples was drawn into icd EDTA vacutainers containing 10% volume of 5,000 KU Trasylol and 0.1 mM diprotin A; after processing (1–33)-GLP-2 quantification was performed using RIA (J. J. Holst, Panum Institute, Copenhagen, Denmark, antibody no. 92160) (21). To determine the role of VIP in mediating the GLP-2 response, we used the specific VIP receptor antagonist [Lys3-Pro37–Arg13]-Tyr3-VIP7–28 (VIP Hybrid; Sigma, Oakdale, ON, Canada), dosed at 100 μg·kg−1·day−1·bid, 2 days following the establishment of inflammation. This neurotensin-VIP hybrid selectively blocks VIP receptors types 1 and 2 (19) and has a demonstrated efficacy in a range of in vivo and in vitro models (18, 23). Dosage was based on preliminary studies in which the anti-inflammatory effects of exogenous VIP (10 nmol·animal−1·day−1·ip bid; Peninsula, Oakland, CA; Ref. 2) in TNBS animals was shown to be optimally blocked by 100 μg·kg−1·day−1·ip bid VIP Hybrid (19). In these studies, all animals received TNBS injections into the terminal ileum on day 0, started GLP-2 and/or blocker treatment on day 2, and were studied on day 5. Treatment groups were TNBS (TNBS + saline sc), TNBS + delayed GLP-2 (TNBS + GLP-2 sc), TNBS + delayed GLP-2 + VIP Hybrid (TNBS + GLP-2 sc + VIP-B ip), and TNBS + VIP Hybrid (TNBS + VIP-B ip) (n = 8 per group).

**Tissue sampling and histology.** At the end of the experimental period, animals received the normal morning injection of GLP-2 or saline. One hour later, bromodeoxyuridine was injected (BrDU, 120 mg/kg ip; Sigma, St. Louis MO). Between 2 and 3 h after BrDU injection, animals were euthanized (pentobarbital, 50 mg/kg iv; Huntington Labs, Toronto, ON, Canada). Sections of ileum (distal 10 cm) and colon (distal 7 cm) were resected and flushed with 5 ml of iced saline. The proximal 2 cm was fixed (10% formalin) and processed for regular histology or immunohistochemical studies. Mucosal morphology (villus height, crypt depth, and inflammation scoring) was measured by using previously described techniques (3, 27). In brief, 10 well-oriented crypt-villus units from intact mucosa were reviewed by an observer blinded as to tissue origin, measuring villus and crypt dimensions with a graduated eyepiece (27). Inflammation was rated on a 0–4 scale, reviewing all areas of mucosa, both intact and ulcerated zones (3). Similarly, from areas of intact mucosa, BrDU-specific labeling was used to quantify dividing crypt cells and activated caspase-3 immunostaining for detection of apoptotic crypt cells in 10 well-oriented crypt units; in each crypt column total cells were also counted (7, 27). Values are reported as BrDU-labeled (antibody, 1:100 rabbit anti-BrDU; Serotec, London, UK) or activated caspase-3-labeled (1:500 rabbit anti activated caspase-3 antibody; BD Pharmingen, San Jose, CA) cells per crypt column divided by the total cells per crypt column (crypt proliferation or apoptotic index, respectively). The next 2 cm of specimen was frozen at −80°C for myeloperoxidase (MPO) assay (22, 29), and the remaining mucosa was scraped and frozen for cytokine assays.

**Cytokine ELISA and inducible nitric oxide synthase analysis.** IL-1β, IFN-γ, IL-10, and TNF-α content in mucosal scrapings were determined by using commercially available rat-specific sandwich ELISA kits, following the manufacturer’s instructions (rat TNF-α and IL-1β from Assay Designs, Ann Arbor, MI; IL-10 and IFN-γ kits from BioSource, Camarillo, CA). Inducible nitric oxide synthase (iNOS) content was determined by using Western blot analysis (anti-iNOS rabbit polyclonal antibody; Upstate, Lake Placid, NY), developed and expressed as percentage of β-actin expression.

**Immunohistochemical localization of GLP-2-activated VIP neurons.** To examine whether GLP-2 activated VIP neurons, the pattern of activation and the neurotransmitter expression of enteric neurons activated by exogenous GLP-2 was assessed by using cFos and neurotransmitter immunohistochemical labeling. Fasted, naïve animals were stimulated with injection of saline or GLP-2 (50 μg/kg, 0.5 ml sc; n = 5 in each group). After 1 h, animals were euthanized and sections of distal ileum were prepared for whole mount immunohistochemistry (34). In brief, the ileal tissue was opened and fixed in Zamboni’s fixative overnight at 4°C. The mucosa and submucosa were dissected, exposing the submucosal plexus. Tissues were washed with PBS and were incubated overnight with 1:100 dilution of rabbit anti-VIP (Dr. John Walsh, UCLA, CURE, code no. 7913) at 4°C. After three washes with PBS, tissues were then incubated with 1:200 FITC-conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Once VIP staining was confirmed, segments were sequentially labeled with goat anti-cFos (1:100; Jackson) overnight at 4°C, washed three times with PBS, and 1:100 Cy3 conjugated to rabbit anti-goat IgG (Jackson) in PBS for 2 h.Slides were then rewashed twice with PBS, mounted, and examined with a Zeiss fluorescence microscope. Photographs were taken with a digital imaging system (SenSys; Photometrics, Tucson, AZ) coupled to image-analysis software (V for Windows; Digital Optics, Auckland, New Zealand). Whole mount images of submucosal ganglia (5–10 per preparation) were counted for the number of cFos-immunoreactive nuclei as a marker of generalized enteric nervous system activation, and then in separate tissues the number of cFos-immunoreactive cells that coexpressed VIP were counted (n = 5 ganglia per preparation from each animal). Each set of counts from one animal...
were averaged and were treated as one value for statistical comparisons.

Statistical analyses. Data are expressed as means ± SE. Statistical significance between groups was determined by using ANOVA, with Bonferroni post hoc testing of significance for parametric data and Kruskal-Wallis testing for nonparametric values (cell counts, inflammation scores). A P value < 0.05 was considered statistically significant.

RESULTS

Effects of GLP-2 on intestinal inflammation. Our initial studies examined the effects of GLP-2 on preventing the development of TNBS ileitis (GLP-2 given concurrently with TNBS) or in healing established inflammation (GLP-2 delayed until 2 days after TNBS). Animals given ethanol (Fig. 1A) or saline (data not shown) gained weight, and the ileum had a normal macroscopic and microscopic appearance (Fig. 2; Table 1). Baseline levels of MPO and cytokines were similar to previously reported values (28) in the two groups throughout the study, and so only the values from the ethanol groups are reported. TNBS treatment induced a consistent ileitis with a 10% weight loss (Fig. 1), alterations in tissue architecture, crypt cell proliferation, apoptosis, and increased cytokine and iNOS expression. GLP-2 therapy, whether given concomitantly with TNBS or after 2 days of inflammation, significantly ameliorated weight loss (Fig. 1), improved histological measures of inflammation (Fig. 2, Table 1), and reduced mucosal levels of inflammatory cytokines (Fig. 1). The most profound effects appeared to occur in the delayed GLP-2 group, in which treatment was started 2 days after induction of inflammation. (Table 1; Figs. 1 and 2). In the noninflamed controls given

![Fig. 1. Effects of glucagon-like peptide-2 (GLP-2) in trinitrobenzene sulfonic acid-induced (TNBS) ileitis. Animals were injected with saline (not shown), ethanol (Con), or TNBS intraluminally in ileum. Groups were treated with daily subcutaneous injection of saline (Con, TNBS) or GLP-2 immediately (TNBS + GLP-2) or 2 days after inflammation was induced (TNBS + D-GLP-2) (n = 8 in each group). Animals were studied on day 5. A: all animals lost weight after TNBS treatment (*P < 0.05 vs. controls), but treatment with GLP-2 (both immediate and delayed) attenuated the degree of weight loss in TNBS-treated animals (†P < 0.05 vs. TNBS). B: TNBS treatment increased crypt proliferation index (CPI), whereas GLP-2 treatment (both immediate and delayed) reduced CPI to approximately control values. C: TNBS treatment increased apoptosis index (*P < 0.05), whereas GLP-2 reduced rates of apoptosis to levels similar to controls after delayed treatment. D–G: effects of GLP-2 on cytokine and inducible nitric oxide synthase (iNOS) expression in TNBS ileitis. Cytokine and iNOS content of mucosal scrapings was determined by using Western blot for iNOS and ELISA assays for IL-1β, TNF-α, and IFN-γ. TNBS induced a generalized increase in inflammatory cytokines and iNOS, which was normalized by GLP-2 treatment, whether given immediately or with established inflammation. All values are means ± SE; n = 6 or more in each group. *P < 0.05 vs. controls and †P < 0.05 vs. TNBS by ANOVA.

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GLP-2, the effects on mucosal cytokines were not significant in either ileum or colon (data not shown).

Interestingly, untreated TNBS-induced ileal inflammation resulted in an increase in both crypt cell proliferation and apoptosis within the inflamed ileal tissue; in the colon, proliferation was increased but apoptosis was not (Table 1, Fig. 1). GLP-2 therapy in control animals resulted in an increase in crypt proliferation index (CPI) in the noninflamed ileum and colon, with a decrease in apoptosis in the ileum. In animals with induced inflammation and GLP-2 therapy, both CPI and apoptosis in the inflamed ileum were normalized with no additional effects in the colon (Table 1). Morphology followed the pattern expected from these changes in kinetics, with an increase in ileal crypt depth in all GLP-2-treated animals; however, the villus height was significantly increased only in GLP-2-treated ileitis animals and the colonic controls (Table 1). In the colon, crypt depth was increased in the GLP-2-treated, noninflamed controls, paralleling the increase in proliferation (Table 1). In both colitis models, delayed GLP-2 treatment normalized mucosal IL-1β, TNF-α, and IFN-γ levels, whereas IL-10 levels were not affected (Fig. 3). In the DSS colitis model the TNF-α and IL-10 contents of the mucosa were normalized in the GLP-2-treated animals, whereas IL-1β and IFN-γ were not affected (Fig. 4).

In the groups studied, GLP-2 levels drawn at 1 h after injection averaged 400 ± 50 pmol/l, with no differences between controls and ileitis animals. Control animals showed normal fasting levels of GLP-2 (12.6 ± 0.9 pmol/l) with no differences in fasting GLP-2 in rats with ileitis (12.4 ± 1.7 pmol/l) or colitis (13.7 ± 1.2 pmol/l). In separate studies we have shown that at 1, 4, and 6, and 12 h following GLP-2 sc ileal inflammation did not alter GLP-2 metabolism, and by 6 h after injection, levels declined to baseline (data not shown).

Effects of VIP blockade. The contribution of VIP to the anti-inflammatory effects of GLP-2 was studied by using the VIP antagonist VIP Hybrid. In preliminary studies, the effects
of exogenous GLP-2 and VIP were compared in the TNBS ileitis model. GLP-2, given at a dose of 14 nmol·kg⁻¹·day⁻¹ (50 μg/kg) sc bid and VIP (5.0 nmol·animal⁻¹·day⁻¹ ip bid) significantly improved weight loss and mucosal MPO activity [weight loss in TNBS-treated controls = −34 ± 4.3 vs. −20 ± 4.5 in GLP-2 and −14 ± 1.6 in VIP-treated animals (as mean ± % starting weight ± SE; n = 6–4 in each group)]. MPO scores were similarly improved: 15.7 ± 4.8 for TNBS controls vs. 6.4 ± 1.9 in GLP-2-treated and 2.1 ± 0.4 in VIP-treated animals. Combined treatment with VIP and VIP Hybrid at a VIP Hybrid dose of 50 μg·kg⁻¹·day⁻¹ ip bid partially blocked these effects (weight loss, −28 ± 4.5%; MPO score, 12.5 ± 5.1), whereas VIP + VIP Hybrid (100 μg·kg⁻¹·day⁻¹ ip bid) completely blocked the protective effects of VIP (weight loss, −32 ± 6.2%; MPO score, 16.2 ± 4.3). Higher doses of VIP Hybrid (200 μg·kg⁻¹·day⁻¹ ip bid) did not further improve MPO scores.

On the basis of these findings, a dose of 100 μg·kg⁻¹·day⁻¹ ip bid was chosen for the VIP Hybrid. Following the induction of inflammation, treatment with GLP-2 (TNBS + delayed GLP-2), GLP-2 and VIP Hybrid (TNBS + delayed GLP-2 + VIP-B), or TNBS + VIP Hybrid was initiated at 48 h.

The untreated TNBS ileitis animals developed the expected weight loss and evidence of ileal mucosal inflammation, which was normalized by the administration of delayed GLP-2 (Fig. 5, A and B). The coadministration of VIP Hybrid blocked the anti-inflammatory effects of GLP-2 (Fig. 5). In the combined TNBS + delayed GLP-2 + VIP Hybrid group, animal weights, crypt cell apoptosis index, and the levels of IL-1β, TNF-α, and IL-10 were unchanged from TNBS-treated controls (Fig. 5), whereas crypt proliferation was significantly decreased but was still greater than in the VIP Hybrid-treated group. MPO activity was significantly increased vs. TNBS controls. Conversely, the additive treatment of TNBS + VIP Hybrid alone did not significantly affect inflammatory indices or weight loss compared with TNBS-treated controls (Fig. 5, A and B). Interestingly, treatment with VIP Hybrid alone decreased cell-proliferation rates in the inflamed ileum but did not affect inflammatory cytokines, except for a reduction in mucosal IFN-γ content, which was noted in all treated groups (Fig. 5, C and E).}

**Neuronal activation by GLP-2.** Having shown that VIP seemed to mediate the anti-inflammatory effects of GLP-2, we next investigated whether neurons that release VIP were activated by GLP-2. We assessed neuronal activation by using cFos, as previously described (8, 34). In these studies, the numbers of activated neurons within the enteric nervous system and the numbers expressing VIP were quantified by immunohistochemical staining by using naïve animals following injection of GLP-2 sc or control vehicle (Fig. 6). Both the number of cells in the submucosal ganglia expressing nuclear Fos activity, as a marker of neuronal activation, and the number staining positively for VIP increased following GLP-2 stimulation (Fig. 6). Representative preparations are shown, which clearly show colocalization of VIP and cFos in GLP-2-activated ganglia (Fig. 6, A–F). The proportion of cFos-positive cells that colocalized VIP was 39.1 ± 10.3% in controls vs. 57.7 ± 1.7% in GLP-2-stimulated animals (P = 0.10 by Kruskal-Wallis test). In addition, the cFos-expressing neurons activated by GLP-2 tended to be central in the ganglia, which is the site described for VIP-expressing neurons (Fig. 6) (17).

**DISCUSSION**

These results demonstrate that exogenous GLP-2 acts as an effective anti-inflammatory peptide in three models of intestinal inflammation in the ileum and colon. These effects were not mediated by increases in progenitor cell proliferation but...
rather appeared to be due a specific reduction in mucosal inflammatory cytokine production, mediated by VIP.

Previous studies (6, 24) noted anti-inflammatory effects using the long-acting analog [Gly2]GLP-2; the mechanism of action was thought to be the associated increase in the CPI, which presumably aided healing of lesions and consequently reduced the production of inflammatory cytokines. In both the previous studies, and in the present results, mucosal inflammation resulted in an increase in the rates of CPI and epithelial cell apoptosis (6, 13, 24, 36). The differential changes in these two aspects of crypt cell kinetics provide an interesting reference point for the effects of inflammation and the compensatory mechanisms activated. Inflammation in both the small and large intestine triggers an intrinsic increase in crypt proliferation; the mechanisms that control this are not clear, but they appear to be related to local effects of inflammation (16, 25, 30). In addition, inflammation increases rates of apoptosis; this has been shown to be mediated by inflammatory cytokines and improved by administration of anti-TNF-α antibodies (36). In the present study, GLP-2 therapy in uninfamed intestinal tissues resulted in a decrease in CPI and a reduction of apoptosis in the ileum (Table 1), as expected. However, in the various models of inflammation, GLP-2 consistently reduced the CPI in the inflamed tissue (Table 1). For the effects of GLP-2 on apoptosis, we noted a reduction in apoptosis rates in the ileum in all GLP-2-treated groups, but in the colon only the GLP-2-treated TNBS colitis animals showed a significant effect (Table 1, Fig. 4). In noninflamed tissue, GLP-2 induced the expected changes in morphology associated with the changes in epithelial cell kinetics. However, there were only minimal
effects on morphological measures of mucosal proliferation in the primary inflamed tissue; villus height (in ileitis) and crypt depth were not changed with these doses of native GLP-2, aside from the increases associated with TNBS treatment itself (Table 1). In all of these sites, there was a significant reduction in inflammation scores, MPO activity, and inflammatory cytokines in GLP-2-treated animals (Table 1, Figs. 1–4). We suggest that this is due to a fundamental anti-inflammatory effect of GLP-2 in these inflamed tissues, which then reduces cytokine-induced apoptosis and the inflammation-induced increase in CPI. This also implies that the mechanisms by which GLP-2 increases CPI, under normal conditions, is different, or has alternative regulatory pathways from the mechanisms that increase proliferation with inflammation. Overall, these results suggest that GLP-2, at the doses used in these studies, does not improve the healing of inflamed intestinal mucosa by directly increasing proliferation rates (12, 27).

The differences noted in crypt cell proliferation with GLP-2 treatment in inflammation from those reported in previous studies may be due to differences in potency of the GLP-2 ligand used (6, 13, 24). The studies quoted used DPP-IV degradation-resistant human [Gly2]GLP-2, which has been shown to increase intestinal weight and villus height two to three times more than an equivalent dose of native GLP-2 in a murine model (11). In the present study, peak levels of GLP-2 in the treated animals were roughly five times the peak postprandial levels but by 6 h were basal, whereas the effects of [Gly2]GLP-2 are much prolonged, due to its delayed metabolism (10). Further comparative studies may be useful; nonetheless, the results of the present study suggest that the anti-

Fig. 4. Effects of delayed GLP-2 in dextran sodium sulfate-induced (DSS) colitis. Animals were treated with 5% DSS in drinking water or were untreated controls, starting on day 0. On day 5 of the protocol, DSS-treated animals were randomly assigned to treatment groups, with continued DSS in the drinking water to the end of the study (day 8). Groups were treated with daily subcutaneous injection of saline (Con, DSS) or GLP-2 from day 5 (n = 8 in each group). Animals were studied on day 8. A: all animals lost weight after DSS treatment (*P < 0.05 vs. controls), and delayed treatment with GLP-2 attenuated the degree of weight loss in DSS-treated animals (†P < 0.05 vs. DSS). B: MPO activity was increased by DSS, reflecting neutrophil infiltration. This was substantially attenuated by GLP-2 treatment. C: DSS treatment and GLP-2 treatment reduced CPI values in colon. D: Neither DSS treatment or GLP-2 therapy affected apoptosis in the crypt compartment. E–H: effects of GLP-2 on cytokine expression in DSS colitis. DSS induced a generalized increase in inflammatory cytokines and a reduction in IL-10 levels. TNF-α and IL-10 levels were normalized with GLP-2 treatment; IL-1β and IFN-γ were not affected. All values are means ± SE; n = 6 or more in each group. *P < 0.05 vs. controls and †P < 0.05 vs. DSS by ANOVA.
inflammatory effects of GLP-2 occur independently of an increase in CPI.

The improved healing and reduced inflammatory cytokine levels seen with delayed GLP-2 administration may provide clues as to potential mechanism(s) of action. Given the effects on established inflammation, GLP-2 is not simply washing out or diluting an inflammatory insult. Secondly, the effect with established inflammation also argues against GLP-2 having its effects via an influence on initial inflammatory cell recruitment, i.e., the initial chemokine response. However, the observed reduction in MPO activity strongly suggests that ongoing neutrophil recruitment is significantly reduced by GLP-2 therapy (Figs. 2–5) (22). This suggests that GLP-2 treatment decreases the local cytokine and chemokine output of an established inflammatory milieu and that these effects occur at a dosage threshold below that required to stimulate an increase in CPI and apoptosis, may also explain the observation that delayed treatment with GLP-2 appeared to be more beneficial than immediate treatment. If GLP-2 modulates this intrinsic re-

Fig. 5. Effects of VIP blockade and GLP-2 treatment in TNBS ileitis. Beginning on day 2 after TNBS induction of ileitis, groups were treated with daily subcutaneous injection of saline (TNBS) or GLP-2 (50 μg·kg⁻¹·day⁻¹ bid; d-GLP-2 and D-GLP-2 + VIP-B). VIP-B animals were treated with VIP blocking agent [Lys¹-Pro³,Arg⁴,Tyr⁶]VIP (VIP Hybrid; 50 μg·kg⁻¹·day⁻¹ sc) 2 days after the establishment of inflammation; n = 8 in each group. Animals were studied on day 5. A: all animals lost weight after TNBS treatment. Delayed GLP-2 treatment attenuated weight loss in TNBS-treated animals (*P < 0.05 vs. TNBS). d-GLP-2 + VIP-B showed equivalent weight loss to TNBS alone (†P < 0.05 vs. TNBS + D-GLP-2 group). VIP-B when given to TNBS-treated animals had no effect on weight loss compared with the TNBS group. B: MPO activity was increased by TNBS treatment. This effect was substantially attenuated by GLP-2 treatment (⁎P < 0.05 vs. TNBS). The effect of GLP-2 was reversed to levels above control by the VIP-B treatment (†P < 0.05 vs. TNBS + D-GLP-2 group). C: GLP-2 treatment reduced CPI values in ileum. The GLP-2 effect was reversed by VIP Hybrid. VIP Hybrid alone also reduced crypt proliferation in this model. D: GLP-2 reduced rates of crypt cell apoptosis, and this effect was reversed after treatment with VIP-B, which alone had no effect on rates of apoptosis. E–H: effects of GLP-2 on cytokine expression in TNBS ileitis. Cytokine content of mucosal scrapings determined by using ELISA assays for IL-1β, TNF-α, IFN-γ, and IL-10 are shown. TNBS + GLP-2 reduced the raised levels of inflammatory cytokines and increased the level of IL-10. Blockade of the VPAC receptors reversed the effects of delayed GLP-2 on IL-1β, TNF-α, and IL-10, but an effect of GLP-2 on IFN-γ persisted despite VIP Hybrid treatment. VIP Hybrid alone also reduced IFN-γ levels. All values are means ± SE; n = 6 or more in each group. *P < 0.05 vs. TNBS controls and †P < 0.05 vs. TNBS + D-GLP-2 group by ANOVA.
GLP-2 ANTI-INFLAMMATORY ACTIONS

How then is GLP-2 affecting inflammation in these models? The present study builds on previous evidence that shows GLP-2 receptor expression on neurons, specifically expressing VIP (4, 20). We showed by immunohistochemical staining that GLP-2 activates neurons in the submucosa and increases the number of neurons expressing VIP and that coadministration of a VIP antagonist abrogates the anti-inflammatory effects of GLP-2 (Figs. 5 and 6). This suggests that GLP-2 acts via a VIP-mediated pathway. This represents a unique overlap in the combined hormonal, neuronal, and immunological systems of the gut. GLP-2 has been clearly shown to activate specific GLP-2 receptors in the enteric nervous system; indeed, the GLP-2 receptor appears to be confined to the central and enteric neuronal systems, enteroendocrine cells, and myofibroblasts (4, 33). Recent work has shown that GLP-2 receptors are colocalized with VIP-positive neurons within the submucosal and myenteric plexus (20) and that GLP-2 stimulation acutely increases blood flow. We have attempted to stain directly for the GLP-2 receptors in the submucosal and myenteric plexus in the rat without success; however, our present findings show functionally that GLP-2 activates neurons and increases the number of neurons with detectable VIP within the submucosal plexus. A limitation of our study is that this was shown only in noninflamed tissue, because attempts to label whole mount preparations in inflamed mucosa were not successful due to their thickness and the infiltration of immune cells. Also, we did not attempt to double label activated neurons for neurotransmitters other than VIP. Despite these limitations, it appears likely that GLP-2 activation of VIP-expressing neurons can act as an immune modulator by decreasing the production of inflammatory cytokines and possibly by increasing the production of anti-inflammatory cytokines in inflamed mucosa. Which neurons actually express the GLP-2 receptor is not known at this time; the VIP effector neurons may be “downstream” from the actual neurons that GLP-2 activates.

VIP-expressing neurons have been shown to be widely distributed throughout the gut but typically have been ascribed a role in regulating secretory and motor functions (14, 17). VIP nerve fibers can be traced to the tip of the villus, with frequent interactions with immunocompetent cells; the density of VIP-immune cell interaction increases in models of acute inflammation (15) and may act to reset the secretory and motor activity of the gut in a protective fashion.

The immunomodulating effects of VIP are profound; indeed, VIP has been proposed as a type 2 cytokine (9). Exogenous VIP has been shown to reduce chemokine production, neutrophil recruitment, and inflammatory cytokine production by activated T cells and macrophages in Th1 inflammation (1, 2). These were the specific effects noted with GLP-2 administration in the present study; in the pilot studies to determine the dosage of VIP blockers, exogenous VIP mirrored the effects of GLP-2 in this same model. Interestingly, besides the reduction in the proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) in the TNBS ileitis and DSS colitis models treated with GLP-2, there was also an increase in the production of the anti-

**Table 1.** Immunoreactive cell counts

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<th>Control</th>
<th>GLP-2</th>
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<td>cFos cells/ganglia</td>
<td>4.0±0.7</td>
<td>8.2±1.6*</td>
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<tr>
<td>VIP cells/ganglia</td>
<td>1.5±0.2</td>
<td>4.7±0.5*</td>
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*P < 0.05 vs. controls.

**Fig. 6.** GLP-2 activation of submucosal neurons. Double-labeled whole mount preparation of the ileal submucosal plexus showing cFos-immunoreactive nuclei (red; A and B) in neurons expressing VIP (green; C and D). Double-labeled cells are readily seen in the merged panels (E and F). Animals were injected with saline (control) or GLP-2 (50 μg/kg sc) 1 h before death. Bottom shows average counts of labeled cells detected per ganglion, from 5–10 ganglia per animal, 5 animals per group (means ± SE). Comparisons were performed by using Kruskal-Wallis testing; *P < 0.05 vs. controls.
inflammatory cytokine IL-10. This has been a hallmark of exogenous VIP in similar models (9) and may be important in regulating local cell responses. These effects were not likely due to a direct effect of GLP-2 on epithelial or inflammatory cells. Extensive direct investigation has not shown a receptor or direct effect of GLP-2 on any of these cell populations, but they do have receptors for VIP (14, 26).

In summary, the results of this study show that native GLP-2 has potent anti-inflammatory effects in models of ileal and colonic inflammatory bowel disease. Labeling and VIP antagonist studies suggest a GLP-2 activated VIP-enteric nervous system pathway linking activation of the enteric nervous system and mucosal anti-inflammatory effects. These findings are supportive both of further studies to examine the mechanisms by which GLP-2 may be altering mucosal inflammation and of future clinical trials.

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DISCLOSURES

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