GLP-2 receptor agonism ameliorates inflammation and gastrointestinal stasis in murine post-operative ileus

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Number of text pages: 23
Number of Figures: 9
Number of Tables: 1
Number of References: 40
Number of words in Abstract: 227
Number of words in Introduction: 699
Number of words in Discussion: 1448

List of Abbreviations: CAM, Cellular adhesion molecules; CLDN, claudin; CNR (CBR), Cannabinoid receptor; COX-2 (PTGS2), Cyclooxygenase-2; CSF-2 (GM-CSF), Colony stimulating factor-2; DPP, Dipeptidyl peptidase; Egr-1, Early growth response gene; FITC, Fluorescein isothiocyanate; GC, Geometric Center; GI, Gastrointestinal; GLP-2, Glucagon-like peptide 2; HO-1 (HMOX-1) Hemoxygenase-1; IgG, Immunoglobulin G; iNOS (NOS2), Inducible nitric oxide synthase; IFN-γ, Interferon-γ; MCP-1 (CCL2), Monocyte chemoattractant protein-1; MIPα (CCL3), Macrophage inflammatory protein; MMP, Matrix metalloprotease; MPO+, Myeloperoxidase-positive; OCLN, occludin; PBS, Phosphate buffered saline; POI, Postoperative ileus; TIMP, Tissue inhibitor of matrix metalloprotease; TLR, Toll-like receptor; TNF, Tumor necrosis factor; VIP, Vasoactive intestinal polypeptide.
Abstract

Glucagon-like peptide 2 (GLP-2) is a pleiotropic intestinotrophic hormone that we hypothesized could lessen gastrointestinal inflammation associated with postoperative ileus (POI). To test this idea, the prophylactic timing and dose of a long-acting variant of human GLP-2 linked to the Fc portion of murine IgG (GLP-2/IgG) was optimized in a murine model of POI. Surgically treated mice received a single dose of GLP-2/IgG, IgG isotype control or PBS 1-48 hours prior to small bowel surgical manipulation. The distribution of orally fed FITC-dextran and histological analyses of myeloperoxidase-positive (MPO+) immune cells were determined 24 and 48 hours post-operatively. TaqMan® qPCR was used to determine early changes in mRNA expression in the muscularis or mucosa. In normal mice, prolonged exposure to GLP-2 increased upper gastrointestinal (GI) transit and mucosal weight. When administered 1 or 3 hours prior to surgery, GLP-2/IgG reduced the leukocyte infiltrate 24 and 48 hours post-operatively, and improved GI transit 48 hours post-operatively. Surgical manipulation rapidly increased gene expression of pro-inflammatory cytokines and enzymes for kinetically active mediators in the mucosa and muscularis. GLP-2/IgG2a impacted the expression of genes associated with mucosal inflammation and barrier function. We conclude that prophylactic treatment with a long-acting GLP-2 agonist ameliorates inflammation and improves intestinal dysmotility associated with surgical manipulation of the bowel. The action of GLP-2 is consistent with a lessening of inflammation leading to a more rapid recovery.
INTRODUCTION:

Glucagon-like peptide 2 (GLP-2) is produced by a subset of enteroendocrine cells residing within the epithelium of the gastrointestinal (GI) tract (Yusta et al., 2000). It functions as a pleiotropic intestinotrophic hormone with wide ranging effects that promote mucosal growth and intestinal homeostasis. The integrative GI responses to GLP-2 are mediated via the GLP-2 receptor, a member of the glucagon/secretin GPCR super-family which is located on enteric (Bjerknes and Cheng, 2001) and vagal (Nelson et al., 2007) nerves, subepithelial myofibroblasts (Orskov et al., 2005) and a subset of intestinal epithelial cells (Thulesen et al., 2000). Activation of GLP-2 receptors regulates epithelial cell growth (Bjerknes and Cheng, 2001), reduces intestinal permeability (Estall and Drucker, 2006), increases mesenteric blood flow (Guan et al., 2006; Stephens et al., 2006), and promotes nutrient absorption (Nelson et al., 2007).

Both cytoprotective and neuroprotective properties have been attributed to GLP-2 (Wallace et al., 2008) suggesting that it may have an important role in intestinal adaptation and repair during inflammatory events. Native GLP-2 given twice daily lessened the enhanced permeability and expression of pro-inflammatory cytokines associated with TNBS colitis (Sigalet et al., 2007). These data are supported by observations in clinical trials, leading to the conclusion that GLP-2 has protective effects in inflammatory bowel disease (Wallis et al., 2007). The importance of GLP-2 in intestinal adaptation is evidenced by Teduglutide (GATTEX™, ALX-0600; NPS Allelix Corp), a GLP-2 analogue developed for treatment of short bowel syndrome (Mardini and de Villiers, 2008), a disorder characterized by impaired intestinal barrier function and poor nutrient absorption. In addition, studies in rodent models of intestinal injury have shown that Teduglutide ameliorates mucosal damage, crypt cell epithelial apoptosis, intestinal lesions, and inflammation (Drucker et al.,...
Thus the GLP-2 receptor is an attractive target for the development of pharmaceutical interventions to promote intestinal adaptation and repair.

Postoperative ileus (POI) continues to be a significant clinical problem following open abdominal surgery, extending the length of hospital stay and contributing to postoperative morbidity and complications. Disturbance to the bowel during abdominal surgery elicits multifactorial responses within the bowel wall that inhibits intestinal smooth muscle contractility. The mechanisms controlling the initiation and maintenance of POI involve a complex array of immune cell interactions, central inhibitory reflexes and neuroimmune interactions (Boeckxstaens and de Jonge, 2009). The cited review describes a number of potential targeted approaches for the pharmacologic management of ileus, including prokinetics, mu opioid receptor antagonists, inhibitors of early signaling inflammatory events, inhibitors of prostaglandin and nitric oxide synthesis, as well as the early and enhanced induction of anti-inflammatory mediators. Although these targeted approaches to the pathways involved in ileus show efficacy, it is attractive to explore whether the integrated intestinal effects of GLP-2 in promoting intestinal homeostasis would reduce the severity of POI.

Native GLP-2 has a short biological half-life (~7 minutes systemic) due to its rapid proteolytic degradation by dipeptidyl peptidase (DPP)-IV (Hartmann et al., 2000). Dosing in preclinical models requires multiple daily injections to maintain adequate systemic exposure. Modification of the 33 amino acid sequence of the natural peptide has produced a few synthetic agonist analogs of GLP-2 with enhanced resistance to DPP-IV cleavage. For example, Teduglutide™ is a synthetic recombinant human GLP-2 variant with an alanine to glutamine substitution (A2G GLP-2) at the second amino acid residue from the
N-terminus of the native peptide, a modification that leads to a modest increase in half-life to ~25 minutes. In this study, we used the same peptide variant linked to the Fc portion of human IgG4 (GLP-2/IgG4) or mouse IgG2a (chimeric GLP-2/IgG2a). As previously described for a GLP-1 construct (Picha et al., 2008), this linkage increased half life dramatically from minutes to days in rodents. During early evaluation of the construct for its capacity to promote mucosal growth, we observed to our surprise that the A2G GLP-2 peptide variant enhanced upper gastrointestinal transit. Based on the known positive effects of GLP-2 in models of intestinal inflammation and the unexpected finding of a prokinetic effect, we hypothesized that GLP-2 receptor agonism would reduce intestinal inflammation and dysmotility associated with POI.

A preliminary description of this study was presented previously in abstract form (Moore et al., 2008).
METHODS

Age-matched male or female CD-1 mice were used in the studies described in this manuscript. The experiment protocols in animals were reviewed and approved by Centocor and Johnson & Johnson Pharmaceutical R&D Institutional Animal Care and Use Committees. Mice were maintained on a 12 hour light/dark cycle with access to rodent chow and water *ad libitum*.

**Potency and Serum Half Life**

The activities of A2G GLP-2, GLP-2/IgG4 and GLP-2/IgG2a peptides were evaluated *in vitro* at 8 concentrations (0.03-3000 nM) using LANCE cAMP assay (Perkin Elmer) in HEK293 cells stably transfected with a mutated form of human GLP-2 receptor.

Serum levels of chimeric GLP-2/IgG2a in mice were detected based on the presence of allotype alleles that are distinct antigenic determinants on homologous proteins specified by allelic forms of immunoglobulin genes. Serum was collected from untreated control CD-1 mice given chimeric GLP-2/IgG2a (4 mg/kg intravenously) and from surgically treated mice 48 hours after intestinal manipulation, where chimeric GLP-2/IgG2a (4 mg/kg) was given 1 hour prior to surgery. Cell culture plates (US Biological, Swampscott, MA) were coated with 2 μg/ml mouse anti-mouse IgG2a<sup>a</sup> allotype-specific (IgH-1a; clone 8.3) antibody (50 μl/well; BD Biosciences) diluted to 2 μg/ml in 50 μl PBS. Plates were then treated for one hour with 200 μl/well blocking solution (PBS, 0.05% Tween, 1% BSA (Sigma, St Louis MO). After washing, purified human GLP-2 peptide conjugated to murine IgG2a<sup>a</sup>, murine IgG2a<sup>a</sup> alone, or sera from treated mice was diluted in blocking solution. Detection was by 1 μg/ml of an in-house generated mouse anti-N-terminal human GLP-2 peptide. The signal was amplified with streptavidin-horseradish peroxidase diluted 1:8000 in blocking solution. Optical densities of TMB substrate reaction product were determined.
(SPECTRAmax Plus 384; Molecular Devices, Sunnyvale, CA) with SoftMax Pro software (Molecular Devices Corporation). Plates were read at 450 nm (excitation) minus 650 nm (emission) and GLP-2/IgG2a was quantified from standard curves generated from known quantities of the construct. We confirmed that this assay detected intact chimeric GLP-2/IgG2a but not murine IgG2a alone or the human construct GLP-2/IgG4 (all added at 20 µg/ml and serially diluted 1:4 down to 0.02 ng/ml in the blocking buffer).

**Intestinotrophic Effects**

The intestinotrophic effects *in vivo* of A2G GLP-2 peptide and GLP-2/IgG4 construct were determined in female CD-1 mice. Mice were randomly assigned (n=7 per group) to receive daily subcutaneous injections of A2G GLP-2 peptide (50 µg in a volume of 500 µl), GLP-2/IgG4 (1.6, 16 and 160 µg in a volume of 500 µl) or equivalent volume of phosphate buffered saline (PBS) control for 10 days. In a separate study, GLP-2/IgG4 (4 mg/kg) was administered as a single dose on day 1 only, daily for 10 days, once every 2 days, or on days 1 and 7. On day 11, animals were euthanized by CO2 inhalation, and a segment of small bowel was harvested from ~6 cm distal to the ligament of Treitz to ~4 cm proximal to the cecum. The length of the unstretched bowel was measured, after which the bowel was opened longitudinally and rinsed with ice-cold PBS. The mucosa was removed by scraping with a glass slide, and mucosal scrapings were collected and weighed.
Post-operative ileus model

Age-matched male CD-1 mice were anesthetized with inhaled isoflurane and the abdomen was opened by midline laparotomy. The small intestine was eventrated and then gently compressed along its entire length using moistened sterile cotton applicators. This procedure was designed to simulate running of the bowel, which is performed commonly during abdominal surgery in the clinical setting. The bowel was repositioned in the abdominal cavity and the incision closed with 2 layers of continuous sutures. The duration of the procedure was approximately 15 minutes, and the animals moved freely about their cage within 20 minutes of anesthetic withdrawal.

Upper Gastrointestinal Transit

In an initial study, upper GI transit was determined 30 minutes after oral gavage of a test meal consisting of carmine red 6% w/v in 0.5% methylcellulose, Methocel® (Dow Chemical, Midland, MI) and 99.5% deionized water. After CO₂ euthanasia and laparotomy, the small bowel was removed intact from the pyloric sphincter to the ileocecal junction. The distance travelled by the marker was measured and reported as the percent of the total length of the unstretched bowel.

In all subsequent POI studies, upper GI transit was determined by calculating the Geometric Center (GC) from the weighted average distribution of a non-absorbable fluorescent marker along the entire GI tract. Fluorescein isothiocyanate (FITC) conjugated to dextran (70,000 MW; FITC-dextran) was dissolved at a concentration of 5 mg/ml in 0.5% Methocel®/99.5% deionized water w/v. FITC-dextran was administered orally by gavage (volume = 0.1 ml/10 g body weight, to a maximum of 0.250 ml). Animals were returned to their home cages for 45 minutes and then euthanized by inhaled isoflurane with exsanguination. The entire GI tract from the lower esophageal sphincter to the terminal...
colon was harvested intact and divided into stomach, small bowel (10 segments of equal length), cecum, and colon (3 segments of equal length). Each segment was opened and minced, and the tissue plus luminal contents were added to 2 ml tubes containing 1 ml PBS. The contents were vigorously vortexed and the solid material pelleted by centrifugation. Aliquots of the supernatant were loaded in duplicate onto a 96 well plate, and the fluorescent signal for each bowel segment was determined using a fluorescence plate reader (Cytofluor™, excitation wavelength 530 nm and emission 590 nm). GC was calculated as: \( \sum (S1x1 + S2x2 + \ldots + S11x11) \), where S is the fraction of the total signal detected in each of the 15 segments (Miller et al., 1981).

**Myeloperoxidase Histochemistry**

The magnitude of the inflammatory cell infiltrate into the intestinal muscularis was quantified by histological analysis. Muscularis wholemounts were prepared from the mid jejunum collected 24 hours after surgery. Segments of intestine were opened along the mesenteric border, stretched to 150% of the length and 250% of the width and fixed in 100% ethanol for 1 hours. After washing in PBS, the mucosa was removed by fine dissection, and the remaining tissue treated with Hanker-Yates reagent (Polysciences, Warrington, PA) for detection of polymorphonuclear neutrophils exhibiting myeloperoxidase (MPO) activity. Tissues were mounted on glass slides, cover-slipped, and inspected by light microscopy at a magnification of 200X. The number of MPO-positive immune cells infiltrating the intestinal muscularis was determined as the average number of cells counted in 5 to 6 adjacent optical fields centered between the mesenteric and anti-mesenteric borders.
Gene expression in small bowel mucosa and muscularis externa

Age-matched male CD-1 mice were divided into experimental groups as follows; naive controls (untreated), and 2 groups treated with either PBS or chimeric GLP-2/IgG2a 3 hours prior to anesthesia and surgical manipulation of the small bowel. Animals were euthanized and tissue harvested 0.5, 1.0, 3.0 and 6.0 hours post-operatively. The small bowel was removed from the ligament of Treitz to the ileocecal junction, placed immediately into ice cold PBS and cut into segments. Bowel segments were flushed with PBS and mounted on a glass rod where the muscularis externa was stripped from the underlying mucosa. Muscularis and mucosal tissues were snap frozen in liquid nitrogen and stored at -80°C.

RNA was isolated from frozen tissues using the RNeasy® Lipid mini protocol (Qiagen, Inc., Valencia, CA). Briefly, 1 ml of QIAzol® reagent (Qiagen, Inc., Valencia, CA) was added per 100 mg of frozen tissue and homogenized for ~20 seconds at maximum speed using a rotor-stator homogenizer. After sitting at room temperature for 5 minutes, 1 ml of lysate from muscularis tissue or 200 µl of lysate from mucosal tissue plus an additional 800 µl of QIAzol® reagent were extracted with 200 µl chloroform. After centrifugation at 12,000 x g for 15 min. at 4 deg C, RNA was purified from the upper aqueous phase by the RNeasy® mini protocol with DNase digestion on the column as per the manufacturer’s instructions. RNA quality and quantity were assessed using a LabChip®GX (Caliper Life Sciences, Inc., Hopkinton, MA). Reverse Transcription of RNA was performed using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer’s instructions, with 1 µg of RNA in 30 µl. Gene expression for markers of inflammation, matrix integrity, and barrier function was determined by TaqMan® analysis using custom low density arrays (Applied Biosystems, Inc., Foster City, CA, Supplemental Table 1), with 100 ng of reverse-transcribed RNA in 100 µl 1x TaqMan® Universal PCR Master Mix loaded per slot. Alternatively, individual reactions were set up with 20 ng of
reverse-transcribed RNA in 10 µl 1x TaqMan® Universal PCR Master Mix. Real-time PCR was performed in duplicate using the comparative Ct method on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer’s instructions. GAPDH was used as the endogenous control. Data are reported as fold change relative to non-operated, PBS-treated, control mice.

**Statistical Analysis**

The statistical analyses performed are noted with the corresponding data, which are reported as mean ± SEM and P<0.05 being considered statistically significant. In general, data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc testing for multiple comparisons. In cases where 2 groups were compared, an unpaired Student’s t-test was performed. For the analysis of mRNA expression at different times after surgery in control and experimental groups a two-way ANOVA was performed followed by Bonferroni post hoc tests.
RESULTS

In cell based cAMP assays, the A2G GLP-2 peptide (EC$_{50}$ = 0.58 nM) was approximately 4 fold more potent than GLP-2/IgG4 and chimeric GLP-2/IgG2a (EC$_{50}$ ~ 2.3 nM). This reduced potency the GLP-2/IgG4 construct was countered by an increased serum half life compared to the A2G GLP-2 peptide, since a single intravenous dose (1 mg/kg) in a Cynomolgus monkey exhibited a serum half life of 5.4 ± 0.9 days when serum samples up to 28 days were analyzed by ELISA for full length peptide.

The reported protective effects of GLP-2 in inflammation models led us to test the hypothesis that a long lived GLP-2/IgG4 construct would ameliorate inflammation in murine POI. Initial experiments evaluated the trophic effects of the A2G GLP-2 peptide and the GLP-2/IgG4 construct on the intestinal mucosa to determine appropriate doses of GLP-2/IgG construct in mice. As expected, daily administration of A2G GLP-2 peptide (50 µg/mouse) for 10 days increased mucosal wet weight (Fig 1A). GLP-2/IgG4 increased mucosal weight dose-dependently, with the highest dose (160 µg/mouse; ~4 mg/kg) eliciting a 4 fold increase in mucosal weight above baseline. Unexpectedly, daily administration of A2G GLP-2 peptide increased upper GI transit (Fig. 1B) as well as a similar increase in mucosal weight to that shown in Fig 1A (data not shown).

Based on these findings, a dose of 4 mg/kg GLP-2/IgG4 was selected for further characterization for both improvements in inflammation and gastrointestinal transit in the mouse surgical model of POI. However, initial experiments using the POI surgical model suggested a potential confounding effect due to non-self discrimination against human IgG4 in this model of inflammation. Therefore, we generated a chimeric construct derived by linking human A2G GLP-2 to murine IgG2a (chimeric GLP-2/IgG2a). After a single intravenous dose (4 mg/kg), full length intact chimeric GLP-2/IgG2a in excess of 1 µg/ml
was detected by ELISA for at least 48 hours using antibodies specific for mouse allotype IgG2a and mouse anti-N-terminal GLP-2 (Fig. 2). In a separate group of mice GLP-2/IgG4 (4 mg/kg) increased mucosal weight to the same extent (0.20 ± 0.02 g vs PBS 0.09 ± 0.01 g; P<0.05) whether administered daily or every other day for 10 days. Further increasing the length of time between dosing (treating only on days 1 and 7) did not result in an increase in mucosal weight above control; therefore the biological duration of action of GLP-2/IgG4 in mice was at least 48 hours. Based on the duration of effect on mucosal growth combined with the detection of serum levels of chimeric GLP-2/IgG2a out to 48 hours, the construct was subsequently administered subcutaneously only once to naïve mice or to operated mice prior to surgery and intestinal manipulation.

Due to the evidence from a model of ischemia-reperfusion injury suggesting that the efficacy of GLP-2 was affected by the timing of GLP-2 pretreatment (Zhang et al., 2008), we tested different pretreatment times at 1, 3, 24, or 48 hours prior to surgery (Fig. 3). Cellular infiltrate into the intestinal muscularis (MPO+ cell counts) and upper GI transit were determined at 2 time points: 24 hours post-operatively to determine whether chimeric GLP-2/IgG2a pretreatment inhibited the peak postoperative manifestations of ileus, and 48 hours post-operatively to determine effects on the recovery phase.

The effects of GLP-2/IgG2a administered 1 hour prior to abdominal surgery and induction of POI are shown in Fig 4. GLP-2/IgG2a treatment did not have a significant effect on GI transit 24 hours post-operatively (data not shown), but by 48 hours post-operatively transit was improved to the point where it was not significantly different from that of naive mice (Fig. 4A). Digital photomicrographs of representative muscularis wholemounts collected 24 hours post-operatively show that MPO+ cells within the small bowel muscularis of surgically manipulated animals are markedly reduced in number in
animals treated with GLP-2/IgG2a compared to PBS and isotype controls (Fig. 4B). These data are summarized in Figures 4C and D, where a significant reduction in the MPO+ cellular recruitment in GLP-2/IgG2a treated animals was observed at both the 48 and 24 hours postoperative time points (Fig. 3C & D). A similar reduction in cellular influx was observed when a higher GLP-2/IgG2a dose (4 mg/kg) was given and responses evaluated 48 hours post-operatively (isotype pretreated = 217±41 MPO+ cells per 200X field; GLP-2/IgG2a pretreated = 62±14; P<0.05). In these mice, terminal blood draws were collected and plasma levels of 1913 ± 387 ng/ml for the fully intact GLP-2/IgG2a (n=8) were detected at 48 hours, indicating that the construct exhibited good plasma exposure at this time point.

GLP-2/IgG2a given 3 hours prior to surgery at a range of doses (0.5 – 2.0 mg/kg), dose-dependently decreased MPO+ immune cells at both postoperative time points, with improvements in upper GI tract transit occurring only at the 48 hours postoperative time point (Fig. 5A & B). Significant reductions in cellular infiltrate were obtained with GLP-2/IgG2a at doses of 1 & 2 mg/kg; however, an improvement in GI motility was obtained only at the 2 mg/kg dose. One explanation for the absence of a positive effect on transit at the 24 hour postoperative time point might be GLP-2 mediated inhibition of GI transit, for example due to stimulation of vagal afferent reflexes (Nelson et al., 2007). Therefore, we tested the effects of chimeric GLP-2/IgG2a administration on upper GI transit in naïve mice (Fig. 5C). The construct had no effect on GI transit measured 24 hours after administration, indicating that the absence of improved GI transit 24 hours after surgery could not be explained by a GLP-2 mediated inhibitory effect.

Chimeric GLP-2/IgG2a (4 mg/kg) given 24 hours prior to surgery maintained the capacity to reduce cellular influx determined 24 hours post-operatively (Fig. 6A), but again no improvement in GI transit was observed (Fig. 6B). When the time of administration of GLP-
2/IgG2a (4 mg/kg) was increased to 48 hours prior to surgery, there was no significant effect on either cellular influx 24 hours post-operatively (isotype pretreated = 118±16 MPO+ cells per 200X field; GLP-2/IgG2a pretreated = 90±33; P>0.05) or upper GI transit (isotype pretreated = 4.29±0.50; GLP-2/IgG2a pretreated = 4.76±0.60, P>0.05).

To gain insight into the mechanisms by which the long-acting GLP-2 agonist given prophylactically decreased inflammatory infiltrate and improved GI transit, TaqMan® RT-PCR was performed on mucosa and muscularis tissues harvested from the small bowel. Of the genes tested, 23 in the mucosa and 10 in the muscularis exhibited statistically significant changes in expression in response to either surgical manipulation of the small bowel or to treatment with GLP-2/IgG2a (Table 1). The pattern of gene expression was time-dependent, with the time of peak expression varying according to the gene and its location. Surgical manipulation rapidly increased pro-inflammatory gene expression in both the mucosa and muscularis (Fig. 7, 8); examples include the transcription factor early growth response gene (Egr)-1, the pro-inflammatory cytokines interleukin (IL)-6 and IL-1β, the chemokines monocyte chemoattractant protein (MCP)-1, macrophage inflammatory factor (MIP)-1α, as well as enzymes that synthesize the kinetically active mediators nitric oxide (inducible nitric oxide synthase, iNOS) and prostaglandin E2 (cyclooxygenase-2, COX-2). Increased expression was also observed for the putative anti-inflammatory mediators IL-10 and hemoxygenase-1 (HO-1).

In the muscularis, pretreatment with chimeric GLP-2/IgG2a resulted in a significant, though modest, reduction in the peak increase in message for the pro-inflammatory mediators Egr-1, MIP-1α, iNOS, and COX2 (Fig 7). Peak HO-1 gene expression was moderately enhanced. GLP-2/IgG2a treatment resulted in a more prominent effect on the expression of pro-inflammatory genes within the intestinal mucosa (Fig. 8). Treatment with
GLP-2/IgG2a significantly enhanced Egr-1 expression relative to control at the 30 minutes time point, followed by a marked inhibition of Egr-1 expression 1 hours post-operatively. Peak expression of IL-6 and iNOS was also markedly reduced. Evidence for an early reduction in COX2 gene expression was apparent at the 1 hours postoperative time point. The effects of GLP-2/IgG2a treatment on IL-1β, MCP-1 and MIP-1α were less clear. Although statistically significant changes were observed at some time points, the effects were minor. The surgically induced induction of IL-10 and HO-1 in (not shown) was not altered by GLP-2/IgG2a treatment in the mucosa. In general, expression of genes for tight junctional proteins (occludin, claudin 1, claudin 3) was not altered by surgical manipulation (Fig. 9), although statistically significant changes in expression were observed in GLP-2/IgG2a treated animals.
DISCUSSION

This study provides the first evidence that treatment before surgery with a single dose of a long acting GLP-2 receptor agonist reduced cellular inflammation associated with intestinal manipulation and enhanced recovery from the surgically-induced delay in gastrointestinal transit. Insight into the mechanisms by which GLP-2 receptor agonism mediates these beneficial effects was obtained from examining changes in the mRNA expression profiles of genes known to be altered within the muscularis and mucosa following small bowel manipulation. GLP-2 receptor agonism did not markedly alter the resident macrophage driven molecular inflammatory response within the muscularis, a mechanism that is normally attributed to induction of cellular inflammation in that tissue. Instead, GLP-2 may be exerting its protective effect by impacting expression of genes associated with mucosal inflammation and barrier function. Its potential therapeutic effects on inflammation and motility are discussed in the context of the known literature.

The mechanisms controlling the initiation and maintenance of POI involve a complex series of events involving altered mucosal barrier function, production of inflammatory mediators, and neuroimmune interactions (Boeckxstaens and de Jonge, 2009). Briefly, disturbance to the bowel during abdominal surgery activates sympathetic inhibitory reflexes, suppressing GI motility within the first few hours after bowel manipulation. At this early time point a molecular inflammatory response is initiated via the activation of resident macrophages by the endproducts of arachidonic acid metabolism, extracellular matrix fragments, and the mucosal translocation of luminal antigen. The induction of macrophage derived transcription factors (EGR-1, STAT3, NFκB), the expression of cellular adhesion molecules (ICAM-1), and the production of chemokines (MCP-1, MIP-1α) elicits the recruitment of neutrophils, monocytes and mast cells to the intestinal muscularis and
mucosa producing a cellular inflammatory response. Mediators released from mast cells and cytokines (IL-6, IL-1β), nitric oxide (iNOS derived) and prostaglandin E2 released from resident macrophages, and recruited immune cells impair neuromuscular communication, inhibit smooth muscle contractility, and enhance central inhibitory neural reflexes, resulting in suppression of GI motility over the subsequent 72 hours.

The cellular inflammatory response is initiated 6 hours after bowel manipulation, reaching its peak by 24 hours and persisting up to 72 hours. Administration of the chimeric GLP-2/IgG2a construct 3 hours prior to surgery markedly reduced cellular inflammation within the muscularis 24 and 48 hours post-operatively. This pretreatment protocol was selected to provide sufficient time for the GLP-2 construct to reach the site of action during the early molecular changes in response to surgery and to provide measurable plasma exposure out to 48 hours post-treatment. In the intestinal muscularis, small bowel manipulation increased expression (> 100 fold) of the pro-inflammatory genes EGR-1, IL-6, IL-1β, MCP-1, MIP-1α, and colony stimulating factor (CSF)-2. Increases in TNFα, cannabinoid receptor (CNR)-2, COX2 and iNOS were also noted. In general, the effects of GLP-2/IgG2a treatment were relatively modest, reducing the expression of some pro-inflammatory genes (e.g. EGR-1, MIP-1α, iNOS, CNR2 and COX2), while having no effect on others (e.g. IL-6, IL-1β, or MCP-1). In particular, the lack of an inhibitory effect on IL-6 and MCP-1, would tend to argue for a cellular inflammatory response, since these have been linked to the upregulation of adhesion molecules and to cellular recruitment (Turler et al., 2002; Wung et al., 2005). Expression of the anti-inflammatory molecules, HO-1 and IL-10 was increased in surgically manipulated animals, but GLP-2/IgG2a treatment only enhanced HO-1 expression, which may have contributed to the reduced inflammatory response within the muscularis. GLP-2 treatment had little effect on IL-10 gene expression,
consistent with the observation that the anti-inflammatory effect of GLP-2 persists in IL-10 knockout mice with colitis (Ivory et al., 2008). In conclusion, although it cannot be ruled out that the GLP-2/IgG2a construct is affecting translation of protein, the modest amelioration of the muscularis molecular inflammatory response may contribute to, but is unlikely to be, the major underlying cause of the reduced cellular inflammation.

One way by which the GLP-2 construct exerts anti-inflammatory effects in POI may involve early modulation of gene expression of multiple signaling pathways in the mucosa. Chimeric GLP-2/IgG2a inhibited early pro-inflammatory gene expression of IL-6, IL-1β, MCP-1, MIP-1α, and COX2, indicative of an overall blunting of the molecular inflammatory response. GLP-2 treatment also reduced mucosal MPO and inflammatory cytokine protein levels (INF-γ, TNF-γ, IL-1β) in ileitis/colitis (Sigalet et al., 2007). However, the positive effects of GLP-2 receptor agonism in the mucosa did not appear to involve the enhancement of anti-inflammatory mechanisms, since the surgically-induced increase in IL-10 and HO-1 expression were not altered by GLP-2/IgG2a treatment. Rather, the GLP-2 construct ameliorated a prominent effect in the mucosa, which was the enhanced mRNA expression of Egr-1. In addition to its role in inflammatory events associated with POI (Schmidt et al., 2008), Egr-1 has been linked to protection against intestinal epithelial cell injury and impaired barrier function (Moon et al., 2007). Surgical manipulation of the bowel transiently increases mucosal permeability (Schwarz et al., 2002; Turler et al., 2007), which allows passage of bacterial cell wall components, enterotoxins and intact bacteria. Native GLP-2 treatment reduces stress-induced mucosal bacteria adherence and translocation induced by enhancing intestinal epithelial barrier function (Cameron and Perdue, 2005). Pro-inflammatory cytokines can also alter the interactions between the tight junction proteins claudins and occludin resulting in increased paracellular permeability (Capaldo...
and Nusrat, 2009). Increased occludin protein expression is reported to enhance trans-
epithelial resistance (Balda et al., 1996) and GLP-2/IgG2a treatment resulted in a small,
though significant increase in occludin gene expression. Finally, endogenous GLP-2 in the
mucosa may contribute to healing responses, as indicated by observations of increased
numbers of GLP-2 positive enteroendocrine cells during and after resolution of ileitis/collitis
(Lomax et al., 2006). Together, these GLP-2 mediated effects may enhance epithelial
barrier function, reducing translocation of luminal materials and limiting the activation of
processes that drive inflammation.

The onset of cellular inflammation correlates temporally with the decline in intestinal
smooth muscle contractility, with both reaching their peak 24 hours post-operatively. In
the present study, consistent and dose-related improvements in upper gastrointestinal
motility were noted only 48 hours post-surgery. The presence of dysmotility at 24 hours, in
the absence of a cellular infiltrate in the muscularis, has been noted previously and was
attributed to a persistent molecular inflammatory response driven by the resident
macrophage cell population within the muscularis (Moore et al., 2007). In the current
study, GLP-2 receptor agonism had little effect on key mediators in the muscularis that are
known to disrupt motility (IL-6, IL-1β, COX-2, iNOS). IL-6 and IL-1β are known to impair
neuromuscular communication and decrease motor function (Natale et al., 2003). COX-2
derived prostaglandins and iNOS derived nitric oxide have direct inhibitory effects on
smooth muscle contractility (Schwarz et al., 2001; Turler et al., 2006). Thus, the failure of
GLP-2/IgG2a treatment to strongly suppress this molecular response may account for the
persistent intestinal dysmotility observed 24 hours post-operatively.

Another factor contributing to dysmotility could be through GLP-2 mediated
activation of neural inhibitory reflexes within the first few hours after bowel manipulation.
Acute administration of GLP-2 peptides delays gastric emptying and slows intestinal transit within a few hours after treatment (Wojdemann et al., 1998; Shibata et al., 2001), and raises the question whether this inhibitory effect could carry out to 24 hours. However, the administration of the chimeric GLP-2/IgG2a construct in normal mice had no effect on transit at this time point. Therefore, the dysmotility seen 24 hours post-operatively in manipulated mice is unlikely to be explained by a direct effect of the construct on smooth muscle or central neural-inhibitory reflexes.

The increase GI transit after longer exposure to GLP-2 in normal and POI mice is a novel finding. In POI mice this is likely to be the result of the reduced inflammatory cell infiltrate. The molecular inflammatory response within the resident macrophage population begins to decline 6 to 12 hours post-operatively. This, along with the absence of an ongoing molecular inflammatory response from infiltrating cells would be expected to result in a more rapid recovery of bowel motility. The activation of vagal cholinergic anti-inflammatory processes by long-acting GLP-2 receptor agonism may also contribute to this process, whereby efferent cholinergic activity of the vagus nerve ameliorates cellular inflammation and dysmotility associated with POI (de Jonge et al., 2005; The et al., 2007).

In summary, our findings demonstrate that prophylactic treatment with a long-acting GLP-2 receptor agonist attenuates inflammation associated with postoperative ileus and accelerates recovery from bowel stasis. The beneficial effects of GLP-2 receptor agonism is mediated by multiple functional pathways involving reduced gene expression for pro-inflammatory mediators and the enhanced expression of genes involved in maintaining mucosal integrity and repair. These findings extend those reporting the beneficial effects of GLP-2 in other models of gastrointestinal injury and confirm that the mechanism of action involves the induction of genes that support bowel homeostasis.
Acknowledgments:

The investigators appreciate the insights of Peter Bugelski and Karyn O’Neil (Centocor R&D) and the support of Patricia Andrade-Gordon (J&J PRD), Frederic Baribaud, Eva Emmel, and Anuk Das (Centocor R&D).
References


Legends for Figures

Figure 1.  A. Small intestinal weight of mucosa after daily administration of PBS, A2G GLP-2 peptide (50 µg/day), or GLP-2/IgG4 construct (1.6-160 µg) for 10 days. Data are mean ± SEM, n = 7 per group. * = P<0.05 compared to PBS; # = P<0.05 compared to peptide alone by ANOVA and Bonferroni’s multiple comparisons test. B. Daily administration of human A2G GLP-2 peptide increased upper GI transit (calculated by determining the leading edge of a carmine red meal 30 minutes after oral administration). Number in column is animals per group. * = P<0.05 by unpaired t-test.

Figure 2. Serum levels of intact chimeric GLP-2/IgG2a detected by ELISA using antibodies specific for mouse allotype IgG2a and mouse anti-N-terminal GLP-2 0.15 to 168 hr after a single intravenous dose of 3 mg/kg (n=3 mice per time point).

Figure 3. Summary of the protocols used to illustrate the different treatments and times relative to surgery, involving laparotomy and small intestinal manipulation, and their endpoint measures. The effects of chimeric GLP-2/IgG2a at a range of concentrations (0.5-4.0 mg/kg) were compared with isotype control (IgG2a) and/or PBS given prior to laparotomy (1, 3, 24 and 48 hr). The distribution of FITC-dextran (upper GI transit) was determined by calculating Geometric Center and quantification of the numbers of myeloperoxide positive cells infiltrating small bowel muscularis whole mounts were performed 24 or 48 hr after surgery (n≥5 per group). Chimeric GLP-2/IgG2a (2 mg/kg, sc) or PBS was administered 3 h prior to surgery for mRNA analysis of muscularis externa and mucosal layers of small intestine harvested at 0.5, 1.0, 3.0 and 6 hr post-surgery (n=5 per group).
Figure 4. Chimeric GLP-2/IgG2a (2 mg/kg) administered 1 hr prior to surgical manipulation of the small bowel improves upper GI transit 48 h post-operatively (A). Digital images demonstrate that the influx of myeloperoxidase–positive (MPO+) immune cells within small bowel muscularis whole mounts is reduced by chimeric GLP-2/IgG2a pretreatment whereas IgG2a isotype (2 mg/kg s.c.) and PBS were without effect (B). Chimeric GLP-2/IgG2a (2 mg/kg s.c.) significantly reduced the inflammatory cell infiltrate 48 hr (C) and 24 hr (D) post-operatively. * P<0.05 compared to naïve controls; ^ P<0.05 compared to isotype and PBS by ANOVA and Bonferroni’s post-test. Data are mean ± SEM, n=5-14 per group.

Figure 5. Illustrates the dose-related effect of chimeric GLP-2/IgG2a (0.5-2.0 mg/kg) administered 3 hour prior to intestinal manipulation on MPO+ immune cells in small bowel whole mounts (A) and upper GI tract transit (B) measured 48 hr post-operatively. Data are mean ± SEM, n = 5-8 per group. * P<0.05 compared to naïve; # P<0.05 compared to control (PBS); ^ P<0.05 compared to 0.5 mg/kg GLP-2/IgG2a by ANOVA and Bonferroni’s test. In naive mice (C) there was no direct inhibitory effect on upper GI transit at 24 hr after 2 mg/kg GLP-2 IgG2a administration (n=10 per group).

Figure 6. Chimeric GLP-2/IgG2a (4.0 mg/kg) administered 24 hr prior to intestinal manipulation improves the cellular infiltrate (A) but not upper GI transit (B) in small bowel whole mounts harvested 24 hr post-operatively. * P<0.05 compared to PBS # P<0.05 compared to isotype control by ANOVA and Bonferroni’s test, Data are mean ± SEM, n≥5 per group.

Figure 7. Chimeric GLP-2/IgG2a given 3 hr prior to surgery altered mRNA levels (relative to GAPDH) of selected inflammatory-related mediators that were increased in small
bowel muscularis externa 0.5 to 6 hr following surgery. Data are shown as fold change over untreated controls, mean ± SEM, n=5 animals per group. # = P<0.05 compared to PBS treatment at the same time point by 2 way ANOVA and Bonferroni’s test.

Figure 8. Chimeric GLP-2/IgG given 3 hr prior to surgery altered mRNA levels (relative to GAPDH) of selected inflammatory and neurotransmitter mRNA levels in small bowel mucosa 0.5 to 6 hr following surgery shown as fold change over untreated controls. Data are mean ± SEM, n=5 animals per group. # = P<0.05 compared to PBS treatment at the same time point by 2 way ANOVA and Bonferroni’s test.

Figure 9. Chimeric GLP-2/IgG given 3 hr prior to surgery altered mRNA levels (relative to GAPDH) of selected tight junction mRNA levels in small bowel mucosa 0.5 to 6 hr following surgery shown as fold change over untreated controls. Data are mean ± SEM, n=5 animals per group. # = P<0.05 compared to PBS treatment at the same time point by 2 way ANOVA and Bonferroni’s test.
Table 1: Genes altered by GLP-2/IgG2a treatment. Abbreviations NC: No change in gene expression at any of the time points studied, ↑: Increased expression, ↓: Decreased expression

<table>
<thead>
<tr>
<th>Location</th>
<th>Gene</th>
<th>Peak Fold Increase in Response to Surgical Manipulation</th>
<th>Peak Postoperative Time Point (hr)</th>
<th>Effect of GLP-2/IgG2a P&lt;0.05</th>
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<tr>
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A. Small Bowel Mucosa Weight

B. Upper GI Transit

Figure 1
Figure 2

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Figure 3

**Treatments:**
-48  -24  -3  -1
PBS or IgG control sc
GLP-2/IgG (0.5 - 4 mg/kg sc)

**Surgery**
-3

**Endpoints:**
24  48 (h)
MPO +ve cells counts
Upper GI transit

-3
PBS or GLP-2/IgG (2 mg/kg sc)

0.5  1  3  6 (h)
mucosa & muscularis mRNA analysis
Figure 4

A. GI transit 48 hours after surgery

B. MPO-positive leukocytes
24 hr after surgery

C. Cell infiltrate 48 hours after surgery

D. Cell infiltrate 24 hours after surgery
Figure 5

A. Cell infiltrate 48 hours after surgery

B. GI transit 48 hours after surgery

C. GI transit in naive mice 24 hours after treatment
Figure 6

A. Cell infiltrate 24 hours after surgery

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24 hour prior to surgery (4 mg/kg)

B. GI transit 24 hours after surgery

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24 hour prior to surgery (4 mg/kg)
Figure 7

Muscularis Externa

EGR-1

Fold Increase

MIP-1α (CCL3)

Fold Increase

iNOS

Fold Increase

COX2

Fold Increase

HO-1

Fold Increase

Postoperative Time Point

Fold Increase

Control 0.5 hr 1.0 hr 3.0 hr 6.0 hr

Fold Increase

Control 0.5 hr 1.0 hr 3.0 hr 6.0 hr

Fold Increase

Control 0.5 hr 1.0 hr 3.0 hr 6.0 hr

Fold Increase

Control 0.5 hr 1.0 hr 3.0 hr 6.0 hr

PBS

GLP-2/IgG2a (2 mg/kg 3 hr prior to surgery)
Figure 8
Mucosa

EGR-1

IL-6

IL-1β

MCP-1

MIP-1α (CCL3)

iNOS

COX2

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Postoperative Time Point

# denotes statistical significance.

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Figure 9

**Claudin 3**

- **PBS**
- **GLP-2/IgG2a (2 mg/kg 3 hr prior to surgery)**

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**Occludin**

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**Claudin 1**

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