Stress Impairs Murine Intestinal Barrier Function: Improvement by Glucagon-Like Peptide-2

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ABSTRACT

Stress-induced intestinal barrier dysfunction may be involved in chronic intestinal disorders. Glucagon-like peptide-2 (GLP-2) is an intestinotrophic growth hormone that can rapidly improve intestinal epithelial barrier function. Here, we investigated whether mouse intestine is responsive to chronic psychological stress and whether pretreatment with GLP-2 can ameliorate stress-induced changes. Mice were subjected to water avoidance stress (WAS; 1 h/day for 10 days) with GLP-2 or saline administered 4 h before each WAS session. After the final stress period, the intestine was removed for assessment of physiological/morphological changes. Compared with controls (sham-stressed mice), stressed mice demonstrated enhanced ion secretion and permeability in the jejunum, ileum, and colon. In addition, increased numbers of bacteria were observed adhering to and/or penetrating the epithelium, associated with infiltration of mononuclear cells into the mucosa. GLP-2 treatment improved intestinal barrier function in stressed mice and ameliorated other aspects of impaired host defense. Our study extends previous findings in rats of stress-induced intestinal dysfunction and provides insights into potential novel therapeutics.

Psychological stress has been shown to influence the clinical course of chronic intestinal disorders such as inflammatory bowel disease (Levenstein et al., 2000; Collins, 2001; Ringel and Drossman, 2001) and irritable bowel syndrome (IBS) (Mayer, 2000). Clinical and experimental studies have documented significant gut pathophysiology in response to acute and chronic psychological stress. In particular, stress-induced changes described in rats include increased secretory state, altered colonic motility, increased epithelial permeability to small and large probes, damaged mitochondria in epithelial cells, altered epithelial/bacterial interactions, and increased inflammatory infiltrate (Saunders et al., 1997, 2002; Kiliaan et al., 1998; Santos et al., 2000; Mazzon et al., 2002; Soderholm et al., 2002). Together, the evidence suggests that intestinal dysfunction and inflammation can be initiated by psychological stress in a naïve host (Soderholm et al., 2002). The mechanism of stress-induced onset or relapse of intestinal dysfunction is largely unknown, but one possible link is mucosal barrier dysfunction.

Mucosal barrier function is maintained largely by the epithelial lining of the gastrointestinal tract: gut epithelial cells (enterocytes) joined at their apical poles by tight junctions to form a physical barrier. This barrier is not inert but rather is regulated by neuroendocrine and immunological factors. Crohn’s disease is associated with increased intestinal permeability (Olaison et al., 1990; Meddings et al., 1994; Soderholm et al., 1999), which precedes relapse (Wyatt et al., 1993). In addition, long-term sustained stress increases the number of relapses in patients with ulcerative colitis (Levenstein et al., 2000). Defective barrier function has been described for at least one subgroup of individuals with IBS, and mild inflammation has also been described in IBS (Spiller et al., 2000). That increased epithelial permeability precedes intestinal inflammation supports the concept of barrier dysfunction as an early or initiating event. Increased permeability could expose the mucosal immune system to an increased load of luminal antigens from foods and/or bacteria, increasing immune stimulation that may then lead to intestinal inflammation. Improvement of intestinal barrier function may be a novel strategy to prevent or ameliorate stress-induced intestinal dysfunction.

Glucagon-like peptide-2 (GLP-2) is an intestinotrophic growth hormone that promotes many aspects of intestinal function, including enhancement of mucosal growth and promotion of nutrient absorption (for review, see Drucker, 2002). Importantly, a unique property of this growth hormone is its
ability to rapidly enhance mucosal barrier function, reducing intestinal permeability of the epithelial barrier via both transcellular and paracellular routes (Benjamin et al., 2000). Given that altered mucosal barrier function is an early event in stress-induced intestinal pathophysiology, and the known importance of mucosal barrier function in maintaining intestinal integrity, we sought to examine whether enhancement of epithelial barrier function with GLP-2 could prevent or ameliorate stress-induced mucosal pathophysiology. For this purpose, we developed a mouse model of chronic psychological stress based on our established model of water avoidance stress (WAS) in rats (Santos et al., 2000, 2001; Soderholm et al., 2002). We found that exposure of mice to chronic stress dramatically altered barrier function, impaired host defense, and initiated inflammation in the jejunum, ileum, and colon. Furthermore, we documented that GLP-2 treatment in this model was able to ameliorate the stress-induced intestinal abnormalities. This information may be useful in designing novel therapeutic strategies for treating various gastrointestinal disorders.

Materials and Methods

Animals

Male BALB/c mice were housed in pairs and maintained on a 12-12-h dark-light cycle and provided with food and water ad libitum. Mice were allocated at random to one of four groups: control, control + GLP-2, stress, and stress + GLP-2. On the day of the experiments, mice were sacrificed by cervical dislocation immediately after the final stress or sham-stress procedure. All procedures were approved by the Animal Care Committee at McMaster University.

Stress Protocol

Mice were handled daily by the same investigator for 7 days before the study and then submitted to WAS or sham stress (control). Mice were given a subcutaneous injection of protease-resistant GLP-2 [h[Gly2]-GLP-2, 5 μg (in 0.5 ml); Astrazeneca AB, Mölndal, Sweden] (or saline) 4 h before WAS or sham stress. The dose of GLP-2 was based on our previous experiments showing enhanced barrier function 4 h after a single dose of GLP-2 (Benjamin et al., 2000; Cameron et al., 2003). The procedure involved placing the mouse on a platform (3 x 6 cm) in the center of a container (56 x 50 cm) containing 3 cm of room temperature water. Mice avoided the aversive stimulus (water) by remaining on the platform for 1 h. Control mice (sham stress) were placed on the same platform in a waterless container for 1 h where mice were free to move off the platform and explore the container. Mice were subjected daily to 1 h WAS or sham stress for 10 consecutive days. Body weight, as an index of growth was measured daily (expressed in grams).

Mucosal Physiology

Ussing Chamber Studies. The intestine was excised and cut into segments of proximal jejunum (distal to the ligament of Treitz), ileum, and colon. Each segment was immersed in 37°C oxygenated Kreb’s buffer and opened along the mesenteric border into flat sheets. Two adjacent pieces of jejunum and a single piece of ileum and colon from each mouse were mounted in Ussing chambers (WPI, Sarasota, FL). The chamber opening exposed 0.6 cm² of tissue surface area to 8 ml of circulating oxygenated Kreb’s buffer at 37°C. The buffer contained 115 mM NaCl, 1.25 mM CaCl₂, 1.2 mM MgCl₂, 2.0 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.35. In addition, the serosal buffer contained 10 mM glucose as an energy source that was osmotically balanced by 10 mM mannitol in the mucosal buffer. The chambers contained agar-salt bridges to monitor the potential difference across the tissue and to inject the required short-circuit current (I_s) to maintain a zero potential difference as registered via an automated voltage clamp (WPI). I_s (microamperes per square centimeter) was recorded by a computer connected to the voltage-clamp system. Tissue conductance (G), a measure of passive permeability to ions, was calculated according to Ohm’s law and expressed as millisiemens per square centimeter.

HRP Flux. Permeability to macromolecules was assessed by measuring the mucosal-to-serosal flux of protein horseradish peroxidase (HRP) (44 kDa). HRP (type II; Sigma-Aldrich, St. Louis, MO) was added to the luminal buffer 15 min after the tissues were mounted at a final concentration of 4.5 x 10⁻⁶ M and allowed to equilibrate for 30 min. Serosal samples (0.5 ml) were obtained at 30-min intervals for 2 h and were replaced with Kreb’s buffer to maintain a constant volume in the chambers. HRP activity was determined using a modified Worthington method as described previously (Kiliaan et al., 1998) using a kinetic assay in a 96-well plate reader. Mucosal-to-serosal fluxes of HRP were calculated according to standard formulas and expressed as picomoles per hour per square centimeter.

Electron Microscopy. Tissue segments from jejunum, ileum, and colon to be used for transmission electron microscopy were immediately fixed in 2.5% glutaraldehyde in 0.1mol/l sodium-cacodylate buffer, pH 7.4, for 2 h at room temperature, rinsed for 18 h (4°C) with 0.05 Tris buffer, pH 7.6, washed three times, 5 min each time, and subsequently processed for routine transmission electron microscopy. For quantification, bacterial-epithelial cell interactions (defined as bacteria in contact with or inside epithelial cells, associated with disappearance of microvilli, and/or condensation of the cytoskeleton) were evaluated in 30 fields per mouse in a blinded manner by one investigator (H. L. Cameron).

Histology. Full-thickness segments of jejunum and colon adjacent to the tissue segments mounted in Ussing chambers were coded and processed for morphological microscopic analysis. Tissues were fixed in paraformaldehyde, embedded in paraffin, and subsequently stained with H&E. In the light microscopy studies, tissues from four mice per group were scrutinized, three tissues per mouse, and for each tissue, 12 contiguous nonoverlapping areas above the muscularis mucosa were evaluated in a blinded manner by investigator H. L. Cameron. As a marker of inflammation, mononuclear cells were identified. Cells were counted at 400x magnification and expressed as number of cells per square millimeter.

Statistical Analysis. Results are expressed as mean ± S.E.M. For all statistical comparisons, when several observations were obtained from the same mouse, the mean value was calculated before group means were obtained; i.e., n values represent the number of mice in each group. One-way analysis of variance was used with Newman-Keuls as a subsequent multiple comparison test. p values <0.05 were considered significant.

Results

Body Weight

Throughout the sham/stress protocol, weight was monitored in all mice. Weight gain was not significantly different between sham-stressed (1.1 ± 0.1 g) and sham-stressed + GLP-2-treated mice (1.1 ± 0.2 g). In stressed mice, weight gain was significantly reduced over the 10-day period (0.5 ± 0.1 g; p < 0.05). In GLP-2-treated stressed mice, the value for weight gain over the 10-day period (0.7 ± 0.2 g) was not significantly different from either sham-stressed or stressed mice.

Mucosal Physiology

Ion Transport. After the final stress/sham stress procedure, baseline I_ac, a measure of active ion transport was recorded (Fig. 1A). WAS induced a significant increase in I_ac
in all regions of the gut. GLP-2 treatment of sham-stressed mice had no effect on baseline $I_{\text{sc}}$. In contrast, GLP-2 treatment of stressed mice ameliorated the stress-induced increase in $I_{\text{sc}}$ for all regions of the gut.

**Permeability.** $G$, a measure of passive permeability to ions was measured for each tissue (Fig. 1B). Stress induced a significant increase in conductance for all regions of the gut compared with sham-stressed controls. GLP-2 treatment of stressed mice ameliorated the stress-induced increase in conductance in the jejunum but not in the ileum or colon. In addition, GLP-2 treatment reduced conductance in the small intestine in control mice.

**Macromolecular Permeability**

Chronic stress caused a dramatic permeability defect in all regions of the gut (jejunum, $27.1 \pm 0.6$; ileum, $23.0 \pm 1.2$; and colon, $17.1 \pm 0.9$ pmol/h/cm$^2$) compared with sham-stressed controls (jejunum, $11.2 \pm 2.1$; ileum, $10.5 \pm 1.0$; and colon, $8.4 \pm 1.3$ pmol/h/cm$^2$; $p < 0.001$) (Fig. 2). GLP-2 treatment eliminated the stress-induced permeability defect ($p < 0.001$) and reduced flux values below those of sham-stressed mice ($p < 0.01$) (jejunum, $7.0 \pm 1.6$; ileum, $6.4 \pm 1.9$; and colon, $4.0 \pm 1.2$ pmol/h/cm$^2$). GLP-2 treatment of sham-stressed mice also significantly reduced the amount of HRP transported across all segments of the gut (jejunum, $5.3 \pm 0.5$; ileum, $5.9 \pm 0.3$; and colon, $2.6 \pm 0.5$ pmol/h/cm$^2$) compared with sham-stressed controls ($p < 0.01$).

**Altered Host Defense to Bacteria**

Segments of jejunum, ileum, and colon were processed for transmission electron microscopy after the final stress/sham-stress procedure. Figure 3 shows a representative electron photomicrograph of the colonic mucosa from control (Fig. 3A) and stressed mouse ileum (Fig. 3B). In control tissues, interacting bacteria were rarely observed, whereas many bacteria were observed in tissues from stressed mice. We observed a lack of cellular organelles and condensation of cytoskeleton at the site of epithelial-bacterial interaction. When the num-
ber of bacteria directly interacting with the intestinal mucosa was counted and expressed as the number of penetrating bacteria per field (Fig. 3C). Bacteria were rarely observed in sham-stressed controls (jejunum, $0.1 \pm 0.1$; ileum, $0.2 \pm 0.1$; and colon $0.3 \pm 0.1$). WAS significantly increased the number of bacteria interacting with the intestinal mucosa (jejunum, $3.7 \pm 1.2$; ileum, $4.5 \pm 0.4$; and colon, $10.0 \pm 1.0$; $p < 0.01$ compared with sham-stressed controls). Stressed mice treated with GLP-2 also showed an increased number of interacting bacteria compared with sham-stressed controls (jejunum, $0.3 \pm 0.2$; ileum, $2.3 \pm 1.0$; and colon $1.3 \pm 1.0$; $p < 0.001$) but significantly fewer than stressed mice ($p < 0.01$). In addition, we observed changes in mitochondria in stressed mouse intestine, including mitochondrial swelling and loss of...
GLP-2 (solid columns) 4 h before 1 h of sham stress for 10 consecutive days. Sham-stressed mice were given saline (open columns) or GLP-2 (solid columns) 4 h before 1 h of WAS for 10 consecutive days. Mononuclear cells in the colonic mucosa were counted in coded light microscopy sections. Tissues are from four mice per group, two tissues per mouse, and for each tissue, 10 contiguous nonoverlapping areas above the muscularis mucosa were evaluated. Data are presented as means ± S.E. ‡, p < 0.01 versus sham; †, p < 0.05 versus WAS.

**Colonic Inflammatory Cells**

Colonic segments adjacent to those used for Ussing chamber studies were processed for histology after the final stress/sham-stress procedure and the number of mononuclear cells in the colonic mucosa was recorded and expressed as cell number per square millimeter (Fig. 4). WAS increased the number of mononuclear cells in the mucosa of stressed mice approximately 2-fold (500 ± 64) compared with sham-stressed controls (p < 0.001). GLP-2-treated stressed mice showed reduced numbers of mononuclear cells in the colonic mucosa (406 ± 52) compared with stressed mice (p < 0.01). GLP-2 treatment in sham-stressed mice had no effect on mononuclear cell number (228 ± 70) compared with sham-stressed controls (240 ± 47) (NS).

**Discussion**

To our knowledge, this is the first study examining the effects of chronic psychological stress on intestinal function in mice. The findings reported in this investigation indicate that mouse intestine is indeed responsive to chronic psychological stress. Our previous studies in rats demonstrated that psychological stress alters physiology and initiates intestinal inflammation in the ileum (Santos et al., 2000; Soderholm et al., 2002). In the present study, we extended these findings to show intestinal dysfunction in mice in response to chronic psychological stress. We identified the changes induced by 10-day chronic WAS in jejunum, ileum, and colon where stress increased ion secretion and permeability to ions and macromolecules, induced infiltration of mononuclear cells into the mucosa, and increased mucosal-bacterial interactions. In addition, we demonstrated that treatment of mice with GLP-2 can prevent or ameliorate many of the observed stress-induced intestinal changes in host defense.

We examined the ability of a daily GLP-2 treatment during the stress protocol to affect intestinal function in stressed and control conditions. In control (sham-stressed) mice, 10 day GLP-2 treatment enhanced gut function to a small but significant extent as measured by reduced permeability to both ions and macromolecules. In the intestine of stressed mice, GLP-2 treatment restored the baseline secretory state of the epithelium to control values and significantly ameliorated stress-induced barrier dysfunction by reducing passive permeability to ions and dramatically limiting penetration of macromolecules. GLP-2 treatment also ameliorated the stress-induced mononuclear cell infiltration and prevented abnormal bacterial interactions with the epithelium.

In stressed mice, we recorded a decrease in weight gain suggesting a failure to thrive during the 10-day stress period. GLP-2 treatment did not affect weight gain in sham-stressed mice, whereas in the stressed mice weight gain was not significantly different from sham-stressed controls. The stress-induced epithelial cell ion secretion in both small and large intestine creates a driving force for water secretion that may act to flush noxious materials out of the lumen, thus contributing to host defense. However, when prolonged as in chronic situations, ongoing secretion may be detrimental by increasing water loss. Here, chronic stress caused an increase in short-circuit current, whereas GLP-2 treatment in stressed mice significantly reduced this increase in all regions of the gut and further restored ion transport to control levels in the jejunum.

Epithelial permeability as measured by conductance and the penetration of an antigen-sized probe provide information about the integrity of the epithelial barrier. The risk of increased exposure to luminal antigens is most accurately reflected by changes in HRP flux, whereas conductance indicates movement of ions. Intestinal permeability to ions and macromolecules was significantly increased in stressed mouse intestine, indicating that epithelial barrier function is severely compromised by stress in mice. In rats exposed to chronic psychological stress, a similar barrier defect has been described previously (Santos et al., 2000, 2001; Soderholm et al., 2002). GLP-2 treatment of sham-stressed mice enhanced barrier function by reducing passive permeability to ions and penetration of a macromolecular probe. Moreover, in stressed mice, GLP-2 treatment dramatically reduced macromolecular permeability in all regions of the gut to levels significantly below both stressed and sham-stressed values. We have previously demonstrated that GLP-2 enhances barrier function by reducing permeation of the intestine by both transcellular and paracellular pathways (Benjamin et al., 2000). Thus, GLP-2 treatment in stressed mice enhanced barrier function to completely prevent the stress-induced permeability defect effectively reducing the amount of luminal antigen that penetrates to the lamina propria, thereby limiting immune stimulation (Cameron et al., 2003). It is known that GLP-2 reduces fluid-phase endocytosis of macromolecules and decreases paracellular permeability of smaller probes (Benjamin et al., 2000), perhaps due to some effect on cytoskeletal components or tight junction proteins. It is also possible that the antiapoptotic/growth-promoting effects of GLP-2 (Drucker et al., 1996; Tsai et al., 1997) contribute to the amelioration of stress-induced effects by maintaining the integrity of the epithelial lining of the gut.
The ability of bacteria to access and adhere to the intestinal epithelia is a key step in the pathogenesis of translocation and the initiation of infection (Katayama et al., 1997). In this study, we noted a significant increase in bacterial interaction with the intestinal mucosa in stressed mice. We also observed mitochondrial changes in tissues with increased bacterial interactions, including mitochondrial swelling and distortion of cristae comparable with our previous findings in rats (Soderholm et al., 2002). Similar mitochondrial changes have been associated with increased intestinal epithelial permeability after uncoupling of oxidative phosphorylation (Somasundaram et al., 1997). The stress-induced increase in bacterial adherence and penetration is likely due, at least in part, to the decreased protective capacity of the mucus layer. In mouse colon, acute immobilization stress has been shown to induce mucin secretion, leading to goblet cell depletion (Castagliuolo et al., 1998). Therefore, increased bacterial interaction with the epithelium may be the result of increased epithelial exposure due to a thinned mucus layer. Thus, we speculate that the modest ability of GLP-2 to reduce this interaction in stressed mice may in turn be due to some effect on mucus production or release. The ability of GLP-2 to reduce bacterial penetration has previously been documented in a report of reduced bacteremia in a murine model of indomethacin-induced intestinal injury (Boushey et al., 1999), likely due to enhanced mucosal barrier function.

Several experimental studies examining the role of GLP-2 to treat diseases characterized by intestinal inflammation (Boushey et al., 1999; Alavi et al., 2000; Kouris et al., 2001) suggest a role for GLP-2 in ameliorating the severity of inflammation, perhaps indirectly by enhancing the integrity of the intestinal barrier. In this study, GLP-2 treatment of stressed mice reduced the number of mononuclear cells recruited to the mucosa, perhaps by limiting immune stimulation via enhanced barrier function. Although there is some controversy in the literature surrounding the location of the GLP-2 receptor in the gut (Yusta et al., 2000; Bjerknes and Cheng, 2001), it seems likely that one or several downstream mediators mediate the diverse changes in intestinal function that are attributed to GLP-2. Ongoing studies aim to understand the underlying changes involved in GLP-2-induced intestinal improvement and the mediators involved in these changes.

In the setting of stress-induced intestinal dysfunction, where a known permeability defect occurs, prophylactic treatment to enhance barrier function, with GLP-2 can ameliorate the effects of stress on intestinal barrier and therefore prevent barrier dysfunction-related sequelae. Compromised intestinal barrier function may be an initiating or propagating factor in intestinal disease and as such may be a promising target for therapy. Improvement of barrier function may then potentially prevent or delay onset of disease and reduce the severity of disease.

In summary, our study demonstrates that chronic psychological stress induces intestinal dysfunction in mouse intestine. Chronic stress induces changes in epithelial physiology, barrier function, and bacterial interaction. In addition, we showed that GLP-2 treatment in this model attenuates or ameliorates these stress-induced changes. These findings support the concept of altered intestinal permeability as an initiating event in intestinal dysfunction and further support the potential therapeutic usefulness of barrier-enhancing treatment strategies to prevent or ameliorate intestinal dysfunctions.

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