Human [Gly^2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis

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Drucker, Daniel J., Bernardo Yusta, Robin P. Boushey, Lorraine DeForest and Patricia L. Brubaker. Human [Gly^2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G79–G91, 1999.—The pathology of Crohn's disease and ulcerative colitis is characterized by chronic inflammation and destruction of the gastrointestinal epithelium. Although suppression of inflammatory mediators remains the principle component of current disease therapeutics, strategies for enhancing repair and regeneration of the compromised intestinal epithelium have not been widely explored. The demonstration that a peptide hormone secreted by the intestinal epithelium, glucagon-like peptide-2 (GLP-2), is a potent endogenous stimulator of intestinal epithelial proliferation in the small bowel prompted studies of the therapeutic efficacy of GLP-2 in CD1 and BALB/c mice with dextran sulfate (DS)-induced colitis. We report here that a human GLP-2 analog (h[Gly^2]GLP-2) significantly reverses weight loss, reduces interleukin-1 expression, and increases colon length, crypt depth, and both mucosal area and integrity in the colon of mice with acute DS colitis. The effects of h[Gly^2]GLP-2 in the colon are mediated in part via enhanced stimulation of mucosal epithelial cell proliferation. These observations suggest that exploitation of the normal mechanisms used to regulate intestinal proliferation may be a useful adjunct for healing mucosal epithelium in the presence of active intestinal inflammation.

intestine; inflammatory bowel disease; epithelium; growth factor; inflammation

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INFLAMMATION OF THE intestinal epithelium, as exemplified by Crohn's disease and ulcerative colitis, results in considerable morbidity, and current therapeutic strategies, generally directed at suppressing components of the inflammatory response, remain suboptimal (27). The identification of molecules important for maintaining the growth and integrity of the mucosal epithelium has stimulated the development of novel approaches toward enhancement of mucosal protection in the gut. For example, the observation that trefoil peptides are abundantly expressed in the epithelium after intestinal injury was followed by studies demonstrating that mice deficient in intestinal trefoil factor are more susceptible to mucosal injury and recombinant intestinal trefoil factor enhances epithelial healing of the murine colon in vivo (35). Similarly, the demonstration that the keratinocyte growth factor (KGF) stimulates epithelial cell proliferation in the gastrointestinal tract (30), taken together with increased KGF expression in inflammatory bowel disease (IBD) (51), suggests a possible link between KGF and intestinal epithelial function in vivo. We have now examined the therapeutic potential of a recently described intestinal growth factor, glucagon-like peptide-2 (GLP-2), in mice with dextran sulfate (DS)-induced colitis.

Despite ongoing advances in our understanding of the cell biology of the gastrointestinal epithelium, principal strategies for treatment of IBD remain focused on suppression of the cellular and humoral inflammatory response. These approaches involve local or systemic administration of corticosteroids, aminosalicylates, or immunomodulatory agents such as azathioprine, mercaptopurine, cyclosporin, and methotrexate (27). Although these latter agents are generally effective they do not specifically target the intestine and their side effects may be considerable, precluding long-term use in patients with chronic IBD. Newer targeted approaches to immunosuppressive and anti-inflammatory therapy, including use of monoclonal antibodies against lymphocyte antigens (24, 48) or tumor necrosis factor (TNF) (46, 50), interleukin-4 (IL-4) delivery via adenoviral gene transfer (29), and antisense oligonucleotides for suppression of intercellular adhesion molecule activity (ICAM), are currently under evaluation.

The rapid turnover and renewal of differentiated cell types that constitute the mucosal epithelium of the small and large bowel raise the possibility that stimulation of epithelial proliferation may be useful for enhancing repair of epithelial damage in vivo. Identification of growth factors produced locally in the bowel that regulate crypt cell proliferation, such as epidermal growth factor, transforming growth factor-α (TGF-α), and insulin-like growth factor I (18), provides an opportunity to manipulate mucosal epithelial regeneration in experimental models of intestinal damage or resection. The gastrointestinal tract also secretes regulatory peptides such as gastrin and gastrin-releasing peptide, with intestinal growth-promoting activity (28, 31, 56). The observation that injury of the intestinal mucosa is frequently associated with increased secretion of the proglucagon-derived peptides (PGDPs) (4), taken together with increased intestinal growth in patients and rodents with glucagon-producing tumors (20, 26, 47), resulted in the identification of GLP-2 as the PGDP with intestinal growth factor-like activity (20).

GLP-2 administered to normal mice and rats increases growth of the mucosal epithelium in small and

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large intestine (21, 53). The increase in small bowel mass is attributable in part to activation of crypt cell proliferation and inhibition of enterocyte apoptosis (53). GLP-2 also promotes intestinal hexose transport via upregulation of sodium-dependent glucose transporter 1 (SGLT-1) activity (11, 12). The importance of GLP-2 as a trophic factor for intestinal epithelium is illustrated by studies demonstrating that GLP-2 infusion prevents parenteral nutrition-associated mucosal hypoplasia in rats (10). To address the possibility that GLP-2 may be therapeutically useful for enhancing the endogenous reparative response to mucosal epithelial damage, we administered a degradation-resistant human GLP-2 analog, h[Gly^2]GLP-2 (6, 21), to mice with experimental DS-induced colitis.

METHODS

Animals and experimental protocol. Groups of 6- to 8-wk-old female CD1 mice, 22–24 g or 8- to 9-wk-old female BALB/c mice, 18–21 g (Charles River), were housed in plastic bottom, wire-lid cages, maintained on a 12:12-h light-dark cycle, and allowed chow and water containing 0 or 5.0% DS ad libitum throughout the study. The experiments carried out with CD1 mice, designated *experiments A* and *B*, were carried out with treatment groups containing four to five mice housed together. The experiments with BALB/c mice were carried out with five mice per control group for saline- and h[Gly^2]GLP-2-treated mice (not receiving DS) and 10 mice per treatment group for the DS arm of the study, each BALB/c mouse being housed in a separate cage. CD1 mice were injected with either 0.5 ml saline or 750 ng h[Gly^2]GLP-2 in 0.5 ml saline twice daily. BALB/c mice were injected with either 0.5 ml saline or 350 ng h[Gly^2]GLP-2 in 0.5 ml saline twice daily.

For the experiments with BALB/c mice individual water intake was recorded every 2 days. DS (mol wt 40,000–50,000; United States Biochemicals, Cleveland, Ohio, lot 103811) was freshly dissolved in drinking water throughout the study for both CD1 and BALB/c mice. Four days before the start of the study, mice were weighed using a Mettler PJ300 scale and randomly allocated to treatment groups. Subcutaneous injections of PBS or h[Gly^2]GLP-2 were administered twice daily, at 8 AM and 6 PM. Groups of mice received either regular autoclaved drinking water or water supplemented with 5.0% DS. CD1 mice were killed on the morning of *day 11* after receiving 10 full days of water alone or water with DS and the final timed injection of saline or h[Gly^2]GLP-2 was administered 2 h before mice were killed. BALB/c mice receiving DS appeared sicker than CD1 mice; after 9 days of DS treatment two deaths occurred in the saline-treated group and one death in the h[Gly^2]GLP-2-treated group before *day 10*. After consultation with veterinary staff, the remaining groups of BALB/c mice were killed on *day 10*, after receiving ~9⅓ days of oral DS. Two additional deaths in the BALB/c group (1 in saline-treated DS group, 1 in h[Gly^2]GLP-2-treated DS group) occurred on *day 10* on the morning when the mice were killed.

Synthetic h[Gly^2]GLP-2 was obtained from Allelix Biopharmaceuticals (Mississauga, Ontario, Canada). The exact peptide concentrations of different lots of h[Gly^2]GLP-2 used in *experiments A–C* were determined using a combination of amino acid sequencing and HPLC. All animal experiments were carried out following experimental guidelines approved by the Animal Care Committee of the Toronto Hospital. The DS colitis experiments were carried out on several occasions, with similar results, and the data shown here are from three

Experimental analyses. Intestinal weights, morphology, enzymatic activity, and GLP-2 content were assessed as described previously (6, 7, 20, 53). For analysis of tissue PGDP content 2 cm of distal ileum (2 cm from cecum) and distal colon (2 cm from the anus) were homogenized on ice in 5 ml of extraction buffer (1 N HCl, 5% HCOOH, 1% trifluoroacetic acid, 1% NaCl) and extracted as described previously (13). RNA was prepared from homogenates of distal jejunum, ileum, and colon (13) and analyzed as previously described (7). Blood for GLP-2 RIA was collected in a final volume of 10% Trasylol, EDTA, Diprotin A (5,000 KIU/ml:32 mM:0.1 nM), and plasma was stored at −80°C before analysis by RIA (6). Semiquantitative RT-PCR was carried out with aliquots analyzed from a range (20–30) of cycle numbers to ensure linearity for mouse TGF-α mRNA as previously described (8, 9, 34). The PCR conditions were 94°C for 1 min and 68°C for 2 min for 30 cycles. Primers for TGF-α were 5′-TGCAGCACCCTGCGCTCGGAAGAT-3′ and 5′-CCACCTGGCCAAATTCCTCCTCTG-3′. Occult blood testing was carried out using Hematest reagent tablets (Bayer, Etobicoke, Canada), as per the manufacturers' instructions. Myeloperoxidase (MPO) activity was assayed spectrophotometrically as previously described (5). Statistical differences between treatment groups were determined by ANOVA using Tukey's studentized range test for multiple comparisons at $P = 0.05$.

Histological analysis. Intestinal segments for histology were taken from proximal jejunum (8 cm distal to the pylorus), distal jejunum (18 cm distal to the pylorus), proximal ileum (10 cm before the cecum), and distal ileum (just proximal to cecum) and from the colon (1–3, 3–5, 5–7, and 7–9 cm distal to the cecum). Tissues were fixed in 10% buffered Formalin for 48 h and embedded in paraffin using standard techniques. Four- to six-micrometer cross sections were cut and stained with hematoxylin and eosin. Intestinal micrometry was performed using a Leica Q500MC image analysis system. Ten well-oriented villi and 25 well-oriented crypts from each small intestinal section were used to determine villus height and crypt depth. Disease severity was graded on a scale from 0–3 according to a standard scoring system (42): 0, normal bowel; 1, focal inflammatory cell infiltrate; 2, inflammatory cell infiltrate, gland drop out and crypt abscess; and 3, mucosal ulceration. Crypt cell proliferation index as assessed by proliferating cell nuclear antigen (PCNA) staining and colonic epithelial apoptosis index as assessed by percent TUNEL-positive cells was carried out as previously described (20, 53).

RESULTS

Control mice not exposed to DS treated with either saline or h[Gly^2]GLP-2 gained weight over the 9- to 10-day experimental period (Fig. 1). The slightly increased body weight gain in the CD1 h[Gly^2]GLP-2-treated control mice is largely attributable to the relatively greater increase in small bowel mass following treatment with the larger dose (750 ng twice daily) of h[Gly^2]GLP-2 (Figs. 1 and 2).

Mice receiving 5% DS in the drinking water developed loose blood-streaked stools after 4–5 days, became progressively more lethargic, and lost ~20–25% of their body weight at the end of the 9- to 10-day experiment (Fig. 1). In contrast, h[Gly^2]GLP-2-treated mice receiving 5% DS appeared much healthier and lost significantly less weight over the 9- to 10-day experimental period ($P < 0.05$ for both CD1 and BALB/c experiments, DS-h[Gly^2]GLP-2- vs. DS-saline-

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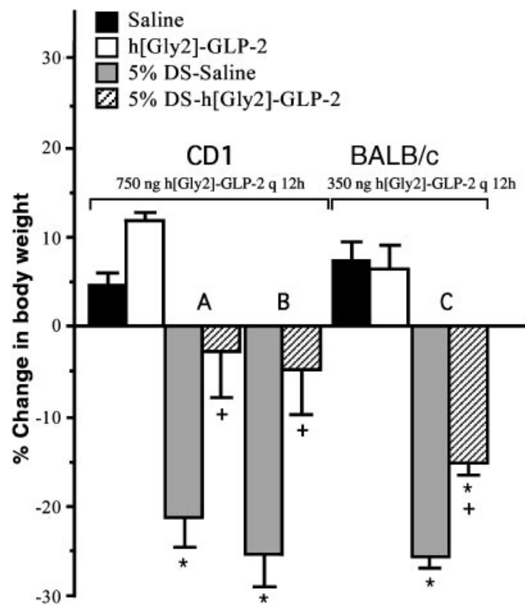

Fig. 1. Change in body weight in groups of CD1 mice (*experiments A* and *B*, $n = 5$ mice for each treatment group) or BALB/c mice [*experiment C*, $n = 5$ mice for each control group and 10 mice for each treatment group receiving dextran sulfate (DS) in water] receiving either drinking water alone or 5% DS and saline vs. human glucagon-like peptide-2 analog (h[Gly²]GLP-2; 750 or 350 ng twice daily for CD1 vs. BALB/c mice, respectively) subcutaneous injections for 9–10 days. *$P < 0.05$ for saline-treated control vs. 5% DS-saline groups. +$P < 0.05$ for 5% DS-saline vs. 5% DS-h[Gly²]GLP-2. Statistical differences between treatment groups were determined by ANOVA using Tukey's studentized range test for multiple comparisons.

GLP-1 has recently been shown to inhibit food and water intake (49, 55), whereas GLP-2 had no effect on food intake in mice over a 10-day experimental period (53). Nevertheless, one potential explanation for the different degree of illness and weight loss in our experiments might be due to theoretical effects of h[Gly²]GLP-2 on reduction of water intake and subsequent cumulative intestinal exposure to DS. Saline-injected control BALB/c mice not receiving 5% DS had a mean daily water intake of 6.5 ± 0.3 vs. 6.7 ± 0.4 ml for h[Gly²]GLP-2-treated control mice (*P* not significant). Furthermore, the cumulative intake of 5% DS water over the entire 9-day experiment, as well as the 5% DS water intake from experimental *days 7–9*, was significantly greater for h[Gly²]GLP-2-treated compared with saline-treated BALB/c mice receiving 5% DS (4.96 ± 0.5 vs. 5.9 ± 0.6 ml/day for saline- vs. h[Gly²]GLP-2-treated mice with DS colitis on *days 7–9*, $P < 0.05$). These observations demonstrate that the difference in disease severity between groups cannot be explained on the basis of any putative effects of GLP-2 on water intake and hence intestinal exposure to DS.

To determine the consequences of h[Gly²]GLP-2 administration in mice with DS-induced colitis, we examined the gastrointestinal tract from the stomach to the colon in control and DS colitis treatment groups. Although no visible or microscopic pathology was detected in the stomach of DS-treated CD1 mice, stomach weight was reduced in the DS-saline-treated group and was restored toward normal in the DS-h[Gly²]GLP-2-exposed to DS but treated with h[Gly²]GLP-2, 750 ng twice daily for 10 days, had a significant increase in small bowel mass (Fig. 2*B*, 2.2 ± 0.04 vs. 1.3 ± 0.01 g h[Gly²]GLP-2 vs. control for *experiment A*, $P < 0.05$). CD1 mice with DS colitis treated with saline alone had a significant reduction in the mass of the small bowel ($P < 0.05$ for *experiments A* and *B*, Fig. 2*B*). In contrast, DS-h[Gly²]GLP-2-treated CD1 mice with colitis (*experiments A* and *B*) exhibited a significant increase in small bowel mass (Fig. 2*B*, 1.85 ± 0.2 vs. 0.86 ± 0.1 g, DS-h[Gly²]GLP-2- vs. DS-saline-treated mice for *experiment A*, $P < 0.05$) and a small but significant increase in small bowel length in *experiment B* (Fig. 2*B*, $P < 0.05$).

Treatment of healthy control CD1 mice with h[Gly²]GLP-2 produced a significant increase in large bowel weight (Fig. 2*B*, 0.27 ± 0.01 vs. 0.35 ± 0.01, $P < 0.05$ saline- vs. h[Gly²]GLP-2-treated animals), consistent with the results of previous studies (19). Similarly, mice with DS colitis (*experiments A* and *B*) treated with h[Gly²]GLP-2 had a significant increment in large bowel weight (0.24 ± 0.04 vs. 0.32 ± 0.02 g, DS-saline- vs. DS-h[Gly²]GLP-2-treated mice, $P < 0.05$, Fig. 2*B*, *experiment A*). Treatment of normal CD1 mice with h[Gly²]GLP-2, 750 ng twice daily, produced a small but significant increment in large bowel length (Fig. 2*B*, *b* and *f*). Mice with DS colitis also exhibited a significant decrease in large bowel length ($P < 0.05$, saline-treated controls vs. mice with DS colitis, Fig. 2*B*). Although large bowel length was greater in h[Gly²]GLP-2-treated mice with colitis (Fig. 2*B*) this difference was statistically significant for mice in *experiment B* (DS-saline- vs. DS-h[Gly²]GLP-2-treated mice, $P < 0.05$) but not in *experiment A* (Fig. 2*B*).

Similar results were observed for BALB/c mice with DS colitis treated with a lower dose of h[Gly²]GLP-2 (350 ng twice daily). The relative magnitude of increase in small bowel weight in wild-type BALB/c mice treated with 350 ng h[Gly²]GLP-2 was smaller than in CD1 mice but still significant ($P < 0.05$, saline- vs. h[Gly²]GLP-2-treated mice, Fig. 2*B*). The BALB/c mice receiving 5% DS appeared more ill than the CD1 mice, and a total of five BALB/c DS mice died during this experiment (3 in the saline-treated and 2 in the h[Gly²]GLP-2-treated group). The small bowel weight was significantly reduced in BALB/c DS-saline mice and increased significantly in mice treated with h[Gly²]GLP-2 ($P < 0.05$, saline- vs. h[Gly²]GLP-2-treated BALB/c mice, Fig. 2*B*). The large bowel weights of saline-treated BALB/c mice with DS colitis were significantly reduced compared with h[Gly²]GLP-2-treated mice with colitis, $P < 0.05$. Furthermore, the large bowel lengths were markedly reduced in both saline- and h[Gly²]GLP-2-treated BALB/c mice with colitis, but large bowel length was significantly greater in the h[Gly²]GLP-2-treated mice ($P < 0.05$, h[Gly²]GLP-2-treated vs. saline-treated BALB/c mice with DS colitis, Fig. 2*B*).

As small and large bowel wet weights in mice with intestinal inflammation potentially reflect cellular infiltration, hyperplasia, increased protein synthesis, and/or

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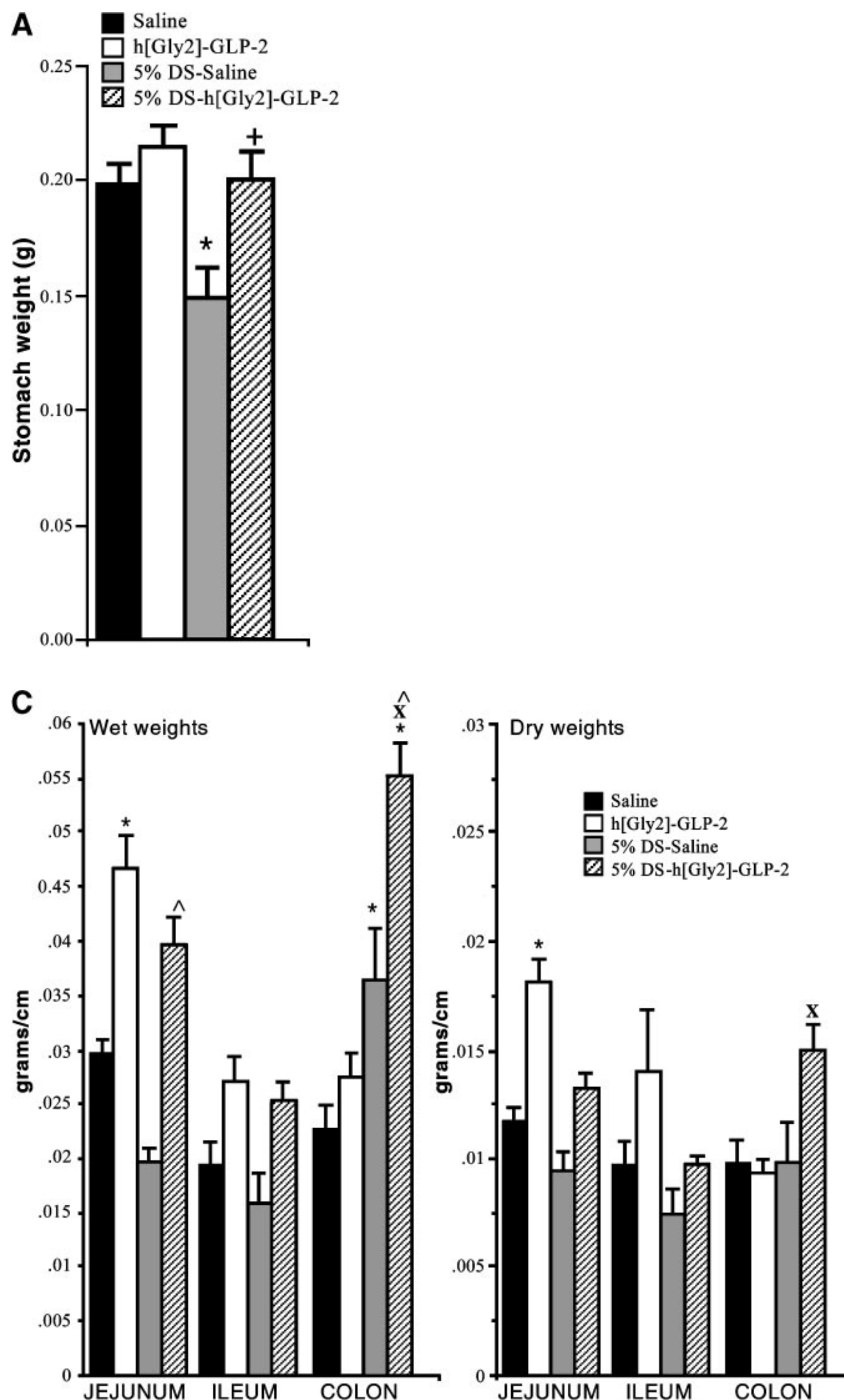

Fig. 2. *A*: stomach weight in groups of CD1 mice (*experiment A*) receiving either drinking water alone or 5% DS and saline vs. h[Gly^2]GLP-2 subcutaneous injections for 10 days. $*P < 0.05$ for DS-saline-treated vs. either saline-treated alone or h[Gly^2]GLP-2-treated mice. $+P < 0.05$ for 5% DS-saline vs. 5% DS-h[Gly^2]GLP-2. *C*: wet and dry weights in 1-cm segments from jejunum, ileum, and colon of control and DS CD1 mice from *experiment A*. $*P < 0.05$ for saline-treated normal controls vs. all other groups. $^{X}P < 0.05$ for h[Gly^2]GLP-2-treated control vs. h[Gly^2]GLP-2-treated DS. $^{\wedge}P < 0.05$ for h[Gly^2]GLP-2-treated DS vs. saline-treated DS mice.

weights in CD1 mice with and without colitis (Fig. 2*C*). The increase in small bowel wet and dry weights in control mice treated with h[Gly^2]GLP-2 was most evident in the jejunum ($P < 0.05$, saline- vs. h[Gly^2]GLP-2-treated control mice, Fig. 2*C*). Both saline- and h[Gly^2]GLP-2-treated mice with DS colitis had increased wet colon weights ($P < 0.05$, control vs. DS colitis groups). In contrast, only the h[Gly^2]GLP-2-treated mice with DS colitis had significantly increased dry colon weights ($P < 0.05$, saline- vs. h[Gly^2]GLP-2-treated mice with DS colitis, Fig. 2*C*).

Control CD1 mice treated with h[Gly^2]GLP-2 exhibited a significant increase in jejunal crypt and villus height that was most prominent in the proximal jejunum ($P < 0.05$, saline- vs. h[Gly^2]GLP-2-treated mice, Fig. 3), consistent with previous experiments (19, 54). Histological analysis of the intestine from DS mice treated with saline injections demonstrated a reduction in small bowel villus and crypt height that was most marked in the jejunum (Fig. 3*A*). In contrast, mice with DS colitis treated with h[Gly^2]GLP-2 exhibited a significant increase in small bowel villus height and crypt

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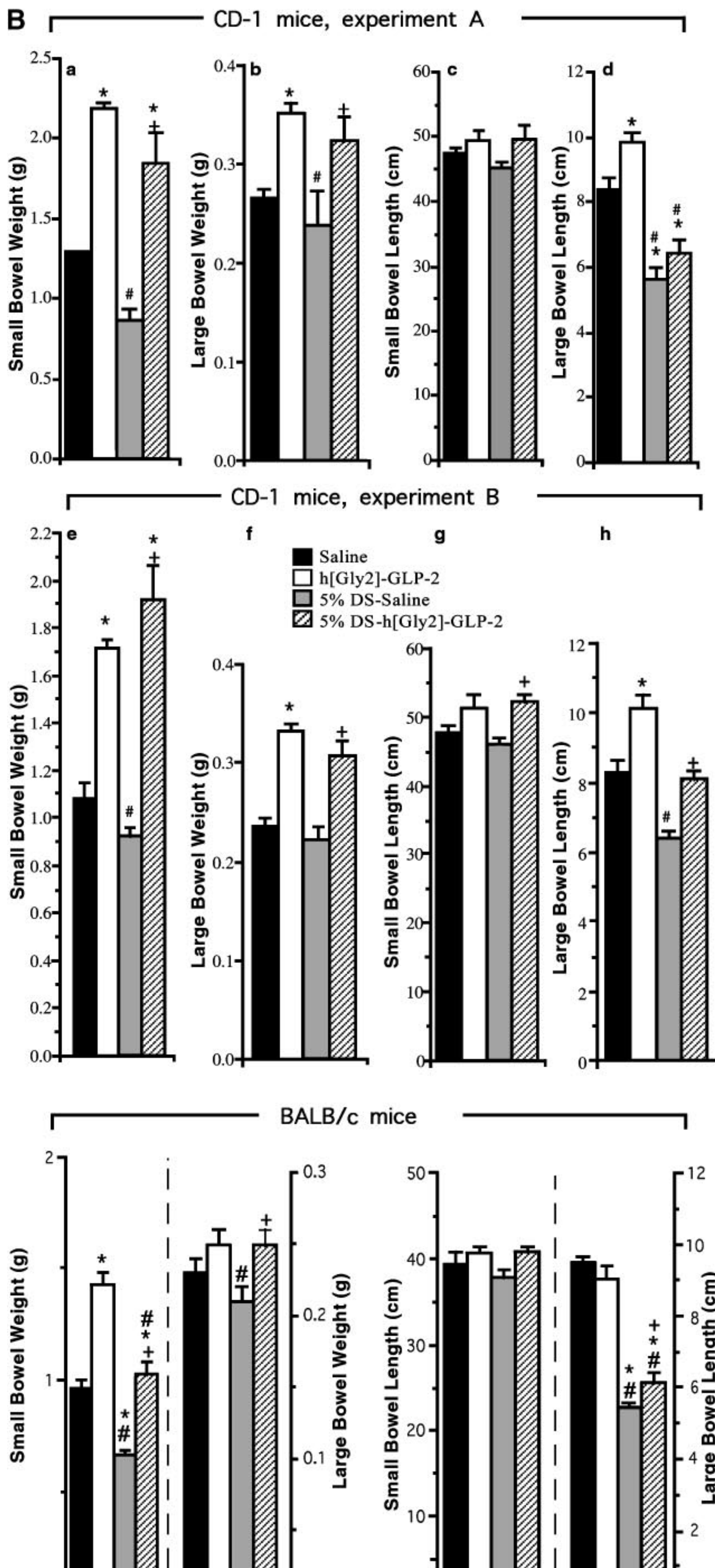

Fig. 2. *B*: intestinal weight and length in mice receiving water alone or 5% DS. Small (*a* and *e*) and large (*b* and *f*) bowel weights and lengths (*c* and *g* and *d* and *h* for small and large bowel, respectively); means ± SE for CD1 mice in *experiments A* and *B* and for BALB/c mice in *experiment C*. * $P < 0.05$ for saline-treated vs. either h[Gly²]GLP-2-treated, DS-saline-treated, or DS-h[Gly²]GLP-2-treated mice. # $P < 0.05$ for h[Gly²]GLP-2- vs. 5% DS-saline-treated mice. + $P < 0.05$ for 5% DS-saline- vs. 5% DS-h[Gly²]GLP-2-treated groups.

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