Insulin-like growth factor I and glucagon-like peptide-2 responses to fasting followed by controlled or ad libitum refeeding in rats

David W. Nelson,1 Sangita G. Murali,1 Xiaowen Liu,1 Matthew C. Koopmann,1 Jens J. Holst,2 and Denise M. Ney1

1Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, Wisconsin; and 2Department of Medical Physiology, Panum Institute, University of Copenhagen, Copenhagen, Denmark

Submitted 9 April 2007; accepted in final form 6 February 2008

IGF-I is a growth hormone-dependent anabolic hormone that stimulates whole body and intestinal growth. Hepatic production of IGF-I, the major source of circulating IGF-I, is regulated by dietary intake of protein and energy (35). The IGF-I system reflects the nutritional state, such that inadequate intake of energy and protein downregulates hepatic IGF-I production and decreases serum concentrations of IGF-I and the IGF binding proteins (IGFBPs) (34). IGF-I has been well characterized as a potent stimulator of intestinal growth via endocrine mechanisms associated with administration of IGF-I, as well as paracrine mechanisms (27, 37). Studies in fasted rats who are refed ad libitum show an association between increased mucosal mass and increased serum IGF-I and jejunal IGF-I mRNA (38, 40).

GLP-2 is a nutrient-regulated intestinotrophic peptide hormone derived from tissue-specific posttranslational processing of proglucagon in the endocrine L cells of the ileum and colon (5, 33). Endogenous GLP-2 is a key mediator of refeeding-induced intestinal adaptive regrowth in mice (32) and resection-induced mucosal adaptive growth in rats (9). Furthermore, exogenous GLP-2 has been reported to increase villus height, crypt depth, intestinal energy absorption, and lean body mass in humans with short bowel syndrome (20, 21). Recent work in mice showed that exogenous GLP-2 improves gut morphology and increases proliferative indexes in wild-type but not IGF-I knockout mice, suggesting that IGF-I is an essential mediator of GLP-2 action (11).

GLP-2 is rapidly inactivated by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV). DPP-IV cleaves two amino acids off the NH2-terminal end of GLP-21–33, leaving the truncated metabolite GLP-23–33. GLP-23–33 has been shown to function both as a weak agonist and as an antagonist to the GLP-2 receptor in mice (32). The ability of GLP-23–33 to antagonize GLP-21–33 action in the rat has not been reported.

Several studies have investigated the effects of fasting and ad libitum refeeding on intestinal adaptive growth (1, 2, 18) and its association with either IGF-I (40) or GLP-2 responses (19, 32). Only one study, however, has isolated the contributions of luminal versus systemic nutrients in stimulating IGF-I responses and the corresponding mucosal atrophy and regrowth induced by fasting and refeeding (38). The objectives of this research were twofold: first, to determine the relationship between luminal nutrients and both IGF-I and GLP-2...
responses in mediating intestinal growth in rats fasted and then refed by continuous intravenous or intragastric infusion and second, to determine whether antagonism of endogenous GLP-2 with GLP-2^{23–33} in rats fasted and refed ad libitum attenuates mucosal growth and IGF-I and GLP-2 responses.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

The University of Wisconsin-Madison Institutional Animal Care and Use Committee approved the animal facilities and protocol. Male Sprague-Dawley rats (Harlan, Madison, WI) initially weighing 200–225 g were housed individually in stainless steel wire-bottom cages with unlimited access to water. The animal facilities were maintained at 22°C on a 12:12-h light-dark cycle. All rats were acclimated to the facility for 5–7 days while being fed a semipurified powdered diet ad libitum (10).

We conducted two experiments to elucidate the roles of the IGF-I and GLP-2 systems in mediating intestinal adaptation in rats fasted for 48 h and then refed for 2 or 4 days by continuous infusion or ad libitum feeding. In the first experiment, we identified systemic and intestinal IGF-I and GLP-2 responses and expression of sodium-glucose transporter-1 (SGLT-1) in response to the intestinal atrophy induced by fasting and differential mucosal regeneration induced by intravenous or intragastric refeeding. Experiment 1 consisted of six groups of rats (n = 6–9 per group) including two baseline groups, fed and 48 h fasted, and four treatment groups subjected to a 48-h fast followed by 2 or 4 days of intravenous refeeding or 2 or 4 days of intragastric refeeding. In the second experiment we administered an antagonist of endogenous GLP-2, GLP-2^{23–33} (32), to rats fasted for 48 h and then refed ad libitum to determine the relationships between attenuation of mucosal regeneration and IGF-I and GLP-2 responses. Experiment 2 consisted of six groups of rats (n = 5 or 6 per group) including baseline fed and 48 h fasted and four treatment groups given subcutaneous injection of PBS or 2.5, 10, or 50 μg/kg body wt^{−1·day^{−1}} of rat GLP-2^{23–33} (California Peptide Research, Napa, CA). The GLP-2^{23–33} was diluted in PBS and injected every 12 h during the 2 days of refeeding.

**Surgical Procedures and Animal Care**

Intravenous and intragastric catheter placement and feeding protocols. Rats were fasted for 48 h before surgical placement of intravenous or intragastric catheters. Rats were anesthetized by inhalation of isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL) via an anesthesia machine before surgery. Intravenous catheters were placed in the superior vena cava as previously reported (10, 24). Intragastric catheters were placed via a midline incision in the abdomen (24). Briefly, the stomach was visualized by a midline incision, immobilized, and pierced with a 20-gauge needle. A Silastic catheter was inserted 1 cm into the greater curvature of the stomach and secured by a purse string suture. In each surgery, the catheter was tunneled subcutaneously to exit the rat between the scapulas. The catheter was passed through a stainless steel spring connected to a backpack harness at one end and a swivel infusion apparatus (Instech, Plymouth Meeting, PA) at the other, allowing continuous infusion and protection of the catheter without restraining the rat. Body weight was determined daily, and urine output was collected during the refeeding period. Urine was collected into vials containing a final concentration of 0.01% boric acid and stored at −20°C for analysis of nitrogen content to determine nitrogen balance (39).

Immediately after surgery (day 0), infusion was started at a rate of 0.5 ml/h. Both intravenously and intragastrically refed rats were infused continuously with a nutritionally complete TPN solution providing 32% nonprotein energy from fat and 68% nonprotein energy from dextrose (10). The TPN solution contained (in g/l) 44 amino acids (Travasol 8.5% with electrolytes, Baxter Healthcare, Deerfield, IL), 180 dextrose (Baxter Healthcare), 28 lipid (142 ml Intralipid, Kabi Pharmacia, Clayton, NC), and vitamins and trace elements (10). The infusion rate was increased to 1.67 ml/h (40 ml/day) on day 1, with full infusion of 2.5 ml/h (60 ml/day) provided on days 2–4. Orally fed rats in the baseline fed groups received a semipurified diet with a macronutrient profile similar to that of the TPN solution (10). This diet contained (in g/kg) 163 casein, 3 α-methionine, 635 dextrose, 70 soybean oil, 70 safflower oil, 12 cellulose, 35 AIN-76 mineral mix, 10 AIN-76A vitamin mix, and 2 choline bitartrate (Harlan Teklad, Madison, WI).

After the 48-h fast in experiment 2, rats received the first of four injections of GLP-2^{23–33} and were offered ad libitum access to a specially formulated semielemental liquid diet previously demonstrated to induce mucosal growth and plasma GLP-2 in intravenously fed rats (25). The diet contained (in g/kg) 161 protein from a blend of soy, whey, and casein; 701 carbohydrate from sucrose and corn maltodextrin; and 42 fat from safflower oil and medium-chain triglycerides (product code TD.07244, Harlan Teklad).

**Intestinal composition, histology, and sucrase activity**. Rats were anesthetized and killed by exsanguination from the heart within 10 min of the end of continuous infusion feeding. The jejunum, defined as bowel between the ligament of Treitz and the ileum, and ileum, defined as the final 25 cm of small bowel proximal to the cecum, were removed for analysis. All sections of bowel were immediately flushed with ice-cold saline and put on a chilled glass plate to be sectioned. In each section of bowel, the proximal 5 cm were collected and mucosa was isolated by scraping for determination of wet and dry mucosal mass. The next 5 cm were weighed and scraped for determination of mucosal protein (bicinchoronic acid protein assay; Pierce Chemicals, Rockford, IL) and DNA (23). Jejunal sucrase activity was assayed from the same section as protein and DNA (9). The remaining tissue from jejunum and ileum was snap-frozen intact in liquid nitrogen and stored at −70°C for RNA extraction. Fixed tissue for histology was paraffin embedded, cut into 5-μm sections, and stained with hema-toxylin and eosin for histomorphology as previously described (10).

**Biochemical Analyses**

**Plasma IGF-I, bioactive GLP-2, and insulin**. Blood was collected in chilled tubes containing a final concentration of 1 mg/ml EDTA, 0.1 mM Diphosphor A (MP Biomedicals, Aurora, OH), and 0.01 mM aprotinin (Calbiochem, La Jolla, CA). Plasma was isolated by centrifugation at 1,800 g for 15 min at 4°C and was stored at −70°C until IGF-I or GLP-2 measurement. Plasma IGF-I was measured by RIA after removal of binding proteins by high-performance liquid chromatography under acidic conditions (29). Plasma bioactive GLP-2 was measured by RIA using an antibody specific to the NH2 terminus of GLP-2 (16). Plasma insulin was measured in experiment 1 with an RIA kit specific for rat insulin (Linco Research, St. Charles, MO).

**SGLT-1 in brush-border membrane**. Approximately 30 cm of distal jejunum (from 17 cm distal to the ligament of Treitz to the ileum) was removed and flushed with ice-cold saline for determination of SGLT-1 expression along the brush-border membrane. To isolate brush-border membrane, mucosa was isolated by cutting the intestine longitudinally and scraping with a glass slide (22). Weighed mucosa was homogenized in 250 ml of cold 2 mM Tris·HCl homogenization buffer containing 50 mM mannitol (pH 7.1) for 2 min. 0.33 g of calcium chloride was added, and the solution was homogenized for an additional 30 s before incubation at 4°C for 20 min. The homogenate was centrifuged at 2,358 g for 15 min at 4°C, the pellet was discarded, and the supernatant was again centrifuged at 23,870 g for 30 min at 4°C. The pellet was collected and resuspended into vesicle buffer in a glass/Teflon Potter Evehjem homogenizer. The homogenate was centrifuged at 23,780 g for 30 min at 4°C, and the resulting pellet containing brush-border membranes was resuspended in vesicle buffer with a 21-gauge needle and syringe and stored at −70°C. The
purity of the brush-border membrane was confirmed by a 10-fold increase in sucrase activity in the membranes compared with the mucosal homogenate. SGLT-1 expression was quantified by Western immunoblotting. A 75-µg aliquot of the brush-border membrane preparation was fractionated on a 10% SDS-PAGE gel, and bands were transferred to a polyvinylidene fluoride membrane. After blocking with 5% milk in PBS-Tween, the blots were incubated overnight at 4°C in 1:1,000 dilution of SGLT-1 (Alpha Diagnostics International) and 1:1,600 β-actin (Sigma) antibody. Bands were visualized after 1-h incubation in 1:3,000 dilution of secondary antibodies conjugated with horseradish peroxidase followed by enhanced chemiluminescence (Amersham Biosciences). Data were analyzed with OptiQuant software (OptiQuant; Packard Instrument), and band intensities were expressed relative to β-actin levels.

**IGF-I, IGFBP-3 and -5, and Proglucagon mRNA**

Total RNA was extracted from intact jejunum, ileum, and liver with TRIzol reagent (GIBCO-BRL Life Technologies, Grand Island, NY) and quantified spectrophotometrically at 260 nm. Integrity was confirmed by visualization of 18S and 28S rRNA on an agarose-formaldehyde gel with ethidium bromide staining.

**RNase protection assay.** IGF-I, IGFBP-3, and IGFBP-5 mRNAs were measured by RNase protection assay according to the procedure described previously (14). Briefly, DNA templates were derived from cDNA fragments cloned into pGEM-4Z vectors and linearized with appropriate restriction enzymes. 32P-labeled antisense RNA probes were synthesized from each template with T7 RNA polymerase. An antisense pTRI RNA 18S control template (Ambion, Austin, TX) was used to generate a labeled RNA probe to measure ribosomal 18S RNA along with mRNA as an internal as well as experimental control. Thirty micrograms of total RNA from intestine was cohybridized with labeled IGF-I, IGFBP-3, or IGFBP-5 and 18S probes. Protected bands were observed at 238 (IGF-I), 550 (IGFBP-3), or 300 (IGFBP-5) nt and at 80 nt corresponding to 18S. The IGF-I, IGFBP-3, and IGFBP-5 vectors were kindly provided by Dr. M. L. Adamo (Univ. Texas Health Science Center, San Antonio, TX). Protected bands were quantified by phosphorimaging (OptiQuant, Packard Instrument). Relative band intensities were calculated by dividing the IGF-I band intensity by the 18S band intensity in each sample.

**Reverse transcriptase-real-time PCR.** Proglucagon mRNA expression was measured in a two-step reverse transcriptase-real-time quantitative PCR (RT-qPCR) using a SYBR Green detection method. Ten micrograms of intact total RNA was treated with DNase (TURBO DNA-free kit, Ambion) to eliminate genomic DNA and reverse transcribed with random hexamers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Control reactions without reverse transcriptase were performed to confirm the specificity of transcription reaction. cDNA was diluted to a concentration of 10 ng/μl based on a template titration assay, and RT-qPCR was performed with SYBR Green PCR Master Mix (ABI). No-reverse transcriptase control and no-template control reactions were done with every assay to ensure the specificity of the reaction and the absence of any contamination. Sequences for forward and reverse primers were 5′-GAA TTC ATT GCT TGG CTG GT-3′ and 5′-TTC CTC AGC TAT GGC GAC TT-3′, respectively (Integrated DNA Technologies, Coralvile, IA), making a 72-bp size amplicon. A four-step hot-start real-time PCR was performed with an Applied Biosystems 7000 Real-Time PCR instrument with conditions as follows: step 1, 50°C for 2 min; step 2, 95°C for 10 min; step 3, 50 cycles of 95°C for 0.15 min followed by 55°C for 1 min; step 4 (dissociation), 95°C for 0.15 min, 60°C for 0.20 min, and 95°C for 0.15 min. The dissociation step was performed to confirm the uniformity of amplicon size and the absence of any primer dimers. 18S qPCR was performed under similar conditions with QuantumRNA 18S Internal Standards (Ambion). Equal efficiency of amplification was verified for all assays by the serial dilutions technique. Data were analyzed with 7000 system software (Applied Biosystems), and relative quantification was done by the ΔΔCt method (where Ct is threshold cycle) (26) with 18S as the internal control and fed group as the reference control.

**Statistical Analyses**

Treatment groups were analyzed with general linear models, and individual differences between the treatment groups were identified by one-way ANOVA followed by the protected least significant differences technique (SAS, SAS Institute, Cary, NC). Main effects resulting from the route of refeeding or the duration of refeeding or their interactions were assessed by two-way ANOVA. All data are presented as means ± SE. P < 0.05 was considered statistically significant.

**RESULTS**

**Body Weight and Nitrogen Balance**

Initial body weights were similar at the beginning of each experiment (240–250 g, experiment 1; 230–235 g, experiment 2). Baseline fed rats gained weight (5–6 g/day), and fasted rats lost ~12% of body weight (24–30 g/48 h) during the experiments. After 2 days of intravenous or intragastric refeeding in experiment 1 (40% and 67% of energy requirements provided on days 1 and 2, respectively) rats regained 1 ± 4 g (Table 1). After 2 days of ad libitum feeding in experiment 2, rats regained all of the body weight lost during the 48-h fast. After 4 days of intravenous or intragastric refeeding (with the last 2 days providing 100% of energy requirements), rats regained 13–16 g or ~50% of body weight lost due to fasting. Refeeding by either the intravenous or the intragastric route led to similar body weight gain that was associated with nitrogen balance consistent with accretion of body protein. Rats were in positive nitrogen balance (186 ± 18 mg/day intravenous, 191 ± 7 mg/day intragastric) by the second day of refeeding, in agreement with the incremental increase in nutrient infusion rate over days 0–2.

**Table 1. Baseline, fasted, and final body weight and body weight change after refeeding (experiment 1)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Baseline BW, g</th>
<th>Fasted BW, g</th>
<th>Final BW, g</th>
<th>BW Change, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Day IV</td>
<td>6</td>
<td>243 ± 4</td>
<td>213 ± 4</td>
<td>214 ± 6b</td>
<td>1 ± 4b</td>
</tr>
<tr>
<td>4-Day IV</td>
<td>7</td>
<td>248 ± 7</td>
<td>217 ± 5</td>
<td>229 ± 7a</td>
<td>13 ± 4a</td>
</tr>
<tr>
<td>2-Day IG</td>
<td>7</td>
<td>245 ± 4</td>
<td>217 ± 3</td>
<td>219 ± 4b</td>
<td>1 ± 4b</td>
</tr>
<tr>
<td>4-Day IG</td>
<td>9</td>
<td>246 ± 5</td>
<td>216 ± 5</td>
<td>231 ± 6a</td>
<td>16 ± 2a</td>
</tr>
</tbody>
</table>

Values are means ± SE for n rats. Body weight (BW) change is difference between fasted and final BW, i.e., after 2 or 4 days of refeeding. IV, intravenous refeeding; IG, intragastric refeeding. Means with different superscripts within a column are significantly different (P < 0.0001). A main effect due to duration of refeeding was significant (P < 0.0001) for final BW and BW change.
Mucosal Adaptive Growth

Significant mucosal atrophy was present throughout the small bowel in response to fasting, and mucosal regeneration was noted with intragastric or ad libitum refeeding (vehicle-treated and 10 μg GLP-23–33 groups) but not intravenous refeeding. Responses were most pronounced in the jejunum, where a 48-h fast resulted in a significant 25–65% decrease in mucosal mass, protein, and DNA compared with the baseline fed groups (Figs. 1 and 2). Mucosal atrophy in the jejunum was not altered with 2 or 4 days of intravenous refeeding, such that mucosal mass and concentrations of protein and DNA were not significantly different compared with fasted rats. However, intragastric refeeding resulted in significant mucosal regrowth after only 2 days of infusion of an elemental diet, despite little change in body weight, and mucosal mass continued to increase from 2 to 4 days with intragastric refeeding. A constant protein-to-DNA ratio in the jejunum after refeeding-induced mucosal regeneration indicates that the increased cellularity is due to cellular hyperplasia. Ileal mucosal mass was significantly decreased by 25% with fasting and rebounded with intragastric refeeding, but protein and DNA responses were not significantly different compared with fasting (data not shown).

Jejunal villus height was significantly decreased (10–18%) and crypt depth showed a nonsignificant decrease (1–3%) in the baseline fasted compared with baseline fed groups in both experiments (Table 2; experiment 2 data not shown). Intravenous refeeding for 2 or 4 days induced additional jejunal mucosal atrophy, resulting in a significant 15% further decrease in villus height and a nonsignificant 7% decrease in crypt depth compared with fasting. Intragastric refeeding for 2 or 4 days increased villus height and crypt depth, resulting in histology that was not significantly different from the baseline fed group.

Administration of GLP-23–33 to antagonize endogenous GLP-2 showed partial attenuation of jejunal and ileal (data not shown) mucosal regeneration only in the refed groups given 2.5 or 50 μg/kg body wt GLP-23–33 (Fig. 2). Jejunal mucosal protein and DNA were significantly lower in the 2.5 and 50 μg groups compared with the baseline fed group. Indexes of jejunal mucosal regeneration were not significantly different in the refed groups compared with the baseline fed group, indicating mild attenuation of mucosal regeneration with administration of GLP-23–33.

Sucrase Activity

Jejunal mucosal sucrase activity reflected changes in mucosal cellularity in both experiments. Segmental sucrase activity (μmol·min⁻¹·cm⁻¹) decreased a nonsignificant 6% after the 48-h fast in experiment 1 (Fig. 1). However, 2 or 4 days of intravenous refeeding led to a further decrease in sucrase segmental activity, resulting in significantly lower jejunal sucrase activity compared with the fed group. Intragastric refeeding maintained sucrase segmental activity at the fasted level after 2 days and resulted in further significant increases in activity to a level significantly above the baseline fed level at 4 days. There were no significant differences in sucrase specific activity (μmol·min⁻¹·mg protein⁻¹) among the groups.
In experiment 2, which showed greater mucosal atrophy with the 48-h fast than experiment 1, jejunal sucrase segmental activity was significantly decreased (data not shown) and sucrase specific activity was significantly increased in the baseline fasted compared with fed groups (Fig. 2). Sucrase segmental activity was not significantly different in refed groups (except for the 2.5 µg group) compared with the baseline fed group. Interestingly, there was significantly greater sucrase specific activity in the 2.5 µg refed group in association with attenuation of mucosal regeneration, which suggests a delay in the maturation of enterocyte enzyme expression.

Plasma IGF-I and Insulin

The concentration of IGF-I in plasma was significantly decreased by 34–39% after the 48-h fast in both experiments (Fig. 3). Controlled refeeding for 2 or 4 days increased plasma IGF-I by only 11% overall in association with a regain of 50% of the body weight lost due to fasting in experiment 1. There was a significant main effect of route of refeeding, such that intravenous refeeding increased plasma IGF-I more than intragastric refeeding. The liver is the primary source of circulating IGF-I, and, as expected, liver IGF-I mRNA was significantly decreased 36% with fasting (34) and returned to the fed level after 2 or 4 days of intravenous or intragastric refeeding (data not shown). The significant increase in IGF-I message in the liver relative to the modest 11% increase in the plasma concentration of IGF-I is consistent with posttranscriptional regulation of IGF-I expression (34). Four days of continuous intravenous refeeding resulted in insulin levels significantly greater than those after 4 days of intragastric refeeding (data not shown).

Ad libitum refeeding in experiment 2, where rats regained the loss of body weight induced by the 48-h fast, increased plasma IGF-I by 45% in the vehicle control group, a level not significantly different from the baseline fasted group. Attenuation of mucosal regeneration in the group treated with 2.5 µg GLP-23–33 was associated with the smallest increase in plasma IGF-I. Interestingly, all three refed groups treated with GLP-23–33 showed an increase in plasma IGF-I of only half that observed in the vehicle control group. Thus, despite regain in body weight, plasma concentration of IGF-I was not significantly different in refed groups treated with GLP-23–33 compared with the baseline fasted group.

![Fig. 2. Jejunal mucosal dry mass (A), protein (B), DNA (C), and sucrase specific activity (D) in rats fed orally, fasted, or fasted and then refed ad libitum for 2 days with administration of vehicle or 2.5, 10 or 50 µg/kg body wt GLP-23–33, an antagonist of endogenous glucagon-like peptide-2 (GLP-2) (experiment 2). Values are means ± SE; n = 4–6 rats/group. Means with different superscripts are significantly different (P < 0.05).](graphic-url)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Villus Height, µm</th>
<th>Crypt Depth, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>452±19a</td>
<td>91±5b</td>
</tr>
<tr>
<td>Fasted</td>
<td>406±18a</td>
<td>90±3b</td>
</tr>
<tr>
<td>2-Day IV</td>
<td>338±18a</td>
<td>84±3b</td>
</tr>
<tr>
<td>4-Day IV</td>
<td>342±7a</td>
<td>83±2b</td>
</tr>
<tr>
<td>2-Day IG</td>
<td>448±18ab</td>
<td>94±6a</td>
</tr>
<tr>
<td>4-Day IG</td>
<td>437±10b</td>
<td>89±2b</td>
</tr>
</tbody>
</table>

Table 2. Jejunal histology in rats fed orally, fasted, or fasted and then refed by continuous intravenous or intragastric infusion for 2 or 4 days (experiment 1)

Values are means ± SE; n = 6–9 rats. Means with different superscripts within a column are significantly different (P < 0.0001). A main effect due to route of refeeding was significant (P < 0.0001) for villus height.
Jejunal IGF-I, IGFBP-3, and IGFBP-5 mRNA

Local jejunal IGF-I mRNA expression was significantly decreased by 25–45% after fasting in both experiments (Fig. 3). Jejunum IGF-I mRNA was significantly greater at 4 versus 2 days of refeeding in both intravenous and intragastric groups. However, only 4 days of intragastric refeeding increased jejunal IGF-I expression to a level not significantly different from the fed group. In contrast, ad libitum refeeding for 2 days in experiment 2 restored jejunal IGF-I expression to levels not significantly different from the baseline fed group. Jejunal expression of IGFBP-3 mRNA was reduced by 54–75% due to fasting in both experiments, and 2 days of refeeding did not restore levels (data not shown). There were no differences in jejunal IGFBP-5 expression in experiment 1. In experiment 2, fasting induced a significant 25% decrease in IGFBP-5 that was not restored with 2 days of ad libitum refeeding (data not shown).

Plasma Bioactive GLP-2 and Ileal Proglucagon mRNA

The concentration of bioactive GLP-2 in plasma and the expression of ileal proglucagon mRNA in response to fasting and 4 days of intravenous or intragastric refeeding (Fig. 4) or 2 days of ad libitum refeeding (Fig. 5) were determined. Plasma bioactive GLP-2 showed a significant 41–60% decrease after the 48-h fast in both experiments. Intragastric refeeding or ad libitum refeeding with administration of GLP-2 restored plasma concentrations of GLP-2 to the fed levels. Intravenous refeeding did not significantly increase plasma GLP-2 concentration compared with fasting, consistent with the lack of mucosal regeneration due to the absence of luminal nutrients. Ileal proglucagon expression, the major source of plasma GLP-2, showed a significant 25–50% decrease after the 48-h fast in both experiments. Mucosal regeneration after 4 days of gradual intragastric, but not intravenous, refeeding resulted in proglucagon expression that was not significantly different compared with the fed level. Plasma GLP-2 concentration and proglucagon expression were restored to baseline fed levels with 2 days of ad libitum refeeding in all four groups.

Sodium-Glucose Transporter-1

Since SGLT-1 immunoreactivity is tightly correlated ($R = 0.998$) to SGLT-1 enzyme activity (12), we measured SGLT-1 immunoreactivity in the jejunal brush-border membrane. SGLT-1 expression decreased nonsignificantly (33%) after fasting, similar to sucrase activity (Fig. 6). Like sucrase activity, SGLT-1 expression continued to decline after 4 days of intravenous refeeding, resulting in a significantly lower level than in the fed state. Intragastric refeeding for 4 days returned SGLT-1 expression to a level virtually identical to that in fed rats.
DISCUSSION

Luminal nutrients are the primary stimulus for intestinal growth, and IGF-I and GLP-2 are potential nutrient-regulated mediators of the intestinal adaptive growth induced by fasting-refeeding (32, 38, 40). Moreover, recent data suggest that IGF-I is an essential mediator of the intestinotrophic actions of GLP-2 (11). We characterized the roles of the IGF-I and GLP-2 systems in mediating intestinal adaptive growth in rats fasted and then refed by continuous intravenous or intragastric infusion of identical parenteral nutrition solutions or by ad libitum feeding of a semielemental diet.

Many studies have used fasting (48–96 h) followed by oral ad libitum refeeding of a stock diet to characterize intestinal adaptive growth in rats (1, 2). Our study differs from previous work in several ways, such as the use of a brief 48-h fast and controlled gradual refeeding of identical nutrient solutions by both intravenous and intragastric routes for 2 or 4 days. Gradually increasing the rate of nutrient infusion allows for adaptive regrowth without the complication of hyperphagia present with postfasting ad libitum food intake. Infusion of identical elemental TPN solutions by the intravenous or intragastric route allows us to isolate the effect of luminal nutrients in the stimulation of intestinal adaptation. Complications due to different diet compositions and eating patterns, present in pair-feeding studies, were thus eliminated. Moreover, experiment 2 provided a complementary, more physiological feeding approach as rats regained the body weight lost due to fasting with 2 days of ad libitum refeeding.

The nutritionally regulated hepatic IGF-I endocrine system controls whole body growth and circulating IGF-I. Fasting for 48 h reduced circulating immunoreactive IGF-I and hepatic IGF-I mRNA by ~35%, consistent with previous reports (17, 38). Interestingly, intravenous refeeding increased plasma IGF-I to a significantly greater extent compared with intragastric refeeding, possibly due to greater circulating insulin in intravenously refed rats (34, 38). All four ad libitum refed groups demonstrated regain of body weight lost due to fasting; however, only the vehicle-treated group showed recovery of plasma IGF-I to fed levels. In contrast, rats given GLP-23–33 showed plasma IGF-I levels not significantly different from fasting, suggesting an unknown link between antagonism of endogenous GLP-2 and reduced circulating IGF-I that warrants further investigation.

A 48-h fast resulted in significantly lower intestinal cellularity that was most pronounced in the jejunum based on a 25–65% decrease in jejunal mucosal mass, protein, and DNA. Histomorphology confirmed the fasting-induced mucosal atrophy, because villus height decreased significantly consistent with an increase in villus apoptosis (32). Crypt depth decreased nonsignificantly after fasting, consistent with another study utilizing a 48-h fast (3). However, studies utilizing longer

Fig. 4. Plasma concentration of bioactive GLP-2 (A) and ileal proglucagon mRNA expression (B) in rats from experiment 1. Ileal proglucagon and 18S RNA levels were determined by RT-qPCR. Data were analyzed by the ΔΔCt method (where C is threshold cycle) with 18S RNA as internal control and expressed relative to the fed group. Efficiency of primers was checked with a serial dilution method. Values are means ± SE; n = 4–6 rats/group. Means with different superscripts are significantly different (P < 0.05).

Fig. 5. Plasma concentration of bioactive GLP-2 (A) and ileal proglucagon mRNA expression (B) in rats from experiment 2. Ileal proglucagon and 18S RNA levels were determined by RT-qPCR. Data were analyzed by the ΔΔCt method with 18S RNA as internal control and expressed relative to the fed group. Efficiency of primers was checked with a serial dilution method. Values are means ± SE; n = 4–6 rats/group. Means with different superscripts are significantly different (P < 0.05).
periods of fasting (72–96 h) have observed significant decreases in both villus height and crypt depth throughout the small bowel (6, 15).

As expected, intestinal adaptive regrowth was present after intragastric and ad libitum (vehicle treated), but not intravenous, refeeding. Moreover, intravenous refeeding induced further decreases in villus height and segmental sucrase activity compared with fasting. Strikingly, after only 2 days of intragastric refeeding of an elemental diet, when infusion rates were supplying 67% of energy requirements and body weight had not yet rebounded, intestinal regrowth was so robust that jejunal mucosal mass, protein, and DNA had returned to fed levels. Additionally, after 4 days of intragastric refeeding villus height had returned to fed levels and sucrase segmental activity surpassed fed levels. Interestingly, the increase in sucrase activity occurred despite the absence of sucrose feeding, because glucose provided the only source of carbohydrate in the elemental refeeding solution. Previous research suggests that nonspecific carbohydrate loading in the intestine is monitored by glucose transport rates, and thus glucose refeeding would be expected to stimulate sucrase activity (4).

Studies in mice have shown that GLP-2 3–33 is an antagonist to exogenous and endogenous GLP-21–33 in mice (32, 36). This study demonstrates that GLP-21–33 is a weak inhibitor of endogenous GLP-2 action in the rat by using a range of three doses that were extrapolated from the mouse (32). The lack of response to the middle dose of GLP-21–33 is unexplained and suggests that investigation with a broader range of GLP-21–33 doses is needed. Partial attenuation of mucosal cellularity was observed with the lowest and highest doses of GLP-21–33; however, the decreased cellularity was not evident in the histology. Moreover, there was no association between the attenuation of mucosal cellularity and plasma concentration of GLP-21–33, ileal proglucagon mRNA, or jejunal IGF-I mRNA. Similarly, Shin et al. (32) noted no change in plasma GLP-21–33 30 min after GLP-22–33 injection (1.2 μg/kg) relative to saline controls. Greater endogenous DPP-IV activity in the rat compared with the mouse may explain why GLP-22–33 did not strongly inhibit GLP-2 action in the present study.

IGF-I stimulates intestinal growth by endocrine actions, as demonstrated by the dramatic intestinal growth induced by infusion of exogenous IGF-I (10), as well as paracrine mechanisms (37). Expression of IGF-I mRNA in jejunum showed a significant 25–45% decrease after a 48-h fast, consistent with previous reports that a 72-h fast induced a 35–80% decrease in jejunal IGF-I mRNA (38, 40). Jejunal expression of IGF-I mRNA showed a positive association with intestinal regeneration with 4 days of intragastric refeeding or ad libitum refeeding based on restoration of IGF-I expression and mucosal cellularity to fed levels. The significant 54–75% decrease in jejunal expression of IGFBP-3 due to fasting is similar to a previous report (38). Our data suggest that 2 days of refeeding is not sufficient to restore IGFBP-3 or -5 mRNA; however, mRNA levels do not always correlate with protein secretion, and the IGFBP may play a role in mucosal regeneration (14). Previous reports demonstrate that jejunal IGF-I mRNA is not reduced in rats fed by nutritionally adequate intravenous compared with enteral feeding despite mucosal atrophy due to intravenous feeding (13, 30, 38). Taken together, an increase in IGF-I expression in small bowel is not sufficient to stimulate intestinal growth in the absence of luminal nutrients and possibly an increase in circulating bioactive GLP-2.

Changes in mucosal cellularity due to the absence or presence of luminal nutrients were positively associated with changes in the plasma concentration of bioactive GLP-2. Plasma GLP-2 decreased with fasting-induced mucosal atrophy and rebounded to fed levels in association with mucosal regeneration after 2 days of ad libitum refeeding or 4 days of intragastric refeeding. These findings demonstrate in the rat, as shown in the mouse (32), that endogenous GLP-2 is a key mediator of refeeding-induced mucosal growth. Dube et al. (11) concluded that IGF-I is an essential mediator of GLP-2 action based on the absence of intestinal growth in IGF-I knockout mice given exogenous GLP-21–33 compared with wild-type mice. Our observation that refeeding-induced mucosal growth was associated with restoration of both endogenous plasma bioactive GLP-2 and intestinal expression of IGF-I mRNA in a physiological rat model supports the finding in a genetic mouse model that IGF-I is a downstream mediator of GLP-2 action. However, further research is needed to understand the mechanisms underlying the intestinotrophic interactions between GLP-2 and IGF-I.

Immunoreactive SGLT-1 decreased after fasting and rebounded to the fed level after intragastric but not intravenous refeeding. This response parallels the mucosal regeneration and increase in plasma concentration of GLP-2 induced by enteral feeding. Our finding is in agreement with previous reports that coinfusion of exogenous GLP-2 with TPN solution increases mucosal growth and SGLT-1 expression in pigs (8, 31) and resected rats (28).

Perspectives and Significance

Our data suggest a strong association between stimulation of mucosal growth by luminal nutrients and induction of the
nutritionally regulated intestinotrophic hormones GLP-2 and IGF-I. This was reflected in mucosal regrowth and elevated levels of plasma bioactive GLP-2 and jejunal IGF-I mRNA in rats fasted and then refed by intragastric and ad libitum feeding. Lower plasma IGF-I levels in rats administered an antagonist of GLP-2 (GLP-2-33) suggest a link between GLP-2 and IGF-I, although only partial attenuation of the mucosal regrowth was observed. The range of GLP-2-3–33 doses used in this study appear to be less effective in inhibiting mucosal regrowth compared with the mouse. A possible explanation may be greater endogenous DPP-IV activity, which may result in higher tolerance to GLP-2 and IGF-I may interact to stimulate mucosal growth. Overall, mucosal growth in response to luminal nutrients is directly associated with increased expression of both GLP-2 and IGF-I.

ACKNOWLEDGMENTS

We thank Dr. George R. Flentke in the Department of Nutritional Sciences, University of Wisconsin-Madison for his help in developing the RT-qPCR methodology.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants RO1-DK-42835 and T32-DK-07765 and by funds from the US Department of Agriculture Cooperative State Research, Education and Extension Service, project WISO 3433, University of Wisconsin-Madison.

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