GLP-2 rapidly activates divergent intracellular signaling pathways involved in intestinal cell survival and proliferation in neonatal piglets

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Burrin DG, Stoll B, Guan X, Cui L, Chang X, Hadsell D. GLP-2 rapidly activates divergent intracellular signaling pathways involved in intestinal cell survival and proliferation in neonatal piglets. Am J Physiol Endocrinol Metab 292: E281–E291, 2007. First published September 5, 2006; doi:10.1152/ajpendo.00129.2006.—We previously demonstrated the dose-dependent glucagon-like peptide (GLP)-2 activation of intracellular signals associated with increased epithelial cell survival and proliferation in the neonatal intestine. Our current aim was to quantify the acute, temporal GLP-2 activation of these key intracellular signals and relate this to changes in epithelial cell survival and proliferation in the neonatal intestine. We studied 29 total parenteral nutrition-fed neonatal piglets infused intravenously with either saline (control) or human GLP-2 (420 mol·kg⁻¹·h⁻¹) for 1, 4, or 48 h. GLP-2 infusion increased small intestinal weight, DNA and protein content, and villus height at 48 h, but not at 1 or 4 h. Intestinal crypt and villus apoptosis decreased and crypt cell proliferation and protein synthesis increased linearly with duration of GLP-2 infusion, but were statistically different from controls only after 48 h. Before the morphological and cellular kinetic changes, GLP-2 rapidly activated putative GLP-2 receptor downstream signals within 1–4 h, including phosphorylation of protein kinase A, protein kinase B, extracellular signal-regulated kinase 1/2, and the transcription factors cAMP response element-binding protein and c-Fos. GLP-2 rapidly suppressed caspase-3 activation and upregulated Bcl-2 abundance within 1 h, whereas there was an increase in apoptosis inhibitors X-linked inhibitor of apoptosis at 1 h and cellular inhibitor of apoptosis-2 at 4 and 48 h. We also show that the increased c-Fos and reduced active caspase-3 immunostaining after GLP-2 infusion was localized in epithelial cells. We conclude that GLP-2-induced activation of intracellular signals involved in both cell survival and proliferation occurs rapidly and precedes the trophic cellular kinetic effects that occur later in intestinal epithelial cells.

The biological effects of GLP-2 are mediated via activation of a G protein-linked membrane receptor (GLP-2R) expressed mainly in the gastrointestinal tract and brain (27, 47). The cellular mechanisms of GLP-2 action subsequent to binding and activation of the GLP-2R are poorly understood. Reports have demonstrated the presence of the GLP-2R in human enteroendocrine cells (47), murine enteric neurons (1), and subepithelial myofibroblasts (33). Recently, we showed the GLP-2R to be localized in both enteroendocrine cells and enteric neurons in the human and piglet intestine (14). Apart from the cellular localization in vivo, the detailed understanding of the intracellular signal pathways engaged by GLP-2R activation has been impeded by the lack of suitable cell culture models. Two reports using primary cells isolated from rat intestinal (43) and brain (42) tissue have demonstrated the presence of GLP-2R expression and that GLP-2 induced cAMP production, c-Fos expression, and [³H]thymidine incorporation. However, the evidence to date indicates that the GLP-2R is not expressed in most intestinal epithelial cell lines (Caco-2, IEC-6, HT-29; see Refs. 35 and 47), but was recently detected in a human epithelial and fibroblast line (37). Despite this, however, studies with Caco-2 cells showed that GLP-2 increases cell proliferation in association with decreased cAMP production but increased extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and the response is suppressed by inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase) and mitogen-activated protein kinase (MAPK; see Refs. 17 and 35).

The most detailed characterization of the GLP-2R intracellular signaling pathways has been reported in studies with GLP-2R-transfected fibroblasts (45, 46, 48) and most recently cervical carcinoma (HeLa) cells (24). The GLP-2R-transfected BHK fibroblast studies showed that GLP-2 induces cAMP production, immediate early gene (c-fos) expression, and cell proliferation and survival (45, 48). Subsequent work with the BHK cells showed that GLP-2 prevents apoptosis via protein kinase A (PKA)-dependent phosphorylation of Bad, protein kinase B (PKB), and glycogen synthase kinase-3 (GSK-3) and downstream inhibition of caspase-3 activity, but does not involve PI 3-kinase or MAPK pathways (46). Recent studies with GLP-2R-transfected HeLa cells confirmed previous reports showing that GLP-2 increases cAMP levels and suppresses apoptosis via a PKA, but ERK1/2-independent mechanism (24). In contrast, however, the HeLa cell studies found an effective therapy for treatment of adult short-bowel syndrome (20–22).

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that GLP-2 also increased cell proliferation and ERK1/2 activation, but these phenomenon were inhibited by dominant-negative Ras expression and PD-98059, implicating a Ras/Raf/ MAPK-dependent pathway. More importantly from these studies was that cell proliferation and ERK1/2 activation was independent of cellular cAMP, PI 3-kinase, and PKA activity. The consistent findings that have emerged from these and other cell culture studies suggest that GLP-2R activation functions via divergent signaling pathways, one involving a cAMP, PKA-dependent suppression of apoptosis and the other that mediates cell proliferation via ERK1/2 activation in the Ras/Raf/MAPK pathway.

To establish the physiological relevance of these cell culture studies, we recently characterized the intestinal activation and expression of these putative GLP-2R signaling pathways in neonatal piglets treated in vivo with GLP-2 (4). This work confirmed that GLP-2 dose dependently suppresses caspase-3 activity and activates PKB, GSK-3 phosphorylation, and Bcl-2 expression in close association with increased epithelial cell survival, especially at physiological circulating GLP-2 concentrations. However, despite the recent evidence from cell culture studies, there are no in vivo studies confirming whether GLP-2 activates intestinal ERK1/2 signaling and whether this is associated with changes in crypt cell proliferation. Thus the aim of the current study was to further establish the physiological relevance of these signaling pathways in vivo by quantifying their temporal activation in piglets treated with GLP-2 for 1, 4, and 48 h. We were especially interested in the early activation of these two apparently divergent pathways and their association with intestinal epithelial cell survival and proliferation because previous evidence showed that GLP-2 maximally increases small intestinal blood flow within 30 min after infusion in neonatal piglets (14, 15, 40).

MATERIALS AND METHODS

Animals, surgery, and study design. The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services publication no. (National Institutes of Health; NIH) 85–23, revised 1985, Office of Science and Health Reports, NIH, Bethesda, MD]. Two-day-old, crossbred piglets were obtained from the Texas Department of Criminal Justice (Huntsville, TX), transported to the animal facility at the Children’s Nutrition Research Center (Houston, TX), and immediately placed in heated cages (25 °C) until surgery the following day. The piglets (3 days old) were surgically catheterized under isoflurane general anesthesia, and Silastic catheters were inserted in the jugular vein and carotid artery as previously described (4, 6). Catheters were secured in a jacket that was attached to a tether, which allowed free movement and secure administration of TPN to the piglets in their cages. Pre- and postoperatively on each day, piglets received enrofloxacin (2.5 mg/kg body wt, Sigma Aldrich, St. Louis, MO) 1 h before the pigs were killed to estimate crypt cell proliferation (see below). In addition, a bolus dose of [13C]phenylalanine (1.5 mmol/kg phenylalanine containing 0.15 mmol/kg [13C6]phenylalanine; Cambridge Isotope Laboratories, Andover, MA) was given 30 min before pigs were killed to measure the rate of tissue protein synthesis (4). Piglets were killed with a venous injection of pentobarbital sodium (50 mg/kg body wt) and sodium phenytoin (5 mg/kg body wt, Benadrylsia-D; Schering-Plough Animal Health, Kenilworth, NJ). The abdomen was opened, and the whole small intestine was excised and quickly flushed with ice-cold saline, weighed, and divided in parts of equal length, designated as jejunum and ileum. Samples of jejunum were placed in formalin for morphological and BrdU analysis. Samples of intestinal tissues were frozen in liquid nitrogen and used for subsequent measurements.

Morphometry, cell proliferation, and apoptosis. Morphometry analysis of intestinal mucosal tissue was performed on formalin-fixed, hematoxylin- and eosin-stained sections as described previously (29). In vivo crypt cell proliferation was measured as described previously (4, 29). BrdU-labeled cells were detected by immunohistochemistry and expressed as a percentage of total nuclei per crypt observed in ~15–20 well-oriented crypt sections from 2–3 tissue sections from each animal. Measurements of apoptosis were based on cell morphology observed in ×400 images by a single, trained observer that was blinded of the treatments, as described previously (29). Apoptotic cells were expressed as a percentage of the total epithelial cell numbers in the villus and crypt compartment of the same section; in this study, we report the number of ~1,000–1,500 total epithelial cells counted from 2–3 tissue sections/animal. Tissue samples were assayed for DNA content as previously described (29).

In vivo protein synthesis and mass spectrometry. Samples of jejunum tissue were homogenized and deproteinized with 2 M perchloric acid, and the perchloric acid-soluble (tissue free pool) and acid-insoluble (protein-bound pool) fractions were subjected to mass spectrometric analysis similar to that described previously (4, 29). The acid-insoluble fraction was hydrolyzed with 6 N HCl for 24 h before gas chromatography-mass spectrometry (GC-MS) analysis. The isotopic enrichment of [U-13C]phenylalanine (M + 6 isotopomer) in the two tissue pools was determined by GC-MS analysis of the n-propyl ester heptafluorobutyramide derivative using methane-negative chemical ionization. The analyses were performed with a 5890 series II gas chromatograph linked to a model 5973B (Hewlett-Packard, Palo Alto, CA) quadrupole mass spectrometer. The isotopic enrichment of phenylalanine was determined by monitoring ions at a mass-to-charge ratio of 383 to 389. Protein synthesis was calculated as described previously as the fractional synthesis rate (FSR, %/day),

\[
FSR = \left( \frac{IE_{\text{bound}}}{IE_{\text{free}}} \right) \times \left( \frac{1,440}{t} \right) \times 100
\]

where IE_{\text{bound}} and IE_{\text{free}} are the isotopic enrichments (mol% excess) of [13C6]phenylalanine of the perchloric acid-insoluble (protein-bound) and perchloric acid-soluble (tissue-free) pool, respectively, \( t \) is the time of labeling (in min), and 1,440 is the number of minutes in...
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a day. Tissue samples were assayed for protein using the bicinchoninic acid method (Pierce, Rockford, IL).

Western blotting analysis. All antibodies used for Western blotting and immunohistochemistry are listed in Table 1. For Western blots, frozen intestinal tissue samples (100 mg) were homogenized in 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, 5 mg/l phenylmethylsulfonyl fluoride, 5 mg/l aprotinin, 5 mg/l chymostatin, and 5 mg/l pepstatin. The homogenate was then sonicated and centrifuged at 12,000 g for 15 min at 4°C. The protein concentration of all extracts was determined as described previously (4) and diluted precisely such that equal amounts (30–120 μg) of total protein were loaded in each lane and then separated on a 9–15% denatured SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in the Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaOH, pH 7.4). Membranes were incubated with a primary antibody (see Table 1) diluted in the 5% nonfat milk in TBS + 0.1% Tween 20. Membranes were incubated with a secondary antibody (goat anti-rabbit IgG-horseradish peroxidase, or goat anti-mouse IgG-horseradish peroxidase; 1:5,000; Santa Cruz Biotech), and the bands were detected as described below. In most cases, the membranes were then stripped (Restore Western Blot Stripping Buffer; Pierce) at 37°C for 15 min, washed with TBS + 0.1% Tween 20 three times, and reprobed with a primary antibody to α-tubulin (mouse 1:2,000, Sigma-Aldrich) and then secondary antibodies diluted in 5% milk + TBS with TBS + 0.1% Tween 20. The molecular masses of specific proteins are as follows: pro-caspase-3 (34 kDa), active caspase-3 (19 kDa), Bcl-2 (29 kDa), XIAP (57 kDa), PKB-Ser473 and PKB-Thr308 were measured by Western blotting after immunoprecipitation with anti-PKB agarose beads. Extracts were combined with 4 μg of anti-PKB, PH Domain, agarose (mouse monoclonal IgG; Upstate, Charlotteville, VA) and incubated overnight at 4°C. Agarose beads were centrifuged at 12,000 revolutions/min, washed three times with ice-cold 1 × PBS, resuspended in 60 μl 2 × Laemmli sample buffer, and boiled for 5 min. Ten microliters of immunoprecipitation product (~150 μg of total protein) were separated on a 9% denatured SDS-PAGE and transferred to nitrocellulose membranes. Phospho-cAMP response element-binding protein (CREB) and CREB was measured in a nuclear extract after homogenization as described above and centrifuged at 12,000 g for 15 min at 4°C; the pellet was resuspended with the above HEPES buffer, sonicated, and treated as described above. Membranes were blocked with 5% nonfat milk in TBS. Membranes were incubated with primary and secondary antibodies diluted in TBS + 0.1% Tween 20. The signal intensity of specific phosphoproteins on each membrane was quantified as described below. The membranes were then stripped (Restore Western Blot Stripping Buffer; Pierce) at 37°C for 15 min, washed with TBS + 0.1% Tween 20 three times, and reprobed with the appropriate primary and secondary antibodies for the unphosphorylated proteins diluted in 5% milk + TBS with TBS + 0.1% Tween 20. The molecular masses of specific proteins are as follows: PKA (40 kDa), c-Fos (62 kDa), CREB (43 kDa), PKB (60 kDa), GSK-3 (51/46 kDa), ERK1/2 (42 and 44 kDa), and RSK (90 kDa). All Western blots were allowed to react with horseradish peroxidase substrate (ECL-plus; Amersham Biosciences, Piscataway, NJ) and then exposed to X-ray film for 30–300 s, and the image was scanned and quantified by ImageQuant 5.0 software (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). All Western blots were run with six pigs from each treatment group and used for statistical analysis, and treatment means and SEs are shown as bar graphs. We expressed the abundances of specific target proteins relative to that of tubulin measured after stripping and reprobing membranes. These values were then expressed as a percentage of the TPN control group in Figs. 1–10. To quantify the phosphorylated forms of specific proteins, we expressed the calculated densitometric band intensities of the phosphorylated form relative to that of the total protein; the latter was determined after stripping and reprobing the membranes. These values were then expressed as a percentage of the TPN control group in Figs. 1–10. Western blots of pooled samples from six pigs in each treatment also are shown in Figs. 1–10 for the respective proteins.

### Table 1. List of antibodies used for Western blotting and immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPKA-Thr197</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Biosource International</td>
</tr>
<tr>
<td>PKA</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>pPKB-Ser473</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>pPKB-Thr308</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>PKB</td>
<td>Mouse monoclonal</td>
<td>1:1,000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>pGSK-3-Ser17/19</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Mouse monoclonal</td>
<td>1:1,000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>ERK1/2-Thr185/Tyr204</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Rabbit polyclonal</td>
<td>1:2,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>p90RSK-Ser380</td>
<td>Rabbit polyclonal</td>
<td>1:2,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>RSK</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>CREB</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>CREB</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>XIAP</td>
<td>Mouse monoclonal</td>
<td>1:1,000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>cIAP-2</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Goat polyclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Active caspase-3</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>

p, Phospho; GSK, glycogen synthase kinase; ERK, extracellular signal-regulated kinase; CREB, cAMP response element-binding protein; XIAP, X-linked inhibitor of apoptosis; cIAP-2, cellular inhibitor of apoptosis; Bcl-2, B-cell lymphoma-2; RSK, p90 ribosomal S6 kinase.
Table 2. Temporal effect of GLP-2 infusion on intestinal mass and protein and DNA content in TPN-fed neonatal piglets

<table>
<thead>
<tr>
<th>GLP-2 Treatment, h</th>
<th>TPN-0</th>
<th>1</th>
<th>4</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass, g/kg</td>
<td>10.2±0.5</td>
<td>10.3±0.3</td>
<td>10.6±0.3</td>
<td>13.6±0.7*</td>
</tr>
<tr>
<td>Protein, mg/kg</td>
<td>449±35</td>
<td>438±29</td>
<td>497±38</td>
<td>602±47*</td>
</tr>
<tr>
<td>DNA, mg/kg</td>
<td>46.2±2.6</td>
<td>46.8±2.0</td>
<td>50.1±2.6</td>
<td>57.8±3.6*</td>
</tr>
<tr>
<td>Villus height, μm</td>
<td>340±29</td>
<td>370±46</td>
<td>430±34</td>
<td>630±82*</td>
</tr>
<tr>
<td>Villus area, μm²</td>
<td>24,244±1,908</td>
<td>29,855±4263</td>
<td>35,604±3359</td>
<td>51,306±7,585*</td>
</tr>
<tr>
<td>Crypt depth, μm</td>
<td>199±10</td>
<td>146±10</td>
<td>147±12</td>
<td>149±8</td>
</tr>
<tr>
<td>Muscularis, μm²</td>
<td>148±13</td>
<td>140±5</td>
<td>131±10</td>
<td>153±8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–8 pigs/group. GLP, glucagon-like peptide; TPN, total parenteral nutrition. *Mean different from control (0.0 GLP-2) based on Tukey’s means comparison (P < 0.05).

Immunohistochemistry. Frozen intestines were cut into 10-μm sections and fixed with 4% paraformaldehyde on ice for 10 min. Sections were washed two times with 1× PBS at room temperature for 10 min each, permeabilized for 5 min at room temperature in 5% (vol/vol) Triton X-100 in PBS, and then washed with 1× PBS for 10 min at room temperature. Sections were blocked with 10% normal goat/donkey serum in PBS for 10 min at 37°C and then incubated with the following primary antibodies at 4°C overnight: active caspase-3 (rabbit polyclonal antibody against human caspase-3, H-277, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and c-Fos (goat polyclonal antibody against human c-Fos, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and c-Fos (goat polyclonal antibody against human c-Fos, 1:100; Santa Cruz Biotechnology). Sections were washed two times for 10 min each, permeablized for 5 min at room temperature in 5% (vol/vol) Triton X-100 in PBS, and then washed with 1× PBS-Tween 20 for 10 min at room temperature. Sections were blocked with 10% normal goat/donkey serum in PBS for 10 min at 37°C and then incubated with the following primary antibodies at 4°C overnight: active caspase-3 (rabbit polyclonal antibody against human caspase-3, H-277, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and c-Fos (goat polyclonal antibody against human c-Fos, 1:100; Santa Cruz Biotechnology).

Sections were washed two times for 10 min at room temperature with 1× PBS-Tween 20, incubated with secondary antibody goat anti-rabbit Alexa Fluor 488 or donkey anti-goat Alexa Fluor 633 (Invitrogen, Carlsbad, CA) for 30 min at 37°C, and washed two times with PBS + 0.1% Tween 20 for 10 min at room temperature. Sections were then incubated with TOPRO-3 with a 1:1,000 dilution for 5 min at room temperature and washed three times with PBS 5 min each at room temperature. Cover slides were placed over the sections, covered with crystal mount, and baked at 65°C for 40 min until dry. Slides were visualized via laser confocal microscopy (Olympus Fluoview Confocal Microscope; Olympus America, Melville, NY). Specimens were scanned under similar laser settings and photomultiplier tube voltages to allow for direct comparisons of staining intensity among treatments.

**Statistical analysis.** Data for the four treatment groups were analyzed using Minitab statistical software (Minitab, State College, PA). Data were first analyzed by one-way ANOVA with time of GLP-2 treatment as a main effect, followed by a Tukey’s means comparison test. Mean comparisons were done specifically to test for statistical differences between the control group and the three GLP-2-treated groups. Data also were analyzed using multiple-regression analysis with time of GLP-2 treatment as a main effect. Results are expressed as means ± SE, and a P value < 0.05 was considered statistically significant.

**RESULTS**

The body weight of piglets in all four treatment groups was not significantly different; the mean final body weight among all groups was 3.25 ± 0.08 kg. Results shown in Table 2 indicate that GLP-2 infusion for either 1 or 4 h did not significantly affect any endpoints of intestinal growth or morphology. However, pigs infused with GLP-2 for 48 h had...
significantly higher intestinal mass, protein and DNA content, and villus height and area compared with TPN control pigs. There were no differences in crypt depth or muscularis thickness among the four groups. Rates of total mucosal protein synthesis, crypt cell proliferation, and apoptosis in crypt and villus epithelial cells were measured to explain the changes in intestinal protein and DNA content. The fractional protein synthesis rate was unchanged after only 1 or 4 h but was significantly higher than the TPN control after 48 h GLP-2 infusion (Fig. 1). Similarly, the mean rate of villus cell apoptosis was lower and crypt cell proliferation was higher only after 48 h GLP-2 infusion but not at 1 or 4 h (Fig. 2). Based on regression analysis, however, there was a significant linear effect of time of GLP-2 treatment for both villus cell apoptosis and crypt cell proliferation.

We measured the phosphorylation of Thr<sup>197</sup> on the catalytic subunit of PKA as an upstream indicator of cellular cAMP induction by GLP-2 treatment and the abundance of the nuclear transcription factor CREB. Results shown in Fig. 3 indicate that phosphorylation of PKA-Thr<sup>197</sup> was significantly increased (2-fold) above control after 1 h of GLP-2 infusion but was reduced to statistically nonsignificant levels at 4 h (1.4-fold) or 48 h (1.3-fold). The abundance of PKA protein was slightly reduced after 1 and 4 h of GLP-2 infusion. Results in Fig. 3 show that phosphorylated CREB at Ser<sup>133</sup> abundance was significantly higher at 1 and 4 h after GLP-2 treatment, but not at 48 h; CREB protein abundance was increased at 1-, 4-, and 48-h time points. Figure 4 shows the protein abundance of the immediate early gene, c-Fos, which is a known downstream target of both PKA and MAPK signaling pathways. c-Fos expression was significantly higher at 1 and 4 h after GLP-2 treatment, but not at 48 h. Figure 4 shows the immunohistochemical staining of c-Fos localized to villus epithelial cells, especially 1 and 4 h after the start of GLP-2 infusion.

We next examined the phosphorylation of signals that have been shown to be downstream targets of PKA, namely PKB and GSK-3. Results in Fig. 6 show that phosphorylation of PKB at Thr<sup>308</sup> was increased at 1, 4, and 48 h, whereas Ser<sup>473</sup> was increased at 4 and 48 h after GLP-2 treatment. GSK-3 phosphorylation at Ser<sup>21</sup>/Ser<sup>9</sup> was also increased at 1, 4, and 48 h after GLP-2 infusion.
48 h after GLP-2 treatment, suggesting a suppression of GSK-3 activity. The abundances of PKB and GSK-3 protein were not affected by GLP-2 treatment at any time point compared with control.

We measured the activation of signaling intermediates in the MAPK kinase pathway, specifically ERK1/2 and p90RSK. Results in Fig. 7 show that ERK1/2 (p42/44 MAPK) phosphorylated on Thr202 and Tyr204 was significantly increased at 1, 4, and 48 h after GLP-2 treatment; the abundance of total ERK1/2 protein was unchanged by GLP-2. A downstream nuclear target of ERK1/2 is p90RSK, which was modestly, but significantly, decreased after 1 h but upregulated after 48 h of GLP-2 treatment based on protein abundance and relative phosphorylation at Ser380 (Fig. 8).

We measured the abundance of several proteins shown to be involved in execution of cell death, namely caspase-3, and inhibition of caspase-3-mediated cell death, including Bcl-2, XIAP, and cIAP-2. Shown in Fig. 9 are results indicating that GLP-2 rapidly suppressed caspase-3 activation as early as 1 h. The ratio of active to pro-caspase-3 was significantly lower at 1, 4, and 48 h after GLP-2 treatment. Conversely, the abundance of Bcl-2 protein was rapidly increased by GLP-2 treatment at all time points (1, 4, and 48 h). Two proteins shown to specifically inhibit caspase-3 were also coordinately increased by GLP-2 treatment, where XIAP was higher after 1 and 4 h and cIAP-2 was higher after 4 and 48 h. Figure 10 shows localization of active caspase-3 to villus epithelial cells in both control and 48-h GLP-2-treated piglets.

FIG. 5. Localization of c-Fos immunostaining in intestinal epithelial cells in jejunal sections from TPN-fed piglets infused with saline (A and B) or 420 pmol·kg⁻¹·h⁻¹ human GLP-2 for 1 (C and D) or 4 (E and F) h. Frozen tissue sections were incubated with anti-c-Fos goat antibody and detected with secondary donkey anti-goat antibody conjugated with Alexafluor 633 (green staining). Sections were counterstained with TOPRO-3 antibody to label cell nuclei (red staining). A, C, and E: merged images of c-Fos (green) and cell nuclear (red) staining. B, D, and F: images of only c-Fos staining (green). Details of immunostaining are described in MATERIALS AND METHODS.

DISCUSSION

The main aim of this study was to characterize in vivo the temporal activation of intracellular signaling pathways that have been suggested in cell culture studies to be involved with GLP-2-mediated induction of cell survival and proliferation. We previously showed that chronic GLP-2 infusion dose dependently reverses TPN-induced mucosal atrophy and that this is associated with increased epithelial cell survival, suppression of caspase-3 activity, and activation of PKB, GSK-3 phosphorylation, and Bcl-2 expression. However, it is unknown how rapidly these cell signaling events are activated or how quickly the TPN-induced mucosal atrophy is reversed after GLP-2 treatment in vivo. We hypothesized that GLP-2 infusion in TPN-fed piglets would lead to rapid activation of cell signaling based on evidence that intestinal blood flow is significantly increased within 15 min of infusion (14, 40). Our results indicate that GLP-2 infusion rapidly (1–4 h) induced intestinal PKA phosphorylation and putative downstream signals associated with increased cell survival, including PKB/ GSK-3/Bcl-2. In addition, we found that GLP-2 induced a rapid (1-h) and robust increase in ERK1/2 phosphorylation and protein abundance and latent activation (48 h) of a downstream p90RSK. In addition, the acute (1–4 h) activation of these signaling pathways preceded the appearance trophic cellular morphological events, marked by apoptosis and proliferation of intestinal epithelial cells.
Our previous piglet studies demonstrated that chronic treatment (7 days) with therapeutic doses of GLP-2 prevents the mucosal atrophy associated with TPN, and this translates into improved intestinal function (4, 5, 10). Recent studies show that GLP-2 rapidly activates small intestinal blood flow, whereas the transition from enteral to parenteral nutrition leads to reduced intestinal blood flow, which precedes the onset of mucosal atrophy (14, 29, 40). The current results suggest that, within 48 h of treatment, GLP-2 significantly increased most

Fig. 6. Temporal induction by GLP-2 of intestinal protein kinase B (PKB) and glycogen synthase kinase (GSK)-3 phosphorylation in TPN-fed piglets infused with saline or 420 pmol kg\(^{-1}\) h\(^{-1}\) human GLP-2 for 1, 4, or 48 h. Top: results of phospho-PKB-Ser\(^{323}\) and PKB-Thr\(^{389}\), phospho-GSK-3-Ser\(^{321}\), and tubulin abundance from Western blot of pooled samples. Bottom: quantitative results of phospho-PKB-Ser\(^{323}\), PKB-Thr\(^{389}\), and phospho-GSK-3-Ser\(^{321}\) abundance expressed relative to PKB and GSK-3 protein, adjusted for tubulin abundance, and expressed relative to TPN-0 control. There were no significant effects of GLP-2 treatment on the protein abundance of PKB or GSK-3. Results are means ± SE for \(n = 6\) pigs/group. Differences between control and specific GLP-2-treated groups based on Tukey’s means comparison, *\(P < 0.05\).

Fig. 7. Temporal induction by GLP-2 of intestinal extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in TPN-fed piglets infused with saline or 420 pmol kg\(^{-1}\) h\(^{-1}\) human GLP-2 for 1, 4, or 48 h. Top: results of phospho-ERK1/2 and total ERK1/2 from Western blot of pooled samples. Bottom: quantitative results of phospho-ERK1/2 abundance expressed relative to ERK1/2 protein. There were no significant effects of GLP-2 treatment on the protein abundance of ERK1/2 protein. Results are means ± SE for \(n = 6\) pigs/group. Differences between control and specific GLP-2-treated groups based on Tukey’s means comparison, *\(P < 0.05\).

Fig. 8. Temporal induction by GLP-2 of intestinal p90\(^{RSK}\) phosphorylation and protein abundance in TPN-fed piglets infused with saline or 420 pmol kg\(^{-1}\) h\(^{-1}\) human GLP-2 for 1, 4, or 48 h. Top: results of phospho-p90\(^{RSK}\)-Ser\(^{380}\) and RSK protein from Western blot of pooled samples. Bottom: quantitative results of phospho-p90\(^{RSK}\)-Ser\(^{380}\} (filled bars) and RSK protein (hatched bars). Results are means ± SE for \(n = 6\) pigs/group. Differences between control and specific GLP-2-treated groups based on Tukey’s means comparison, *\(P < 0.05\).
endpoints of mucosal growth, including tissue mass and villus height. It is possible that GLP-2 induced mucosal growth in <48 h; however, we suggest that this likely occurs no sooner than 24–48 h based on evidence that the TPN-induced mucosal atrophy requires 24 h (29). In addition, the proportional increase in intestinal tissue mass values after 48 h were 30%, whereas the increases observed previously (4) after 7 days of GLP-2 treatment were 50%. However, the proportional increase in intestinal villus height after 48 h was slightly greater than that observed after 7 days of GLP-2 treatment. However, crypt depth was unchanged after 48 h of GLP-2 in this study but was shown previously to be increased after a chronic 7-day treatment. The changes in intestinal tissue mass and morphology were paralleled by changes in epithelial cell kinetics and mucosal protein synthesis. Villus cell apoptosis was decreased, whereas crypt cell proliferation and protein synthesis were increased significantly, but only after 48 h of GLP-2 treatment. These findings suggest that the GLP-2-induced increases in mucosal morphology are initially (24–48 h) manifested as altered villus elongation and that crypt expansion occurs later. The GLP-2-mediated villus elongation appears to be mediated by both increased epithelial cell survival and crypt cell proliferation.

The induction of mucosal epithelial cell growth by GLP-2 occurs via activation of the GLP-2R, which is localized in various cell types within the intestinal mucosa, including enteric neurons, subepithelial myofibroblasts, and enteroendocrine cells (1, 14, 33, 47). The nature of the paracrine mechanisms that translate the signal from GLP-2R-expressing cells to other target cells, namely enterocytes and vascular smooth muscle cells, is unknown. However, evidence from studies in cell culture and the inherent molecular structure of the glucagon family receptors indicate that an early intracellular event in GLP-2R activation is the association with cellular G proteins, stimulation of adenylate cyclase, and production of cAMP (25, 27, 45, 46, 48). These cell culture studies have shown that GLP-2 increases cellular survival via a cAMP- and PKA-dependent signaling mechanism. Upon cAMP binding to the regulatory subunits of PKA, the catalytic subunits are activated by phosphorylation, translocate to the nucleus, and phosphorylate key nuclear transcription factors, especially CREB (26); phosphorylation at Thr197 has been shown critical for PKA subunit catalytic activity (44). Another key transcription factor involved in cellular survival and proliferation is the immediate early gene, c-Fos, which forms heterodimers with c-Jun to form activating protein 1 complex (16). Previous studies found

Fig. 9. Temporal effects of GLP-2 infusion are markers of apoptosis. Shown is Western blot analysis of active caspase-3 (band on bottom, 19 kDa) to pro-caspase-3 (band on top, 34 kDa) in the pooled jejunal tissue extracts from TPN-fed piglets infused with saline or 420 pmol·kg⁻¹·h⁻¹ human GLP-2 for 1, 4, or 48 h. Percentage values show the calculated ratio of active caspase-3 to pro-caspase-3 expressed relative to TPN-0 control group, where results are means ± SE for n = 6 pigs/group (top left). Also shown is Western blot analysis of Bcl-2 (top right), XIAP (bottom left), and cIAP-2 (bottom right) protein expression in pooled jejunal extracts from the 4 treatment groups. All 4 blots show relative abundance of tubulin in each pooled sample, and the relative quantitative expression of all specific proteins has been corrected for tubulin abundance. Differences between control and specific GLP-2-treated groups based on Tukey’s means comparison, *P < 0.05.
that GLP-2 rapidly stimulates c-Fos gene and protein expression in vivo in mouse intestine and cultured cells expressing the GLP-2R (1, 42, 48). The current results indicate that GLP-2 rapidly (within 1 h) and transiently upregulates not only c-Fos in villus epithelial cells but PKA and CREB (Ser133) phosphorylation in the intestinal mucosa. This evidence supports cell culture studies showing the GLP-2-mediated cell survival is cAMP and PKA dependent.

The cellular signaling mechanism whereby GLP-2 activates PKA, CREB, and c-Fos remains a key unanswered issue. Our results are consistent with previous studies suggesting an intercellular signaling process, since c-Fos and caspase-3 activation was largely localized in mucosal enterocytes, yet the GLP-2R expression in piglets is confined to enteric neurons and enteroendocrine cells. The rapidity of GLP-2-induced blood flow and cell signaling has led to speculation and evidence that paracrine neural mediators are involved in the vasoactive response, particularly nitric oxide (1, 15). Our recent neonatal piglet studies show that GLP-2 upregulates the intestinal Ser1177 phosphorylation and abundance of endothe-
lial nitric oxide synthase within 1 h (14) and that endothelial nitric oxide synthase colocalizes with the GLP-2R in submucosal enteric neurons. Moreover, extracellular nitric oxide has been shown to induce neuronal cell survival via activation of CREB phosphorylation and Bcl-2 expression (8, 9). However, the GLP-2 induction of mucosal epithelial cell survival and proliferation may also involve additional paracrine signals, such as insulin-like growth factor I and keratinocyte growth factor, from other GLP-2R-expressing cell types, including enteroendocrine cells and subepithelial myofibroblasts (13, 33, 47).

We previously showed that putative downstream signals of cAMP/PKA activation, namely PKB and GSK-3, are modulated in the neonatal intestine by GLP-2 treatment in vivo (4). We show here that GLP-2 rapidly (1 h) activates PKB phosphorylation and that phosphorylation and inhibition of GSK-3 subsequently occurs at 4 h. Downstream targets of GSK-3 phosphorylation, especially Bcl-2, have been linked to mitochondrial permeability and cell survival (23). Thus GLP-2-induced inhibition of GSK-3 could lead to reduced Bcl-2 phosphorylation and enhanced prosurvival function. Consistent with our previous report (4), we show here that GLP-2 increased intestinal Bcl-2 protein abundance and that this occurred rapidly and paralleled the suppression of caspase-3 activity, which was localized to villus epithelial cells. In addition to Bcl-2, the current results suggest that GLP-2 upregulates two members of the inhibitors of apoptosis (IAP) family, XIAP and ciAP-2. Both XIAP and ciAP-2 are expressed in intestinal epithelial cells, and these factors inhibit apoptosis via specific binding and inhibition of caspases-3, -7, and -9 (28). Agonist-induced activation of intestinal epithelial cAMP signaling inhibits apoptosis via upregulation of ciAP-2 and phosphorylation of CREB at Ser133, consistent with the events observed in the GLP-2-stimulated intestine (31, 32). The finding of IAP induction suggests a novel alternative mechanism, besides Bcl-2, to explain the GLP-2-induced suppression of caspases-3 activity and epithelial cell apoptosis in the neonatal intestine.

In addition to the promotion of cell survival, GLP-2-induced cAMP/PKA pathway activation has also been linked to cell proliferation in cell culture studies (19, 43, 48), although a recent report questions the link between cAMP and the cell proliferative response (35). What has emerged from studies in Caco-2 cells and recent evidence in GLP-2R-transfected HeLa cells is that GLP-2 may induce cell proliferation via divergent kinase signaling pathways in addition to PKA, particularly the MAPK or ERK pathway (18, 24). Considerable evidence supports the existence of cAMP-induced proliferation of epithelial cells through multiple intracellular signaling pathways, including PKA, ERK, PKB, and PI 3-kinase (7, 30, 36, 41). We show here that GLP-2 induced a rapid (1 h) and robust (2- to 3-fold) increase in the phosphorylation and abundance of ERK1/2 in the neonatal intestine. Moreover, we found an early decline (1 h) and latent increase (48 h) in the phosphorylation and abundance of p90RSK, a downstream ERK1/2 target, after GLP-2 treatment. Whether the GLP-2-induced stimulation of crypt cell proliferation is mediated by ERK1/2 in vivo warrants further study, but the pattern of p90RSK activation after 48 h coincided with the increase in cell proliferation. We explored whether the activation of ERK1/2 was associated with activation of other upstream signals other than PKA, namely B-Raf, but were unable to detect the protein with Western blotting.

In summary, our results suggest that GLP-2 rapidly upregulates multiple signaling pathways that are known to mediate cellular survival and proliferation. We show upregulation of PKA/PKB/GSK-3 phosphorylation believed to function upstream of cell survival targets that inhibit caspases-3 activity, such as Bcl-2 and IAPs. In addition, we found a rapid activation of ERK1/2 and subsequent p90RSK that may be involved with cell proliferation. These studies suggest that the signaling pathways demonstrated in cell culture studies are also activated in the neonatal intestine in vivo. We also show that the rapid activation of intracellular signaling pathways after GLP-2 treatment coincides with the early induction of mucosal vasodilation and blood flow observed previously in neonatal piglets. The rapid activation of downstream intracellular signals and apoptotic targets, such as c-Fos and caspases-3, was localized to mucosal epithelial cells and preceded the cellular kinetic (apoptosis, proliferation, protein synthesis) and morphological (villus elongation) changes associated with mucosal growth in response to GLP-2 treatment. These studies highlight the need for integrative in vivo physiological approaches at the intestinal tissue and whole animal level to establish whether the cellular signals identified here are essential for GLP-2-mediated intestinal epithelial growth (20–22).

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REFERENCES

GLP-2-INDUCED CELL SIGNALING IN THE NEONATAL INTESTINE


