The gastrin-releasing peptide analog bombesin preserves exocrine and endocrine pancreas morphology and function during parenteral nutrition

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bombesin; gut hormones; pancreas; parenteral nutrition; adjuvant therapy

PARENTERAL NUTRITION (PN) prevents malnutrition in patients unable to receive enteral feeding. In these patients, elemental nutrients are administered directly into the vascular system, bypassing the gastrointestinal tract. The lack of enteral nutrition precludes the chemical and physical stimuli that normally promote gastrointestinal hormone release and enteric nervous system signaling, resulting in the atrophy and dysfunction of digestive organs. For instance, PN causes hypomotility of the intestine and gallbladder and structural and functional alterations in the small intestine, including mucosal atrophy, loss of barrier function, increased permeability, and reduced immune function (6, 17, 28, 32, 42, 43, 45, 48). PN also induces histological changes and altered function of the pancreas, an organ with essential roles in digestive enzyme secretion (the exocrine pancreas) and hormonal regulation of glucose homeostasis (the endocrine pancreas, or islets of Langerhans) (7, 23). While PN is necessary in patients otherwise unable to eat, the collective compromise of exocrine and endocrine organ functions following PN has the potential to alter morbidity and mortality risk (38, 44, 63).

Previous studies examined the effect of exogenous agents, including the gut hormone cholecystokinin (CCK) and intravenous fatty acids and amino acids, on pancreatic exocrine and endocrine function following PN; however, these studies showed incomplete protection from the structural and functional pancreatic abnormalities following PN (5, 55, 60). During enteral feeding, digestive organs, including the intestine and pancreas, are stimulated by effenter vagal nerve fibers that broadly excite organ function through the release of gastrin-releasing peptide (GRP) (1, 40, 50). Bombesin (BBS) is composed of the amino-terminal peptide sequence of GRP and binds to the same high-affinity receptors as GRP, BBS receptors 1 and 2 (BB1 and BB2). Our previous work demonstrated that BBS stimulates aspects of intestinal and salivary gland immune function during PN, maintaining proper gut function (13, 18, 27, 46, 62). BBS receptors are also highly expressed in the splanchnic organs, including the pancreatic exocrine cells (31). In addition, several previous studies have indicated that BBS and/or GRP are insulin secretagogues (25, 33, 36, 54). Together, these findings suggest that BBS might improve pancreatic exocrine and endocrine function in PN.

In this study we utilized a mouse model of PN to investigate the effects of exogenous BBS on protection from structural and/or functional abnormalities of the exocrine and endocrine pancreas during PN. We hypothesized that the addition of BBS to PN would stimulate pancreatic weight and protein, amylase, and lipase levels and prevent alterations of beta-cell function compared with animals receiving PN alone. We confirm both
of these findings in vivo, by monitoring circulating markers, and in vitro, by utilizing isolated organs and tissue samples. Interestingly, we show a augmentation mechanism of BBS on pancreatic endocrine function that is different from expected: an indirect effect on the pancreatic islet via the gut-pancreas axis. Our work demonstrates that an exogenous adjuvant might protect exocrine and endocrine pancreas morphology and function during PN, which may have important clinical applications for patients with uncontrolled hyperglycemia or pancreatic insufficiency.

MATERIALS AND METHODS

Antibodies, chemicals, and reagents. Polyclonal guinea pig anti-insulin was obtained from Dako (product no. A056401-2); rabbit monoclonal anti-Ki-67 from Cell Signaling Technology (product no. 91299S); FITC-coupled anti-rabbit antibody (product no. 4156B), Cy3-coupled anti-guinea pig antibody (product no. PA1-28727), and Infinity glucose oxidase reagent (product no. TR15221) from Thermo Scientific; VECTASHIELD mounting medium with DAPI (catalog no. H-1200) and VECTASTAIN guinea pig IgG ABC kit (catalog no. PK-4007) from Vector Laboratories; Permount mounting medium (catalog no. S70104), aprotinin (catalog no. BP250310), and glucose oxidase (catalog no. TR15221) from Fisher Scientific; active glucagon-like peptide-1 (GLP-1) ELISA (catalog no. EGPL-35K), rat/mouse insulin ELISA (catalog no. EZRM-13K), and dipetidyl peptidase IV (DPP-4) inhibitor (catalog no. DPP4) from Millipore; glucagon enzyme immunoassay (ELA; catalog no. RAB0202), gastrin ELA (catalog no. RAB0200), BBS (catalog no. B4272), collagenase for islet isolation (catalog no. C7657), Ficol (catalog no. F9378), exendin-4 (Ex4; catalog no. EZ1744), and streptavidin-peroxidase polymer (catalog no. S2438) from Sigma; CCK RIA from Alpco Diagnostics (catalog no. 13-CCKHU-R100); somatostatin ELA from Phoenix Pharmaceuticals (catalog no. EK-060-03); monoclonal insulin/proinsulin (catalog no. 10R-I136a) and biotin-conjugated (catalog no. 61R-I136BBT) antibodies from Fitzgerald; Hanks’ balanced salt solution (catalog no. 14065-056) and RPMI 1640 medium (catalog nos. 11879-020 and 22400-105) from Gibco; RNeasy Mini Kit (catalog no. 74106) and RNase-free DNase set (catalog no. 79254) from Qiagen; high-capacity cDNA reverse transcription kit (catalog no. 4368813) from Applied Biosystems; and FastStart Universal SYBR Green Master mix (catalog no. 4919941001) from Roche.

Animals. All protocols were approved by the Institutional Animal Care and Use Committees of the University of Wisconsin-Madison, the William S. Middleton Memorial Veterans Hospital, and the University of Chicago. All facilities were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Male Institute of Cancer Research (ICR) mice were purchased from Harlan (Indianapolis, IN) and housed, five per microisolator-top cage, in a temperature- and humidity-controlled environment with a 12:12-h light-dark cycle. Animals were given a standard pelleted mouse chow (Rodent Diet 5001, LabDiet, PMI Nutrition International, St. Louis, MO) and water ad libitum for 1 wk prior to initiation of the study protocol.

Male ICR mice (7–8 wk old) were anesthetized and randomized to Chow (Sham), parenteral nutrition (PN), or PN + BBS (15 or 45 μg/mouse−1·day−1) for 5 days. Animals were anesthetized by intramuscular injection and weighed, and a silicone-rubber catheter was placed in the vena cava through the right external jugular vein (0.012-inch ID/0.025-inch OD: Helix Medical, Carpinteria, CA). The catheter was tunneled subcutaneously at the midpoint of the tail. The animals were housed individually in metabolic cages with wire floors to prevent coprophagia and bedding ingestion and partially immobilized by tail restraint to protect the catheter during infusion. This technique has proven to be an acceptable method of nutritional support and does not produce physical or biochemical evidence of distress (51).

The catheterized mice were connected to infusion pumps delivering saline (0.9%) at 4 ml/day and received ad libium choc and water during the 48-h recovery period. After 48 h, the Chow group continued to receive 0.9% saline at 4 ml/day and ad libium choc and water. The PN group received PN solution at 4 ml/day (day 1), 7 ml/day (day 2), and 10 ml/day (days 3–5), as a graded infusion period allowed the mice to adapt to the glucose and fluid loads. The PN solution contained 6.0% amino acids and 35.6% dextrose, electrolytes, and multivitamins (1,440 kcal/l total) and had a nonprotein calories-to-nitrogen ratio of 128:1 (Table 1). These values were calculated to meet the nutrient requirements of 25- to 30-g mice. BBS was injected directly into the catheter in equal doses three times a day to reach the appropriate total daily dose of 15 or 45 μg/mouse−1·day−1.

For tissue analysis by histology, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg) and exsanguinated via left axillary artery transection after 5 days of feeding (7 days postcatheterization). Blood was collected for measurement of plasma or serum insulin, glucose, glucagon, somatostatin, gastrin, and GLP-1 levels. For measurement of active GLP-1, tubes contained 10 μl/ml DPP-4 inhibitor to preserve the active GLP-1 form. The pancreas was removed, washed in saline, blotted dry, and weighed. One section of the pancreas was frozen in liquid N2 and stored at −80°C until processing; the other section was fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4°C for histology.

For islet isolation, mice were prepared and euthanized using a previously established protocol (39). Prior to isolation, blood plasma was collected with heparinized capillary tubes by retro-orbital bleeding and treated with 10 μl/ml DPP-4 inhibitor, 10 μl/ml 0.5 M EDTA, and 20 μl/ml aprotinin. Immunohistochemical and immunofluorescent analyses. The fixed pancreas tissue sections were processed (Tissue-Tek VIP, Sakura Finetek, Torrance, CA) and embedded in paraffin. For quantification of exocrine pancreas morphology, the embedded tissues were cut (5 μm thick) and placed on adhesive-coated (white aminosilane) slides (Newcomer Supply, Madison, WI), deparaffinized, rehydrated through graded ethanol washes (twice in 100% ethanol, twice in 95% ethanol, and once in 70% ethanol, for 2 min each), and rinsed in 

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount, per liter</th>
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<tbody>
<tr>
<td>Dextrose</td>
<td>356.0 g</td>
</tr>
<tr>
<td>Amino acids (Clinisol)</td>
<td>60.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>32.0 meq</td>
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<tr>
<td>Sodium phosphate</td>
<td>36 mmol</td>
</tr>
<tr>
<td>Potassium chloride</td>
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<tr>
<td>Calcium gluconate</td>
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<tr>
<td>Potassium acetate</td>
<td>44.0 meq</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
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</tr>
<tr>
<td>Manganese</td>
<td>0.8 mg</td>
</tr>
<tr>
<td>Copper</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>Zinc</td>
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<tr>
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<tr>
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<td>Vitamin D3</td>
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<td>Thiamine</td>
<td>6 mg</td>
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<td>Riboflavin</td>
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<tr>
<td>Pyridoxine HCl</td>
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<tr>
<td>Niacinamide</td>
<td>40 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>600 μg</td>
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<tr>
<td>Biotin</td>
<td>60 μg</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>5 μg</td>
</tr>
<tr>
<td>Vitamin E (dl-a-tocopheryl acetate)</td>
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<tr>
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distilled water. To determine changes in pancreatic acinar cells, slides were stained with hematoxylin and eosin (6).

For quantification of endocrine pancreas morphology, five pancreases were randomly chosen from each group (Chow, PN, or PN + BBS) and sectioned at 5 μm onto positively charged microscope slides. Insulin immunoreactivity was quantified essentially as previously described (34) using a biotin-avidin signal-amplifying system (VECTASTAIN ABC kit). An Axio-Plan automated pan-and-stitch microscope (Zeiss, Oberkochen, Germany) was used to image entire pancreas sections. Two sections separated by ≥200 μm (the average diameter of a pancreatic islet) were analyzed for each mouse pancreas. The Threshold macro in ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify total pancreas area, while islet area was quantified by tracing around insulin-positive regions in ImageJ. Beta-cell fractional area was calculated as the percent insulin-positive area per total pancreas area. The beta-cell mass was calculated by multiplying the pancreas weights by the beta-cell fractional area.

Beta-cell replication was quantified by immunofluorescence of serial sections from the pancreases described above by measuring the number of Ki-67-positive insulin-positive cells as a percentage of total beta cells per islet, essentially as described previously (34). To quantify the total number of beta cells in each islet, an image-based nucleus counter (ITCN) plugin for ImageJ 64-bit was used to circle the region of interest (i.e., the insulin-positive area).

Paraformaldehyde-fixed enteroids were processed by hand and embedded in paraffin. Sections (5 μm thick) of enteroids were rehydrated in xylene and ethanol and stained with hematoxylin and eosin for gross histology or 2) boiled in sodium citrate (10 mM, pH 6) for 10 min, blocked with protein-serum block (Dako) for 1 h at room temperature, and stained with rabbit polyclonal anti-chromogranin A antibody (ab15160, Abcam, Cambridge, MA) for 1 h at room temperature. Slides were washed in Tris-buffered saline-Tween 20 and stained with donkey anti-rabbit IgG (Alexa Fluor 555 nm, Invitrogen) for 30 min at room temperature. Nuclei were counterstained with ProLong Gold with DAPI (catalog no. P36935, Molecular Probes, Eugene, OR).

Measurement of pancreatic protein, DNA amylase activity, and lipase. Approximately 70 mg of frozen pancreas from each animal were homogenized in ice-cold buffer (1×) and resuspended in 1× volume of ice-cold buffer (1×) to bring the final concentration to 1 mg/ml. Then, the homogenate was centrifuged at 13,000 g for 10 min. The supernatant was aliquoted and stored at −80°C until further use. The protein concentration of each sample was measured using a BCA protein assay (Thermo Scientific). The total DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen). For lipase activity measurement, the assays were performed using a modified version of the method of Wijk and Holman (36). For amylase activity measurement, we used the kit from BioAssay Systems that is based on the chromogenic substrate 4-MUP (4-methylumbelliferyl-α-D-1,4-glucopyranoside). The enzymatic activity was expressed as nanomoles of product hydrolyzed per minute per milligram of protein. For proglucagon mRNA expression measurement, each group of islets was grown as described by Sato et al. (49). Briefly, small intestine was gently removed from mice and opened longitudinally. Tissues were rinsed in ice-cold PBS to remove contents. A glass slide was used to gently remove the villi, and the mucosa was chopped into 1- to 2-mm pieces with a scalpel and collected into 10 ml of ice-cold PBS. Pieces were agitated and rinsed using a serum-coated serological pipette. This wash cycle was repeated until the supernatant became clear.

Intestinal pieces were resuspended in 25 ml of ice-cold 2.5 mM EDTA-PBS and rotated at 4°C for 30 min. Once tissue settled, EDTA-PBS was removed and replaced with 10 ml of Advanced DMEM/F12 (ADF) medium (Life Technologies). After it was pipetted up and down three times, the supernatant was discarded and fresh ADF medium was added. This collection step was repeated three more times. Cells were centrifuged at 300 g at 4°C for 5 min, resuspended in 10 ml of ADF medium, and passed through a 70-μm cell strainer to remove debris. Cells were centrifuged at 300 g at 4°C for 3 min and resuspended in complete ADF medium. The complete ADF medium was made by supplementation with Glutamax (Life Technologies), HEPES buffer (Life Technologies), penicillin-streptomycin (Life Technologies), N2 supplement (Life Technologies), B27 Supplement Minus Vitamin A (Life Technologies), murine EGF (50 ng/ml; Life Technologies), noggin (100 ng/ml; Peprotech), jagged-1 (1 μg/ml; Anaspec), Y27632 (10 mM; Cayman Scientific), and R-spondin-1 (500 ng/ml; Peprotech). Isolated crypts were combined with Matrigel (BD Biosciences, San Jose, CA) in a 1:3 ratio, plated onto a prewarmed collagen-coated tissue culture plate, and allowed to solidify for 1 h in 5% CO2 at 37°C. Two milliliters of complete ADF medium were added to each well, fresh growth factors were added every 2 days, and complete ADF medium was changed every 4 days. After 21 days of culture, wells of enteroids (35 per well) were stained with 1 or 100 nM BBS (B-4272, Sigma, St. Louis, MO) for 3 or 24 h and compared with controls (untreated). Wells of enteroids were harvested in paraformaldehyde for histology or TRizol reagent for RNA isolation (see below).

Measurement of intestinal enteroid proglucagon expression. Isolated enteroids were collected and immediately placed in TRizol reagent (Ambion, Austin, TX), vortexed for 2 min, and mixed with chloroform. After centrifugation (10,000 rpm for 15 min), the top aqueous phase was mixed with 100% isopropanol to precipitate RNA. Samples were centrifuged (10,000 rpm for 10 min), and pellets were washed (75% ethanol), dried, and reconstituted in nuclease-free water. RNA purity was validated through UV-visible spectrophotometry (NanoDrop Lite, Thermo Scientific, Wilmington, DE). Then 1 μg of total RNA was reverse-transcribed to cDNA using the Transcriptor first-strand cDNA synthesis kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The relative quantitation of gene expression was performed using the LightCycler 480 real-time PCR system (Roche). Proglucagon gene expression data were presented relative to glyceraldehyde 3-phosphate dehydrogenase. Primer sequences are provided in Table 2.

Measurement of intestine and islet gene expression. Isolated islets were washed once with PBS and snap-frozen in RLT buffer from the Qiagen RNEasy kit. RNA was processed according to the RNEasy protocol, including DNase treatment to ensure RNA purity. After RNA quantification, cDNA synthesis was completed according to the protocol provided by Applied Biosystems. Relative gene quantification to β-actin was performed using SYBR reagents in a StepOnePlus real-time PCR system (Applied Biosystems). Primer sequences are provided in Table 2.

Mouse islet glucose-stimulated insulin secretion and insulin ELISA. Immediately following isolation, islets were transferred to RPMI 1640 medium containing 11.1 mmol/l glucose, 10% FBS, and penicillin-streptomycin. The glucose-stimulated insulin secretion (GSIS) assay was performed using a high-throughput multiple-well plate technique, as described elsewhere (56). Briefly, islets were individually incubated in a tissue culture-treated 96-well V-bottom plate, allowed to adhere for 48 h, and incubated with Krebs-Ringer bicarbonate buffer at 1.7 mM glucose (preincubation) followed by a stimulatory 16.7 mM glucose incubation with indicated compounds.
Table 2. Mouse primer sequences used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Protein (gene)</th>
<th>Primer</th>
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<tbody>
<tr>
<td><strong>Actb</strong></td>
<td>TCTGCGGATGGATGATGCT-<code>GCTTCTGGATGATGCTC-</code>CTGCAATGGCTGATT-`AGGAA</td>
</tr>
<tr>
<td><strong>Nndr</strong></td>
<td>ACCCCTTGGTTATCATGAC-<code>GCTCCTTGATCTGCTTGGT-</code>GTCTTGTCTGCTTGGT-`GGG</td>
</tr>
<tr>
<td><strong>Gpr</strong></td>
<td>GTGACCAAGACGATGCAA-<code>AGATCTGATGAGGGCTTCCC-</code>GGGAGATGGATGATGCTC-`AGT</td>
</tr>
<tr>
<td><strong>Gapdh</strong></td>
<td>GGCAAAATTGCAGGGGAGGAA-<code>ACTTTTGCTCAGAGTGGTAAGGATGAGATGGATGAGATGGTGA-</code>AGT</td>
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<tr>
<td><strong>Glpir</strong></td>
<td>GTATCGGGTCTTACCTTT-<code>AGATCTGATGAGGGCTTCCC-</code>GGGAGATGGATGATGCTC-`AGT</td>
</tr>
<tr>
<td><strong>Preproglucagon</strong></td>
<td>TCTCAACTGGGTGATT-<code>AGATCTGATGAGGGCTTCCC-</code>GGGAGATGGATGATGCTC-`AGT</td>
</tr>
</tbody>
</table>

BB1 and BB2, bombesin receptors 1 and 2; Glpir, glucagon-like peptide 1 receptor.

decreases in pancreas weight and pancreas-to-body weight ratio were observed in the PN group compared with the Chow group, suggesting pancreatic atrophy, a known consequence of PN. The addition of BBS to PN significantly increased both of these measurements: pancreas weight was normalized compared with the Chow group, and pancreas weight-to-body weight ratio was significantly elevated. While there were no significant differences in pancreas protein concentration between any of the groups, PN reduced the mean protein content compared with chow, while the addition of BBS to PN significantly increased pancreatic protein content compared with chow or PN. In addition, DNA concentration was significantly decreased in PN with BBS treatment compared with PN, while DNA content was essentially unchanged among all the groups. Finally, the protein-to-DNA ratio was significantly elevated in PN with BBS treatment compared with chow or PN alone. Taken together, increased protein content with an unchanged DNA content and a significantly augmented protein-to-DNA ratio suggest that changes in pancreas organ size in PN with BBS are due to cell hypertrophy, and not cell hyperplasia.

Acinar cell morphology is augmented in PN with BBS treatment. Both gastrin and BBS have been linked to exocrine pancreas enzyme secretion and/or cell hyperplasia (35). To directly analyze the effect of PN and PN + BBS on pancreas cell morphology, we conducted a microscopic analysis. Acinar cell secretory granules strongly bind the hematologic stain eosin, which is visible under fluorescent excitation in a concentration-dependent manner. Hematoxylin-eosin-stained sections were analyzed by transmitted light and fluorescence microscopy for changes in acinar cell morphology (Fig. 2). PN treatment clearly alters acinar cell morphology and granulation, whether viewed by transmitted light (Fig. 2, A and B) or eosin autofluorescence (Fig. 2, D and E). Acinar cells were smaller in the PN than the Chow group and bound less eosin. In contrast, BBS treatment in PN clearly stimulated acinar cell hypertrophy compared with PN alone, whether viewed by transmitted light (Fig. 2, B and C) or eosin autofluorescence (Fig. 2, E and F). The size and granulation of BBS-treated exocrine cells appeared to exceed that of the Chow group as well. Overall, these results support the presence of exocrine pancreas atrophy and degranulation in PN and confirm that the increased protein-to-DNA ratio shown in Table 1 is a result of acinar cell hypertrophy in PN with BBS treatment.

RESULTS

Gastrin secretion is elevated in PN with BBS treatment. BBS is a gastrin-releasing hormone homolog. Gastrin is released from the parietal cells of the stomach in response to physiological enteral feeding stimuli. These stimuli are lost in PN. Therefore, we aimed to determine the daily dose of BBS that would elevate gastrin secretion in PN. Mice were subjected to logical enteral feeding stimuli. These stimuli are lost in PN.

Statistical analyses. Data are expressed as means ± SE. Statistical significance was determined using one- or two-way ANOVA with Tukey’s or Dunnett’s multiple-comparisons test post hoc or Student’s t-test as appropriate (depending on whether only preplanned comparisons were made). Differences were considered to be statistically significant at P < 0.05. Statistical calculations were performed with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

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Table 3. Body weight and pancreas weight, protein, and DNA of Chow, PN, and PN + BBS mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow (n = 8)</th>
<th>PN (n = 9)</th>
<th>PN + BBS (n = 8)</th>
</tr>
</thead>
</table>
| Body wt, g                 | 36.3 ± 1.0         | 31.4 ± 0.7  
(P < 0.01, 1.7 to 8.1) | 32.4 ± 1.0  
(P < 0.05 vs. Chow, 0.7 to 7.2; NS vs. PN) |
| Pancreatic wt, mg           | 456.4 ± 15.0       | 315.3 ± 16.5 
(P < 0.0001, 85.1 to 197.1) | 512.8 ± 15.9  
(NS vs. Chow; P < 0.0001 vs. PN, −253.5 to −141.5) |
| Pancreas wt/body wt, mg/g   | 12.6 ± 0.5         | 10.1 ± 0.6  
(P < 0.01, 0.6 to 4.4) | 15.7 ± 0.5  
(P < 0.05 vs. Chow, −5.1 to −1.1; P < 0.0001 vs. PN, −7.5 to −3.7) |
| Protein concentration, mg/g tissue | 185.2 ± 8.8      | 61.5 ± 3.3  
(P < 0.05, 1.7 to 43.9) | 112.0 ± 9.7  
(P < 0.05 vs. Chow, −49.4 to −6.0; P < 0.0001 vs. PN, −71.6 to −29.4) |
| Protein content, mg/pancreas | 84.3 ± 3.1         | 61.5 ± 3.3  
(NS) | 216.9 ± 14.7  
(NS vs. Chow or PN) |
| DNA concentration, mg/g tissue | 2.6 ± 0.2         | 3.0 ± 0.2  
(NS) | 2.0 ± 0.1  
(NS vs. Chow; P < 0.01 vs. PN, 0.4 to 1.6) |
| DNA content, mg/pancreas    | 1.19 ± 0.1         | 0.96 ± 0.1  
(NS) | 1.06 ± 0.1  
(NS vs. Chow or PN) |
| Protein-to-DNA ratio        | 74.5 ± 6.8         | 61.4 ± 4.3  
(NS) | 110.0 ± 8.4  
(P < 0.01 vs. Chow, −59.2 to −11.8; P < 0.0001 vs. PN, −71.6 to −25.6) |

Values are means ± SE. PN, total parenteral nutrition; BBS, bombesin; NS, not significant. Data were compared by 1-way ANOVA with Tukey’s multiple-comparisons test post hoc; P < 0.05 was considered statistically significant. 95% confidence intervals of the difference between the means are shown as a range after the P value.

Exocrine pancreas products are enhanced in PN with BBS treatment. The levels of pancreatic amylase and lipase are representative of exocrine pancreas secretory capacity. The addition of BBS during PN significantly increased amylase levels compared with PN alone (5.22 ± 0.58 and 9.15 ± 0.96 for PN and PN + BBS, respectively, n = 8/group, P = 0.0052; Fig. 3A). Similarly, the addition of BBS during PN significantly increased lipase levels (177.1 ± 17.59 and 371.6 ± 58.86 for PN and PN + BBS, respectively, n = 8/group, P = 0.0156; Fig. 3B). The approximate doubling in amylase and lipase content (normalized to DNA) in PN with BBS treatment directly correlated with the increased protein content (normalized to DNA) in Table 1 (61.4 ± 4.3 and 110.0 ± 8.4 for PN and PN + BBS, respectively, n = 8–9/group, P = < 0.0001). These results suggest that BBS enhances digestive enzyme secretory capacity from the pancreas during PN through a general increase in exocrine cell size and, thus, protein content.

Endocrine pancreas morphology is not altered in PN with BBS treatment. Morphology of the rodent endocrine pancreas can be quantified by insulin immunohistochemistry of organ sections, including beta-cell fractional area, beta-cell mass, and islet size. This is because rodent islets are primarily (≥80%) insulin-containing beta cells, forming the core of the islet. Pancreas sections immunostained with an anti-insulin antibody and counterstained with hematoxylin were imaged using an automated pan-and-stitch microscope (Fig. 4, A–C). While there were no statistically significant differences in any of the islet morphological analyses, the insulin-positive area trended toward being increased in the PN compared with the PN + BBS group (Fig. 4D), a trend that was reversed when the
fractional areas were normalized to the total pancreas weight (Fig. 4E). These results suggest that any differences in insulin-positive pancreas area were due to the atrophy of the exocrine pancreas, and not an overall increase in beta-cell number or size. This can be observed in Fig. 4B, where the exocrine pancreas (stained blue) appears condensed and devoid of ductal structure compared with chow-fed (Fig. 4A) and BBS-treated (Fig. 4C) pancreas sections. Finally, comparison of the islet area, which is representative of islet size, shows no apparent differences between the PN and PN + BBS groups. Overall, these results are not surprising because of the short duration of treatment and the slow replication and turnover rates of the beta cell. However the beta-cell replication rate as indicated by Ki-67 activity was significantly enhanced in PN with BBS treatment (Fig. 5), suggesting that, over the long term, beta-cell mass and islet area may be altered in PN with BBS treatment.

**BBS treatment during PN preserves pancreatic endocrine function and incretin responsiveness.** BBS and the related GRP have been suggested to be insulin secretagogues (25, 33, 36, 54); therefore, we aimed to determine whether insulin secretion was elevated in PN with BBS treatment. Circulating insulin levels were approximately twofold higher in the PN + BBS than the PN group (0.84 ± 0.06 vs. 1.71 ± 0.37 ng/ml, n = 20–25/group, P = 0.032; Fig. 6A). Paradoxically, blood glucose concentrations did not differ between the PN and PN + BBS groups (171.2 ± 13.3 and 181.1 ± 15.96 mg/dl for PN and PN + BBS, respectively; Fig. 6B). As BBS has also been shown to stimulate secretion of glucagon, the counterregulatory hormone to insulin, we also tested our samples for circulating glucagon levels. Interestingly, the mean glucagon levels were threefold higher in the PN + BBS than the PN group, although because of variation among the samples, this difference was not statistically significant (Fig. 6C). Most of this variation was due to samples between 100 and 1,000 mg/dl glucagon (the 2 most-concentrated standards on the curve), which were only found in the Chow and PN + BBS groups (1 of 9 for Chow, 0 of 9 for PN, and 4 of 8 for PN + BBS, P = 0.03 for PN vs. PN + BBS). Taken together, our results suggest that BBS in PN elevates both insulin and glucagon secretion from the pancreatic islets, resulting in a blood glucose level that is unchanged. Regulation of blood glucose did not appear to be due to any influence of BBS on somatostatin secretion, as there were no statistical differences between somatostatin levels in PN and PN + BBS samples (Fig. 6D).

To directly quantify whether PN, with or without BBS treatment, impacts beta-cell function, we isolated pancreatic islets from each treatment group and then subjected them to in vitro GSIS assays (Fig. 7A). No differences were observed among groups in terms of the responsiveness of the islets to low (1.7 mM) glucose. Islets from PN mice trended toward having a lower mean responsiveness to stimulatory (16.7 mM) glucose than islets from Chow or PN + BBS mice, but this effect was not statistically significant. Strikingly, however, there was a significant decrement of the response of PN islets to the GLP-1 receptor (GLP1R) agonist Ex4 that was completely reversed when the mice were infused with BBS (Fig. 7A). As a control, no differences in insulin content per islet were found among the groups (636.1 ± 78.61, 749.1 ± 124.1, and 553.7 ± 82.95 (SE) ng/islet for Chow, PN, and PN + BBS, respectively, n = 7–8/group).

To begin to elucidate the mechanism by which BBS treatment preserved pancreatic islet function, we tested the impact of BBS directly on the islet using a concentration near the known affinities of BBS for its high-affinity receptors, BB1 and BB2 (7–9 nM) (2). This concentration (1 nM) was also in the range expected in plasma during the in vivo infusions in a 25- to 35-g mouse [peak concentration 264–370 nM, half-life ~3 min (9), repeated 3 times per day]. Interestingly, even though BBS has been suggested to promote insulin secretion, addition of exogenous BBS directly to islets had no stimulatory effect on GSIS in any of the groups (Fig. 7A).

As an alternative mechanism by which BBS treatment might promote islet incretin responsiveness in PN, islets were isolated from each of the groups and used to generate cDNA for quantitative RT-PCR analysis. There existed a linear relationship between relative GLP1R expression in the islet samples and the response of the islets to Ex4 (Fig. 7B). Individual data points for each group cluster together, with Chow and PN + BBS essentially overlaying each other (Fig. 7B) and PN islet clustering primarily in the low GLP1R/low Ex4 response (Fig. 7B). Next, we looked at the relative expression of BB1 and BB2 in islet cDNA samples. BB1 expression was significantly decreased in PN and PN + BBS vs. Chow. There were no differences in mean BB1 expression between PN and PN + BBS islets (Fig. 7C). Similarly, BB2 expression was reduced in islets from PN vs. Chow mice, and there were no significant differences between the expression in PN and PN + BBS islets (Fig. 7D). Overall, the results shown in Fig. 7 support the increased functionality of BBS-treated PN islets, being indica-
rectly mediated, in particular, by their responsiveness to a hormone involved in the enteroendocrine cell-pancreas axis, GLP-1.

BBS treatment during PN directly stimulates the intestinal cells to promote GLP-1 precursor production. We previously showed that BBS targets the intestine and has a profound effect on gut immunity and function (18, 27, 62). Thus we aimed to determine if BBS also stimulated gut production of GLP-1 and, if so, whether this effect was direct. Besides our previous work, evidence for a direct effect of BBS on the gut during PN is suggested by expression of its high-affinity receptors, BB1 and BB2, in intestine samples from each of the groups (Fig. 8). BB1 expression was below the cutoff for signal-to-noise ratio in all eight of the PN samples, but in 5 of 10 samples...
supplemented with BBS, BB1 expression was restored to the mean found in chow-fed mice (Fig. 8A). On the other hand, BB2 expression was not affected by PN or BBS treatment and remained detectable in all samples (Fig. 8B). Thus, in contrast to the pancreatic islet, BBS supplementation preserves or augments its own receptor expression in the intestine, suggesting preservation of signaling. To confirm the effect of BBS treatment on GLP-1 secretion, we performed an ELISA for active GLP-1 in the plasma of mice from each of the three groups. Our results reveal a significantly enhanced circulating
active GLP-1 level in PN + BBS mice compared with PN mice (Fig. 8C), perhaps through a direct, receptor-mediated mechanism.

To confirm that BBS treatment can stimulate GLP-1 production in intestinal cells, we cultured isolated intestinal enteroids, which fully differentiate into specialized epithelial cells, including the GLP-1-producing L cells. Representative bright-field and hematoxylin-eosin-stained sections are shown in Fig. 9, A and B, respectively. We confirmed the presence of enteroendocrine cells by performing immunofluorescence microscopy for chromogranin A (ChgA), an enteroendocrine cell marker (Fig. 9C, magenta). Finally, we lysed enteroid cultures at 3 and 24 h after BBS treatment to perform quantitative RT-PCR analysis of expression of preproglucagon, the mRNA precursor for GLP-1. At 3 h, addition of 1 or 100 nM BBS significantly elevated preproglucagon mRNA expression compared with control by 1.7- and 1.8-fold, respectively (Fig. 9D), suggesting that preproglucagon is an immediate early gene downstream of BBS receptor signaling. The stimulation of preproglucagon expression was still significantly elevated by 1.4-fold at 24 h with 1 nM BBS stimulation (Fig. 9D), while the elevated (by 1.2-fold) expression with 100 nM BBS was not statistically significant (Fig. 9D).

Taken together, our data support the positive impact of BBS infusion in PN on the exocrine pancreas, endocrine pancreas, and intestinal cell morphology and function, but through very different mechanisms. The effects of BBS on the exocrine pancreas and intestine may be direct, while the effects on the endocrine pancreas appear to be indirect and mediated by the enteroendocrine cell-pancreas axis, exemplified by L-cell-derived GLP-1.

**DISCUSSION**

Digestive organ atrophy and dysfunction contribute to the morbidity and mortality associated with PN. In this study we aimed to determine whether BBS treatment could preserve and/or augment the exocrine and endocrine pancreas during PN in mice. Atrophy of the exocrine pancreas can delay or limit the return to normal enteral feeding (i.e., refeeding syndrome) following prolonged PN (58). Furthermore, hyperglycemia occurs in many PN patients, regardless of diabetic status, and patients requiring PN following trauma, surgery, or critical illness are at even greater risk of hyperglycemia (8). Additionally, the growing proportion of diabetes in the general population only exacerbates the difficulty of adequate glycemic control in PN patients (10, 57). Thus, discovery of adjuvants to ameliorate aspects of pancreas exocrine atrophy and endocrine dysfunction during PN is of significant clinical interest.

Previous work demonstrated that PN without enteral feeding results in pancreatic atrophy, characterized by decreased organ weight, total protein content, and digestive enzyme content (6). Our study confirms these findings (Table 3). Intravenous adjuvants for pancreas acinar cells have been investigated with variable success. A protein-free diet induces pancreatic atrophy (15); however, oral and intravenous amino acids fail to protect the exocrine rat pancreas from atrophy and only partially restore digestive enzyme levels during PN (5, 22). Another adjuvant was the gut hormone CCK, which stimulates pancreatic digestive enzyme release (41). Addition of CCK to PN partially restored pancreatic organ weight and digestive enzyme content compared with PN alone (23, 60); however, CCK receptor-deficient mice revealed that CCK is not required for pancreatic growth following feeding, suggesting other neuroendocrine mediators (55). Interestingly, BBS was studied...
several decades ago as an adjuvant to zero-fat, liquid elemental diets administered enterally for 5 or 10 days (21). Evers and colleagues (21) reported that treatment with an enteral elemental diet plus BBS for 5 days caused an increase in pancreatic weight, RNA, and protein, suggesting hypertrophy, and treatment for 10 days caused an increase in DNA concentrations, suggesting hyperplasia. Our findings confirm that BBS during PN induces marked pancreatic exocrine cell hypertrophy, significantly augments digestive enzyme levels (Table 3, Figs. 2 and 3), and suggests that BBS could be a novel adjuvant to protect or augment pancreas exocrine morphology and function during PN.

We cannot confirm that the effects of BBS on the exocrine pancreas are not at least partially mediated by CCK, as BBS has been shown to promote CCK secretion (16). In our samples, circulating CCK was below the detection limit for the assay in all groups (data not shown), suggesting that, in the PN model and random-fed controls, CCK levels are quite low and BBS does not dramatically change them. It is possible, however, that changes in CCK secretion are transient and were missed by the timing of our sample collection. Furthermore, an increased unbound CCK tracer fraction upon CCK RIA in our highest-BBS-dose group (data not shown) is suggestive of higher, although still very low, circulating CCK levels.

In contrast to the exocrine pancreas, PN appears to have little effect on endocrine pancreas gross morphology (Fig. 4), at least following the 5-day experimental period in our study. In fact, any apparent differences in insulin-positive area in PN appear to be due to exocrine pancreas atrophy, rather than direct endocrine modulation. Despite the lack of endocrine pancreas morphological changes, PN increased beta-cell replication compared with chow, similar to previous reports of short-term glucose exposure upon islet and beta-cell replication (11). The addition of BBS to PN further elevated beta-cell replication rates (Fig. 5). Further work is necessary to determine whether this increased replication actually increases beta-cell mass. This could potentially aid in the treatment of diabetic PN patients, although differentiating such a protective effect from a neoplastic event is of critical importance.

The primary function of pancreatic beta cells is to release insulin in response to glucose. Previous work suggests that PN impairs endocrine pancreas function with reduced basal insulin levels and blunted glucose-induced insulin response (24). Since PN includes intravenous dextrose (glucose) as a carbohydrate source, bypassing the intestine, insulin-secreting beta cells are continually stressed to regulate the steady influx of blood glucose in the absence of signals, indicating enteral nutrient ingestion (7, 52, 53). In our work, circulating insulin was significantly augmented in PN with BBS (Fig. 6A), suggesting improved beta-cell function with BBS. Yet there were no differences in serum glucose between groups (Fig. 6B). An increased insulin-to-glucose ratio can be one indication of insulin resistance. However, a more likely explanation of our results is increased glucagon secretion following BBS, since BBS was demonstrated to stimulate insulin and glucagon secretion from canine pancreas (30). In healthy humans, BBS also promoted increased plasma insulin and glucagon (12). Correspondingly, our data suggest increased glucagon secretion with BBS during PN (Fig. 6C). Thus our phenotypic data are consistent with increased glucagon, as well as insulin, secretion with BBS during PN, and not insulin resistance. An interesting future study would include development of hyperglycemia models, with underlying pathology, and examination of the effect of BBS on islet function during PN in those animals.

To confirm that the in vivo increase in plasma insulin secretion is due to enhanced insulin secretion, we performed in vitro insulin secretion assays on islets isolated from Chow, PN, and PN + BBS mice (Fig. 7A). These experiments demonstrated similar baseline insulin release between groups in response to 1.7 and 16.7 mM glucose. However, after addition of the GLP-1 analog Ex4, islets from PN mice demonstrated a highly significant defect (~40%) in insulin secretion compared with Chow or PN + BBS mice (Fig. 7A). On the basis of earlier work utilizing beta-cell lines, isolated islets, and perfused pancreases (1, 25, 33, 36, 40, 54), we had presumed that BBS would elicit a direct stimulatory effect on islet insulin secretion. This was not the case, however; the addition of BBS did not stimulate insulin release in any of the groups (Fig. 7A). A true lack of an effect in the PN and PN + BBS groups is supported by significantly decreased expression of mRNA for the receptors through which BBS signals (Fig. 7, C and D), BB1 (Nmdr) and BB2 (Grpr). Expression of BB1 and BB2 in human islets is controversial, with some studies confirming (3) and some excluding (26) expression; thus, an indirect mechanism of BBS action on the islet helps preserve the translatability of our results to the human condition.

Our findings are, however, consistent with BBS promoting GLP-1 release from the gut in PN, which may stimulate beta-cell function through the islet GLP1R. Human studies demonstrate that the lack of enteral stimulation in PN patients reduces incretin release, including GLP-1 (7, 29). Our data support this finding and show a significantly elevated release of active GLP-1 into the circulation in PN with BBS treatment (Fig. 8C). Strikingly, islet GLP1R expression is significantly correlated with islet responsiveness to GLP-1 and appears to be enhanced in PN with BBS treatment (Fig. 7B), suggesting a receptor-feedback mechanism. A reciprocal phenomenon has been observed in type 2 diabetes, where the reduction in islet GLP1R contributes to blunted GLP-1-potentiated insulin secretion (61). In contrast to islets, intestinal BB1 and BB2 expression remained intact (Fig. 8, A and B). Therefore, we tested the hypothesis that BBS directly stimulates the enteroendocrine cells to produce GLP-1 by generating intestinal enteroids, isolating BBS effects to the intestinal epithelium (Fig. 9). Enteroids are superior to cell culture models, because they differentiate crypt pluripotent stem cells into enterocytes, Paneth cells, goblet cells, and enteroendocrine cells (49). Previous cell culture work demonstrated that BBS or GRP stimulates the production of glucagon-like insulinotropic peptide from cell lines (4, 20, 37). However, to our knowledge, our results are the first to demonstrate BBS stimulation of enteroendocrine cell products, specifically proglucagon, in epithelium derived from native intestine (Fig. 9).

Limitations of our study are as follows. 1) Gastrin has been shown to activate the L-cell CCK-B receptor to increase biosynthesis and secretion of GLP-1 (14). However, this potential caveat is alleviated by the direct stimulation of enteroendocrine cells with BBS shown in Fig. 8. 2) Islet cells have been shown to produce not only glucagon, but also GLP-1 (19); thus we cannot exclude a role for islet-derived GLP-1 in the effects of BBS in PN. 3) The pancreas produces gastrin, which has
been shown to stimulate islet differentiation and replication (59). An effect of pancreatic gastrin could explain the potentiation of beta-cell replication in PN with BBS (Fig. 5), but not in the isolated pancreatic islets (Fig. 7), as the exocrine pancreas was removed. Regardless of these caveats, an enhanced gut GLP-1-to-islet GLP1R signaling axis is present and active in PN + BBS mice, suggesting that this mechanism is, in part, responsible for the endocrine effect of BBS during PN.

In summary, the current data support the known effect of PN on pancreatic atrophy and reveal that addition of BBS to PN causes a dramatic hypertrophy of the pancreatic exocrine cells. Increased exocrine pancreas enzyme content has direct relevance to the ability of the pancreas to aid in digestion once enteral feeding is resumed. In addition, we demonstrated that BBS preserved pancreatic islet function, in particular, the responsiveness of the islets to GLP-1, a critical hormone for modulating proper insulin response to food intake. Our data are fully consistent with the idea that BBS directly promotes GLP-1 release from the intestine into the circulation, mimicking that aspect of enteral feeding. These data support a growing body of evidence that the addition of BBS can maintain intestinal, salivary gland, and, now, exocrine and endocrine pancreatic function during PN feeding, further supporting its use as a potential adjutant for PN patients.

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DISCLAIMERS

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AUTHOR CONTRIBUTIONS


REFERENCES


Li G, Wollheim CB, Pralong WF.

Lhoste E, Aprahamian M, Pousse A, Hoeltzel A, Stock-Damge C.


Lhoste E, Aprahamian M, Pousse A, Hoeltzel A, Stock-Damge C.


