Original Article

MOLECULAR METABOLISM

The endogenous preproglucagon system is not essential for gut growth homeostasis in mice

Pernille Wismann [1,*], Pernille Barkholt [1], Thomas Secher [1], Niels Vrang [1], Henrik B. Hansen [1], Palle Bekker Jeppesen [2], Laurie L. Baggio [3], Jacqueline A. Koehler [3], Daniel J. Drucker [3], Darleen A. Sandoval [4], Jacob Jelsing [1]

ABSTRACT

Objective: The prevalence of obesity and related co-morbidities is reaching pandemic proportions. Today, the most effective obesity treatments are glucagon-like peptide 1 (GLP-1) analogs and bariatric surgery. Interestingly, both intervention paradigms have been associated with adaptive growth responses in the gut; however, intestinotrophic mechanisms associated with or secondary to medical or surgical obesity therapies are poorly understood. Therefore, the objective of this study was to assess the local basal endogenous and pharmacological intestinotrophic effects of glucagon-like peptides and bariatric surgery in mice.

Methods: We used in situ hybridization to provide a detailed and comparative anatomical map of the local distribution of GLP-1 receptor (*Glp1r*), GLP-2 receptor (*Glp2r*), and preproglucagon (*Gcg*) mRNA expression throughout the mouse gastrointestinal tract. Gut development in GLP-1R-, GLP-2R-, or GCG-deficient mice was compared to their corresponding wild-type controls, and intestinotrophic effects of GLP-1 and GLP-2 analogs were assessed in wild-type mice. Lastly, gut volume was determined in a mouse model of vertical sleeve gastrectomy (VSG).

Results: Comparison of *Glp1r*, *Glp2r*, and *Gcg* mRNA expression indicated a widespread, but distinct, distribution of these three transcripts throughout all compartments of the mouse gastrointestinal tract. While mice null for *Glp1r* or *Gcg* showed normal intestinal morphology, *Glp2r*$^{-/-}$ mice exhibited a slight reduction in small intestinal mucosa volume. Pharmacological treatment with GLP-1 and GLP-2 analogs significantly increased gut volume. In contrast, VSG surgery had no effect on intestinal morphology.

Conclusion: The present study indicates that the endogenous preproglucagon system, exemplified by the entire GCG gene and the receptors for GLP-1 and GLP-2, does not play a major role in normal gut development in the mouse. Furthermore, elevation in local intestinal and circulating levels of GLP-1 and GLP-2 achieved after VSG has limited impact on intestinal morphometry. Hence, although exogenous treatment with GLP-1 and GLP-2 analogs enhances gut growth, the contributions of endogenously-secreted GLP-1 and GLP-2 to gut growth may be more modest and highly context-dependent.

Keywords GLP-1R; GLP-2R; GCG; Preproglucagon; Intestinal volume; Obesity; Diabetes; Bariatric surgery

1. INTRODUCTION

Obesity and type-2 diabetes (T2D) represent increasing health and socio-economic problems worldwide [1]. The currently most effective pharmacological treatments for obesity include peptides stimulating GLP-1 receptor (GLP-1R) function [2,3], while dual agonists for the GLP-1R and other mechanisms targeting anorexigenic receptors are in development [4]. Furthermore, bariatric surgery has become increasingly attractive providing a significant, rapid, and sustainable weight loss with several positive effects on related morbidities, including resolution of T2D. Although Roux-en-Y gastric bypass (RYGB) has historically been the standard bariatric surgery method, less invasive procedures, *e.g.* vertical sleeve gastrectomy (VSG), have comparable beneficial metabolic outcomes with reduced perioperative complications, which explains why VSG is now the fastest-growing weight loss surgery option for the treatment of obesity [5]. The underlying molecular mechanisms leading to these marked metabolic effects are not fully elucidated, but several lines of evidence support an important role for nutrient-stimulated gut hormones, such as GLP-1 and GLP-2 [6–8].

High circulating levels of GLP-1 and GLP-2 have been linked to development of gut hypertrophy following RYGB [9–12]. The rise in GLP-1 and GLP-2 could provide a positive feedforward mechanism rendering the enlarged intestine more predisposed towards glucose disposal [13,14] and the release of a plethora of gut hormones with additional metabolic implications. GLP-1 and GLP-2 are co-secreted from enteroendocrine L cells in the gut and released into the circulation following enzymatic cleavage of the common prohormone proglucagon [15] see reviews [16,17]. While GLP-1 is mainly known for its metabolic effects; i.e. the increase in pancreatic glucose-dependent insulin secretion (the incretin effect), regulation of glucose flux, inhibition of gastric emptying, and reduction of appetite [18,19], GLP-2 is primarily known for its direct

[1]Gubra Aps, Hørsholm Kongevej 11B, DK-2970 Hørsholm, Denmark [2]Rigshospitalet CA-2121, Blegdamsvej 9, DK-2100 Copenhagen, Denmark [3]Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G1X5, Canada [4]University of Michigan, 2800 Plymouth Road, Ann Arbor, MI, USA

*Corresponding author. E-mail: pwi@gubra.dk (P. Wismann).

Received March 30, 2017 • Revision received April 21, 2017 • Accepted April 24, 2017 • Available online 27 April 2017

http://dx.doi.org/10.1016/j.molmet.2017.04.007

actions on the gut. Accordingly, peripheral administration of GLP-2 exerts potent intestinotrophic effects by increasing mesenteric blood flow and stimulating epithelial proliferation and may constitute the molecular link between nutritional status and commensurate adaptation of mucosal absorptive surface area [9,20–23]. Furthermore, GLP-2 reduces gastric emptying and gastric secretion and exhibits anti-inflammatory properties in the intestinal mucosa [24–28]. A growth-regulating role of GLP-1 has also been reported [29], demonstrating that activity of GLP-1R controls mucosal expansion in both the small and large intestine. However, even though the actions of these peptides are well described, the anatomical distribution and functional implications of the endogenous proglucagon system for normal gut growth and development remains poorly understood.

Traditionally, the ileum and colon have been identified as the primary sites containing the majority of the proglucagon expressing L-cell populations in rat, pig, dog, primate, and man [30–34]. This general assumption focused greater attention on studies of proglucagon-related biology in the distal gut, with limited analysis of the full gastrointestinal tract. The GLP-1R is known to be expressed in several tissues, including brain, gastrointestinal tract, pancreatic islets, kidney, heart, and lung [35–38]. However, cellular localization of GLP-1R expression is confounded by the lack of validated, specific antibodies [39–41]. Similarly, GLP-2 receptor (GLP-2R) expression has been reported previously in gastrointestinal tract, mesenteric lymph nodes, fat, spleen, bladder, and hepatocytes, as well as in the central nervous system [24,42–45]. However, the exact local distribution of intestinal GLP-2Rs is disputed and thus remains unresolved [44–48]. Given the current limitations in our understanding of the localization of GLP-1R and GLP-2R expression in the gut, we aimed to provide a detailed map of *Glp1r*, *Glp2r*, and *Gcg* mRNA expression throughout the complete rostral–caudal axis of the mouse gastrointestinal tract. To gain further insight into the functional relevance of the endogenous GLP-1 and GLP-2 system on intestinal growth, we characterized intestinal volumes in $Glp1r^{-/-}$, $Glp2r^{-/-}$, and $Gcg^{-/-}$ mice in comparison to corresponding wild-type littermate controls. Furthermore, since bariatric surgery represents a valuable tool for studying the role of these peptide hormones in intestinal adaptation, we performed a detailed study of intestinal volume in a mouse model of VSG surgery.

2. MATERIALS AND METHODS

2.1. Animals

All animal experiments were approved by the Danish Committee for Animal Research under the personal license of Jacob Jelsing (2015-15-0201-00518) using internationally accepted principles for the use of laboratory animals. All animals were housed in a light-, temperature-, and humidity-controlled room (12-hour light:12-hour dark cycle, lights on/off at 4AM/4PM hour; 22 ± 1 °C; 50 ± 10% relative humidity) and offered domestic quality tap water. Mice bred in Toronto were cared for in accordance with animal protocols approved by the Animal Care Committee, Toronto Centre for Phenogenomics, Mt. Sinai Hospital.

2.2. Compounds

The GLP-1 analog liraglutide was acquired commercially (Hørsholm Pharmacy). Native GLP-1, native GLP-2, and the GLP-2 analog teduglutide were prepared by automated solid-phase peptide synthesis (SPPS) using the Fmoc/tBu strategy on pre-loaded PHB TentaGel resin (Rapp polymere GmbH, Tuebingen, Germany). The couplings were performed using Fmoc-Nα-protected amino acids, *N,N*-diisopropylcarbodiimide and ethyl cyanoglyoxylate-2-oxime (oxyma) in *N,N*-dimethylformamide (Iris Biotech GmbH, Marktredwitz, Germany) for 2 × 2 h. The N-deprotections were performed using 40% piperidine in *N*-methyl-2-pyrrolidione (Iris Biotech GmbH, Marktredwitz, Germany) for 3 min followed by 20% piperidine in *N*-methyl-2-pyrrolidione for 17 min. Finally, the peptide was simultaneously side-chain deprotected and released from the solid support by a TFA cocktail containing trifluoro acetic acid (TFA) (Iris Biotech GmbH, Marktredwitz, Germany), triethylsilane (Sigma–Aldrich, Brøndby, Denmark), and H_2O (95/2.5/2.5) as scavengers for 2 h. The peptide was precipitated by the addition of diethylether (Sigma–Aldrich, Brøndby, Denmark). The peptide was purified by RP-HPLC and identified by LC-MS. The final products were obtained with >95% purity.

2.3. Sub-chronic treatment in C57BL/6J mice

C57BL/6J mice (Janvier Labs, Saint Berthevin, Cedex, France), 8 weeks of age, were fed a regular chow diet (Altromin 1324, Brogaarden A/S, Denmark). Mice were randomized according to body weight into four individual study groups (n = 10 per group): Group 1: Vehicle (SC, BID), Group 2: liraglutide (0.2 mg/kg, SC, BID), Group 3: teduglutide (1 mg/kg, SC, BID), Group 4: liraglutide (0.2 mg/kg, SC, BID) + teduglutide (1 mg/kg, SC, BID). Compounds were dissolved in PBS buffer containing 3% mannitol and 0.6% L-His (pH 9.0), and dosing volume was 5 ml/kg. On day 8, animals were fasted for 4 h before being sacrificed during the light phase. The intestines were collected, and the length of the small and large intestine was measured. Intestines were cleaned by flushing with saline and finally the weight was measured. Intestines were placed in 10% natural buffered formalin until further processing. For description of mice treated with native GLP-1 and GLP-2, see supplementary information.

2.4. Histology and stereology

The gut was dissected into small and large intestine, and the lengths were measured. The intestine was sampled using systematic uniform random sampling (SURS) principles, providing a minimum of 8 systematically placed biopsies from both small and large intestine. All biopsies were embedded in blocks of paraffin enabling later identification of individual biopsies. Paraffin blocks were sectioned into 5 μm thick sections and stained with hematoxylin-eosin for subsequent stereology-based volume estimations. Stereological volume estimations were performed by point-counting on digitally scanned slides using the newCAST system (Visiopharm, Denmark) [49–51]. For studies involving double KO ($Glp1r^{-/-}$:$Glp2r^{-/-}$) mice or mice treated with native GLP-1 and GLP-2 peptides, weights of saline-flushed intestines were used (see supplementary information).

2.5. In situ hybridization (ISH)

Single-cell ISH was performed on paraffin-embedded intestinal tissue biopsies from two C57BL/6J mice using the RNAscope 2.5 HD – RED Assay (Advanced Cell Diagnostics) to visualize cellular mRNA using specific probes directed against selected genes. Slides with tissue biopsies were treated according to RNAscope 2.5 HD – RED Assay user manual. In brief, tissue sections were pretreated, including target retrieval, hydrogen peroxide treatment, and protease treatment. Then, the specific probe was hybridized to the mRNA target, and the signal was amplified and visualized using Fast Red substrate. A probe against bacterial *dapB* mRNA was used as negative control, whereas a mouse probe against *Ppib* was used as positive control. Custom-made specific probes against *Glp1r* (REF418851), *Glp2r* (REF447061), and *Gcg* (REF400601) mRNA were employed on sections covering the entire gastro-intestinal tract (glandular and non-glandular stomach, duodenum with Brunner's glands, caudal duodenum without Brunner's

