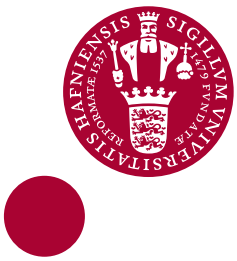

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Investigating Intestinal Glucagon after Roux-en-Y Gastric Bypass Surgery

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Context After Roux-en-Y gastric bypass (RYGB) surgery, postprandial plasma glucagon concentrations have been reported to increase. This occurs despite concomitant improved glucose tolerance and increases in circulating plasma concentrations of insulin and the glucagon-inhibiting hormone glucagon-like peptide 1 (GLP-1).
Objective Investigate whether RYGB-induced hyperglucagonemia may be derived from the gut.
Design and setting Sub-study of a prospective cross-sectional study at a university hospital in Copenhagen, Denmark.
Participants Morbidly obese individuals undergoing RYGB [n=8] with or without type 2 diabetes.
Interventions Three months before and after RYGB, participants underwent upper enteroscopy with gastrointestinal mucosal biopsy retrieval. Mixed meal tests were performed 1 week and 3 months before and after RYGB.
Main Outcome measures 29-amino acid glucagon concentrations in plasma and in mucosal gastrointestinal biopsies were assessed using mass spectrometry-validated immunoassays,

and a new monoclonal antibody reacting with immunoreactive glucagon was used for immunohistochemistry.
Results We observed increased postprandial plasma concentrations of glucagon after RYGB. Small intestinal expression of the glucagon gene increased after surgery. Glucagon was identified in the small intestinal biopsies obtained after, but not before RYGB. Immunohistochemically, mucosal biopsies from the small intestine harbored cells co-staining for GLP-1 and immuno-reactive glucagon.
Conclusions Increased concentrations of glucagon, estimated by two glucagon specific assays, were observed in small intestinal biopsies and postprandially in plasma after RYGB. The small intestine harbored cells immunohistochemically co-staining for GLP-1 and glucagon-like immunoreactivity after RYGB. These findings suggest that glucagon derived from small intestinal enteroendocrine L-cells may contribute to postprandial plasma concentrations of glucagon after RYGB.

After RYGB surgery in obese individuals, we observed increased glucagon concentrations in postprandial plasma samples and gut biopsies, which also harbored cells IHS co-stained for GLP-1 and glucagon.

Introduction

Roux-en-Y gastric bypass (RYGB) surgery has emerged as an effective treatment of obesity and type 2 diabetes[1]. The mechanisms underlying the antidiabetic effect of RYGB are complex and include increased insulin secretion stimulated by exaggerated postprandial secretion of the insulinotropic and glucagonostatic gut hormone, glucagon-like peptide 1 (GLP-1)[2]. However, the surgical procedure has also been associated with postprandial hyperglucagonemia in several studies[3–8]. Postprandial hyperglucagonemia after RYGB is surprising and counterintuitive given the well-known RYGB-induced increases in circulating levels of insulin and GLP-1, which both would be expected to inhibit glucagon secretion[9]. A similar postprandial hyperglucagonemia has previously been observed in totally pancreatectomized patients after an oral glucose load, but not after iv administered glucose[10]. We therefore speculated that excessive postprandial plasma glucagon concentrations after RYGB might be gastrointestinally derived. We hypothesized that the postprandial increments in plasma glucagon concentrations after RYGB might reflect a disturbed processing of the common precursor for GLP-1 and glucagon, proglucagon, which is produced in the intestinal enteroendocrine L cells[11]. To test this hypothesis, we collected gastric and small intestinal mucosa biopsies by upper enteroscopy and performed mixed meal tests (MMTs) before and after surgery in eight individuals undergoing RYGB. We used mass spectrometry-validated immunoassays for glucagon quantification in plasma and mucosal biopsies and a new C-terminal-specific monoclonal glucagon antibody for immunohistochemistry to elucidate whether the postprandial hyperglucagonemia observed after RYGB may reflect postoperative changes in proglucagon processing in the gastrointestinal mucosa.

Research design and methods

This study was a sub-study of a prospective observational study conducted in Denmark between December 2014 and December 2016. All participants gave written informed consent. The study was approved by the Municipal Ethical Committee of Copenhagen (reg. no. H-6-2014-047) performed in accordance with the Declaration of Helsinki II and registered at ClinicalTrials.gov (NCT03093298).

Study participants

Eight morbidly obese Caucasian individuals (sex: 2/6 (male/female); age [mean ± SD]: 48 ± 5.6 years; body weight: 128 ± 21.8 kg; BMI: 42.2 ± 4.4 kg/m^2) scheduled for laparoscopic

RYGB at a Danish public hospital, who met the study inclusion criteria (individuals assessed eligible for RYGB surgery, age ≥ 25 and ≤ 60 years) and exclusion criteria were included in the study. A full list of inclusion and exclusion criteria can be found at ClinicalTrials.gov (trial record NCT03093298). At inclusion, three of the participants were diagnosed with type 2 diabetes and five had HbA_{1c} < 48 mmol/mol (6.5%). The patients with type 2 diabetes were all on metformin monotherapy, which was paused for seven days prior to MMTs performed before RYGB and discontinued at the day of surgery. All participants completed a preoperative diet-induced body weight loss of at least 8%, as required by health care authorities in Denmark.

Surgery was performed using a standard laparoscopic RYGB technique[12] resulting in a gastric pouch with a volume of ~30 mL, a ~150 cm alimentary limb and a ~75 cm biliopancreatic limb (Fig. 1). None of the participants experienced complications perioperatively or during the following three postoperative months.

Experimental procedures

The participants were examined over a total of 6 experimental days. Identical MMTs were performed before the preoperative diet-induced weight loss program was initiated ~3 months (-3mo) and ~1 week preoperatively (-1wk), ~1 week and ~3 months postoperatively (+1wk and +3mo, respectively). In addition, an upper enteroscopy with biopsy retrieval was performed at ~3 months preoperatively and repeated ~3 months postoperatively.

Liquid mixed meal tests.

Each participant arrived at the research facility after 10 hours of fasting and abstinence from tobacco and alcohol as well as strenuous physical activity. Body weight was recorded, and the participant was placed in a hospital bed in a 45° recumbent position. For sampling of blood, a catheter was inserted into a cubital vein of a forearm wrapped in a heating pad. At time 0 min, the participants started ingesting a liquid mixed meal consisting of 200 mL Nutridrink [300 kcal, carbohydrate (50 E%), protein (15 E%), fat (35 E%), Nutricia Nutridrink, Allerød, Denmark] + 30 mL of water with 1,500 mg of dissolved acetaminophen (Pinex®, Actavis, Søborg, Denmark) (for evaluation of gastric emptying). The meal was ingested at an even rate over a 30-minute period at all MMT study days to avoid dumping symptoms after RYGB[13]. Blood was drawn before (time 0 min) and at time points 30, 60, 120 and 240 min. The blood was distributed in chilled tubes and centrifuged. Plasma/serum was either analyzed immediately (glucose) or stored at -80°C until analysis (C-peptide, insulin, glucagon and gut hormones).

Enteroscopy with biopsy retrieval.

Gastrointestinal mucosa biopsies were obtained during propofol sedation using a pediatric colonoscope (PCF-Q180AL, Olympus, Tokyo, Japan). An illustration of the anatomy before and after surgery with indications of biopsy sites is given in Fig. 1. Before RYGB, the enteroscope was introduced 120 cm from cavum oris (without curving in the stomach). Then, using a standard forceps, biopsies were sampled from the small intestine at the expected site of the entero-entero anastomosis (X) and from the proximal part of the lesser curvature of the stomach (G_1), which is preserved after surgery. Approximately three months after RYGB, biopsies were sampled from the remnant gastric pouch (G_2), the alimentary limb (A), the biliopancreatic limb (B) and the common channel (C), respectively (Fig. 1). Eight biopsies were sampled at each location and were snap frozen and subsequently stored at -80°C until analysis or placed in formaldehyde 4% (for immunohistochemical staining) and transferred to ethanol after 24 hours and stored refrigerated.

Biochemical analyses

Blood for bedside measurements was collected in Eppendorf tubes containing sodium fluoride and centrifuged for 30 seconds at 6,720 *g*. Plasma glucose concentrations were

measured using the glucose oxidase method (YSI 2300 STAT plus analyzer, YSI Inc., Yellow Springs, OH, USA). Blood for plasma acetaminophen measurement was collected in tubes added lithium-heparin (Vacuette, Greiner Bio-One GmbH, Frickenhausen, Germany). Blood for serum insulin and C-peptide concentrations was collected in tubes added clot activator (Vacuette, Greiner Bio-One GmbH, Frickenhausen, Germany) and kept at room temperature until complete coagulation. Blood for analyses of gut hormones was collected in iced P800 tubes (BD Biosciences, San Jose, CA, USA) added a proprietary cocktail of protease, esterase and dipeptidyl peptidase 4 inhibitors. Plasma and serum were separated from blood cells and platelets by centrifugation at 2,876 *g* for 15 minutes at -4°C (Hettich ROTINA 420R, Merck, Tuttlingen, Germany) and was kept at -80°C until analysis. Plasma acetaminophen was measured using dry chemistry technology (Vitros 5.1 FS, Ortho-Clinical Diagnostics, Allerød, Denmark). Serum insulin and C-peptide were quantified by routine chemiluminescence immunoassays using the ADVIA Centaur XP analyzer (Siemens Healthcare, Ballerup, Denmark). Total plasma glucose-dependent insulinotropic peptide (GIP), active GLP-1 (GLP-1(7-36)NH_2 + GLP-1(7-37)glycine) and total peptide YY (PYY) concentrations were measured using a custom-made ELISA (Meso Scale Discovery, N45ZA, Gaithersburg, MD, USA). Plasma samples were undiluted, run in duplicates and analyzed on a Meso Scale Discovery MESO QuickPlex SQ 120 plate reader (Meso Scale Discovery, Rockville, MD, USA) according to manufacturer's protocol. Extractable GLP-1 concentrations in the intestinal biopsies were measured using a sandwich ELISA, which equally detects GLP-1(7-36)NH_2 and GLP-1(9-36)NH_2, as previously described[14]. Glucagon concentrations (both plasma and intestinal) were measured using an in-house RIA based on a C-terminal-wrapping antibody (code 4305) and a sandwich ELISA (Mercodia A/S, Uppsala, Sweden) based on N and C-terminal-wrapping monoclonal antibodies as described previously[15]. N-terminally elongated glucagon (proglucagon 1-61), oxyntomodulin and glicentin were measured as previously described[16,17].

mRNA sequencing of gut biopsies.
mRNA sequencing (mRNA-seq) of small intestinal mucosa tissue embedded in Tissue-Tek® O.C.T. Compound (Sakura® Finetek, Torrance, CA, USA) was performed in biopsies obtained before and after RYGB. Total RNA from each biopsy was purified using NucleoSpin® RNA Plus (Macherey-Nagel, Düren, Germany). The quantity of the purified RNA was measured using Qubit® RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quality of purified RNA was determined with a Bioanalyzer using Agilent RNA 6000 Nano Kit (Agilent Technology, Waldbrönn, Germany). Subsequently, cDNA library preparation with 25-100 ng of purified RNA sample using TruSeq® Stranded mRNA Library Prep Kit for NeoPrep™ (Illumina, San Diego, CA, USA) was prepared. The sequencing of cDNA libraries was performed with NS® 500 high Output Kit v2 (75 cycles) (Illumina, San Diego, CA, USA) on a NextSeq 500 platform. The gene expression level is displayed as reads per kilobase million (RPKM), thus quantifying gene expression from mRNA sequencing data by normalizing for total read length and the number of sequencing reads.

Peptide extraction.
Snap-frozen mucosal tissue was weighed and subsequently homogenized in 1% trifluoroacetic acid (cat. no. TS-28904, Thermo Fisher Scientific, Waltham, MA, USA) with a 5 mm-steel bead and a bead mill (TissueLyzer, Qiagen Instruments AG, Hombrechtikon, Switzerland) at 30 Hz for 4 × 2 min. Next, samples were left to stand one hour at room temperature and cleared by centrifugation (3,300 *g*, 10 minutes, room temperature). Total protein concentration was estimated using a bicinchoninic acid assay (cat. no. 23225, Fischer Scientific, Roskilde, Denmark). Extracts were then purified using Sep-Pak pH resistant tc18

cartridges (cat. no. WAT036810, Waters, Milford, MA, USA) and peptides were eluted in 70% ethanol containing 0.1% trifluoroacetic acid and dried under a gentle stream of compressed air overnight. Samples were reconstituted in assay buffer before analysis (100 mmol/l TRIS buffer (cat. no. T-3253 and T-1503, Merck KGaA, Darmstadt, Germany) supplemented with 0.1% (w/v) human serum albumin (cat. no. 12666, Merck KGaA, Darmstadt, Germany), 20 mmol/L EDTA and 0.6 mmol/L Thiomersal (cat. no. T-5125, Sigma Chemical Co., St. Louis, MO, USA) (pH 8.5). More details are described elsewhere[18].

Immunohistochemical staining

Double immunohistochemical staining of gastrointestinal biopsies was performed using a custom-made monoclonal mouse C-terminal-wrapping anti-glucagon mouse antibody (20-7060-01/E6Al 1K, 1:5000, Mercodia A/S, Uppsala, Sweden) and a previously validated rabbit C-terminal (amide requiring) GLP-1 antibody (in-house 89390, 1:1000, University of Copenhagen, Denmark[19]). The specificity of the glucagon antibody was tested in archival sections of human pancreas by addition of 1 µM glucagon or a C-terminal fragment of oxyntomodulin (CKMNTKRNRNNIA, GenScript, Piscataway Township, NJ, USA) to the diluted antibody before double immunohistochemical staining. In these sections, staining was totally abolished with glucagon, but unaffected by the oxyntomodulin fragment (data not shown). For immunohistochemical double-stainings, 5 µm sections of all intestinal biopsies were dewaxed and subjected to microwave antigen retrieval with EGTA buffer, pH 9, for 15 minutes. Sections were then preincubated 15 minutes with 2% BSA and subsequently incubated overnight at 4°C with the primary antibodies. Double-staining for glucagon and GLP-1 was performed using the glucagon antibody anti-glucagon 1:5000 (mouse, Mercodia A/S, Uppsala, Sweden) and the anti-GLP-1 1:1000. Immunohistochemical double-staining for glucagon and prohormone convertase 2 (PC2) was performed using the glucagon antibody 1:5000 and a human specific PC2 antibody (rabbit, ALX-210-529-R100, Enzo Life Sciences, Farmingdale, NY, USA) 1:1000. The sections were washed and incubated with a mixture of Alexa488 goat anti-rabbit antibody (1:500, Life Technologies, Carlsbad, CA, USA) and Alexa568 donkey anti-mouse (1:500, 175700, Abcam, Cambridge, England), washed in buffer and cover-slipped. The stained sections were examined using an Axioscope 2 plus microscope (Zeiss, Jena, Germany). Images were taken using CoolSNAP camera (Photometrics, Tucson, AZ, USA).

Calculations and statistical analysis

Area under the curve (AUC) was calculated by the trapezoidal rule and presented as total and baseline-subtracted AUC (bsAUC). Due to a substantial number of zero-concentration for biopsy samples, all comparisons between gastric samples (G_1 and G_2) and comparisons between intestinal samples (X, A, B and C) for RIA-measured glucagon and proglucagon 1-61 were analysed using *McNemars* test for dichotomized data (concentration = 0 vs. >0). For plasma/serum analyses, basal, C_{max} (maximum concentration (peak) during 4-hour MMT), T_{max} (time-to-peak), AUC and bsAUC values were evaluated. Summary statistics were reported as mean ± SD for normally distributed variables, skew distributions were log-transformed prior to analysis and presented as median (IQR). Changes in outcome between the MMT study days were assessed by linear mixed model analyses[20]. An unstructured covariance was assumed. Goodness of fit was assessed by residual diagnostics. Due to the large number of tests carried out, all *P* values were corrected for multiple testing using the Benjamini and Hochberg procedure[21]. A standard cut-off for the false discovery rate at 0.1 was applied (i.e. adjusted *P* values of <0.10 were considered statistically significant, which limits the rate of false-positives among the reported findings to one in ten). All data were statistically processed using SAS software version 9.1 (SAS Institute, Cary, NC, USA). GraphPad Prism Software version 7 (La Jolla, CA, USA) was used to create graphs.

Results

Clinical characteristics of study participants before and after RYGB

The mean weight loss during the preoperative diet-induced weight loss period was 8.4 ± 3.4 kg, and 3 months postoperatively, the participants had lost another 14.6 ± 5.2 kg, corresponding to decreases in BMI of 2.7 ± 0.9 kg/m^2 and 4.7 ± 1.4 kg/m^2, respectively. After surgery, HbA_{1c}, insulin resistance and sensitivity and cholesterols improved significantly (figshare.com).

Increased secretion of gut hormones and glucagon after RYGB

RYGB increased the acetaminophen absorption rate, a proxy for the transit of the meal to the small intestine (Fig. 2*A*). As expected, postprandial plasma glucose profiles changed 1 week after RYGB (Fig. 2*B*) with reduced time-to-peak (T_{max}) and lower 120 min plasma glucose concentrations (Table 1). The 3-month postoperative plasma glucose profiles were similar to the 1-week profiles (Fig. 2*B*).

The postprandial serum excursions of insulin and C-peptide also changed as a result of surgery (Fig. 2*C*, Fig. 2*D*). Three months after RYGB, basal insulin and C-peptide decreased, and the peak concentrations of insulin nearly doubled postoperatively compared to preoperative concentrations (Fig. 2*C*, Table 1).

For ELISA-measured glucagon, the postprandial peak concentration (Fig. 3*A*, Table 1) and the initial 120 min postprandial response ($bsAUC_{0\text{-}120min}$) were increased 1 week after RYGB. The 120 min postprandial response ($bsAUC_{0\text{-}120min}$) dropped 3 months after RYGB, however, was elevated compared to the preoperative postprandial responses (Fig. 3*A*, Table 1). Similar results were found using a validated C-terminal specific glucagon RIA (figshare.com). The basal and peak concentrations and the postprandial response (AUC) of proglucagon 1-61 increased 1 week after RYGB compared to preoperative concentrations and remained elevated 3 months after RYGB compared to preoperatively (Fig. 3*B*, Table 1).

Postprandial plasma concentrations of active GLP-1 increased 1 week after RYGB compared to 3 months preoperatively with a nearly 12-fold increase in peak concentration, and ~5.4 and ~14 fold greater AUC and bsAUC, respectively (Fig. 3*C*, Table 1). Three months postoperatively, the postprandial GLP-1 responses remained elevated compared to preoperatively (Fig. 3*C*, Table 1).

The basal and peak concentrations and postprandial responses of plasma glicentin and oxyntomodulin increased 1 week after RYGB (Fig. 3*D*, Fig. 3*E*, Table 1). These parameters decreased somewhat 3 months after RYGB, yet remained elevated compared to preoperatively (Fig. 3*D*, Fig. 3*E*, Table 1). The peak and postprandial levels of plasma PYY increased 1 week after RYGB compared to 3 months preoperatively and remained elevated 3 months after surgery compared to preoperatively (Fig. 3*F*, Table 1).

One week after RYGB, the time-to-peak decreased for GIP compared to preoperative levels. The postprandial GIP responses decreased 3 months after RYGB compared to 3 months preoperatively (Fig. 4, Table 1).

Glucagon in gut biopsies after RYGB

The expression of the glucagon gene (*GCG*) (encoding proglucagon) increased in the intestinal mucosa after RYGB in the biliopancreatic limb (Fig. 5*A*). The expression of the *PCSK2* gene (encoding PC2, which in the pancreatic alpha cells cleaves proglucagon to glucagon) was very low in intestinal mucosa and did not change after surgery (Fig. 5*B*).

Before RYGB surgery, glucagon was not detectable in six of the eight biopsies from the gastric mucosa and in five of the eight biopsies from the small intestinal mucosa, and values below the limit of detection (1 pmol/g) were obtained in the remaining biopsies (Fig. 5*C*). After RYGB, glucagon was not detectable in gastric mucosa biopsies, but increased concentrations were identified in the mucosa biopsies from the alimentary and biliopancreatic

limb as well as the common channel (Fig. 5*C*). Similar data were observed using a C-terminal-specific glucagon RIA (figshare.com). Proglucagon 1-61 was undetectable in gastric as well as intestinal mucosa biopsies before RYGB, but concentrations were detectable in the small intestine after surgery (Fig. 5*D*). GLP-1 was not above analytical detection limits in gastric biopsies before and after RYGB (Fig. 5*E*), whereas intestinal GLP-1 was detected before RYGB and the concentrations increased after surgery (Fig. 5*E*).

Glucagon localized to GLP-1-producing cells after RYGB

GLP-1-producing L cells were found in biopsies from the small intestine, and the majority also expressed glucagon as determined using the specific C-terminal monoclonal glucagon antibody. A few cells were only GLP-1 immunoreactive, but no cells were only glucagon immunoreactive (Fig. 6). There were no cells displaying immunoreactivity for glucagon or GLP-1 in the gastric biopsies (data not shown). Also, some cells were displaying immunoreactivity for PC2 in the small intestinal biopsies but not for both PC2 and glucagon (figshare.com).

Discussion

This study suggests that fully processed glucagon 1-29 may be generated by GLP-1-producing enteroendocrine L cells in the proximal part of the small intestine following RYGB surgery in humans.

The hypothesis of extrapancreatic glucagon in humans has been supported by findings of plasma glucagon in totally pancreatectomized patients[22]. The findings were replicated using a RIA directed against the free C-terminus of glucagon, a region which is only exposed after cleavage of proglucagon at the dibasic consensus site (residues 62 and 63 in proglucagon), which in the pancreas is cleaved by PC2[22]. However, such assays will also react with N-terminally elongated glucagon isoforms (e.g. proglucagon 1-61). New sandwich ELISAs targeting simultaneously the C and N-termini of the peptide hormones have been developed, minimizing potential 'cross-reactivities' with such elongated forms reacting with the C-terminal assays[15]. A recent study, using a mass-spectrometry-validated sandwich ELISA, confirmed the presence of circulating glucagon 1-29 after total pancreatectomy in man[10]. Common for these patients and the RYGB-operated individuals included in the present study, is the surgery-induced gastrointestinal rearrangement involving removal of the pyloric sphincter leading to rapid delivery of ingested nutrients and, hence, altered stimulation of enteroendocrine cells in the small intestine. In the present study, we observed an increase of proglucagon-derivatives (GLP-1, glicentin, oxyntomodulin and proglucagon 1-61 in addition to glucagon 1-29) in plasma, using newly developed and mass-spectrometry-validated sandwich ELISAs[15]. Roberts et al. were unable to detect increased glucagon secretion in plasma of lean individuals undergoing gastrectomy when using liquid chromatography-mass spectrometry (LCMS)[23]. Roberts et al. proposed that elevated plasma levels of glucagon after RYGB surgery may be due to cross-reactivity with other proglucagon-like molecules such as glicentin[23]. We therefore used two extensively evaluated antibody-based methods, RIA and ELISA[10,15], with a documented lack of cross reaction with these molecular species. The results obtained with the ELISA were closely mirrored by results obtained with the C-terminally directed glucagon RIA. In addition, we included immunohistochemistry using a new monoclonal antibody reacting exclusively with the C-terminus of the glucagon molecule (and therefore capable of reacting only with molecules exposing this sequence, i.e. fully processed glucagon 1-29 or proglucagon 1-61) – and found positive immunoreactivity in many L cells, indicating that cleavage leading to the formation of one or both of these two moieties did occur in the GLP-1-producing cells identified in the biopsies.

An important limitation to the study is the small number of participants, which limits the statistical power. Regarding the meal-stimulated glucagon response in plasma, the 0-120 min of the bsAUC for glucagon was evaluated (in addition to the 0-240 min) because the meal-induced response had a duration corresponding to this period (a complete return to basal levels was reached at 120 min on all days). The postprandial glucagon response increased numerically after RYGB compared to before surgery (for bsAUC at time 0-240 min and 0-120 min). Increasing postprandial plasma concentrations of glucagon after RYGB are surprising in view of the concomitant, high concentrations of insulin and GLP-1, both known to inhibit glucagon secretion. Another study limitation is the mix of participants with and without type 2 diabetes in small groups of $n = 3$ (T2D) vs. $n = 5$ (NGT), respectively. Statistical analysis of such small groups is not meaningful and was therefore not performed. Nevertheless, we observed a trend for higher concentrations of glucagon, proglucagon 1-61 and GLP-1 in the biopsies of the NGT group.

GLP-1 and glucagon are both processed from the *GCG* gene product, proglucagon (Fig. 7). The proglucagon-processing PCs and their peptide products are traditionally considered tissue-specific (i.e. PC1/3 occurs in and acts on proglucagon in gut and brain forming GLP-1, while PC2 occurs in and acts on proglucagon in pancreatic alpha cells forming glucagon). However, the absolute tissue specificity of proglucagon processing has been challenged by previous findings of intestinal *PCSK2* mRNA expression and PC2-positive cells in human gut biopsies [24,25]. In addition, the two processing enzymes may have overlapping specificities [26]. From these data, we suggest that in the intestine, processing of proglucagon to glucagon may therefore not be PC2-dependent and may occur on the basis of unspecific processing of proglucagon from PC1/3 or other prohormone convertases.

Contradictory results have been found regarding presence of gastric glucagon in humans. Ravazzola et al.[27] identified glucagon-positive cells in the fetal human stomach that showed ultrastructural features consistent with pancreatic alpha cells, whereas Holst et al. were unable to detect glucagon in gastric mucosal biopsies from adult humans[28]. In the gastric mucosa biopsies from the present study, glucagon concentrations were almost undetectable, and we saw no signs of increases in glucagon content following RYGB. Furthermore, no glucagon immunoreactivity was identified by immunohistochemistry in the gastric biopsies (data not shown). Accordingly, we find it unlikely that the stomach contributes to RYGB-induced hyperglucagonemia.

Although we cannot exclude that the increase in plasma glucagon may be of pancreatic origin (perhaps via accelerated amino acid absorption), our findings do support a potential involvement of the intestinal tract in the postprandial hyperglucagonemia observed after RYGB surgery and - possibly - other conditions characterized by postprandial hyperglucagonemia. It is of importance to recognize the limitations of antibody-based methods as used in this study as they may suffer from inaccuracy and therefore future studies combining antibody-dependent and independent approaches are warranted. Whether increased circulating glucagon after RYGB surgery has any physiological implications (e.g. protection from hyperinsulinemic hypoglycemia) remains to be established.

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Author contributions

T.J., B.M., M.F., P.B.M., S.T., P.J.L., N.V., J.J. and F.K.K. contributed substantially to the concept and design of the study; T.J., M.M.C., D.W., R.K.S., A.A. and C.B.J. recruited participants; T.J., M.M.T., B.M., E.W., E.L. and S.F. performed experimental study days and provided clinical samples; molecular characterization, analysis of certain gut hormones and immunohistochemistry were performed in the laboratories at the Panum institute (N.J.W.A., J.J.H. and C.Ø.); T.J., N.J.W.A., M.M.C., B.M., D.W., C.Ø., R.K.S., A.A., C.B.J., J.J.H., N.V., J.J., N.V., T.V. and F.K.K. contributed substantially to the analysis and interpretation of the data; F.S. and J.L.F. performed statistics; T.J. and FKK drafted the manuscript; N.J.W.A., M.M.C., B.M., E.W., E.L., S.F., D.W., C.Ø., R.K.S., A.A., C.B.J., F.S., J.L.F., M.F., P.B.M., S.T., P.J.L., J.J.H., N.V., J.J. and T.V. critically revised the manuscript for important intellectual content. All authors have provided approval of the final version to be published. F.K.K. and T.J. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure summary

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DATA AVAILABILITY:

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.;

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Figure 1. Illustration of the gastrointestinal anatomy before (left) and after (right) RYGB with black circles indicating biopsy sites (A, alimentary limb; B, biliopancreatic limb; C, common channel; G1, proximally at the lesser curvature of the stomach; G2, remnant gastric pouch; X, the expected site of entero-entero anastomosis (black line)). RPKM, reads per kilobase million

Figure 2. Plasma responses for glucose, insulin and C-peptide . Plasma/serum excursions for acetaminophen (**A**), glucose (**B**), insulin (**C**) and C-peptide (**D**) from 4-hour liquid mixed meal tests (MMT) performed in participants (n = 8) before (MMT -3mo, full black circles) and after (MMT -1wk, open black circles) a diet-induced weight loss and ~1 week (MMT +1wk, full blue triangles) and 3 months (MMT +3mo, open blue triangles) after RYGB surgery. Data are mean ± SD.

Figure 3. Plasma responses for glucagon, proglucagon 1-61, GLP-1, oxyntomodulin, glicentin and PYY. Plasma responses of glucagon (**A**), proglucagon 1-61 (**B**), active glucagon-like peptide 1 (GLP-1) (**C**), oxyntomodulin (**D**), glicentin (**E**) and total peptide YY (PYY) (**F**) from 4-hour liquid mixed meal test (MMT) performed in participants (n = 8) before (MMT -3mo, full black circles) and after (MMT -1wk, open black circles) a diet-induced weight loss and ~1 week (MMT +1wk, full blue triangles) and 3 months (MMT +3mo, open blue triangles) after RYGB surgery. Data are mean ± SD for normally distributed variables (**A**, **B**, **C**, **D**, **E**), geometric mean ± 95% CI for skewed distributions (**F**).

Figure 4. Plasma response for GIP. Plasma response for total glucose-dependent insulinotropic polypeptide (GIP) from 4-hour liquid mixed meal test (MMT) performed in

participants (n = 8) before (MMT -3mo, full black circles) and after (MMT -1wk, open black circles) a diet-induced weight loss and ~1 week (MMT +1wk, full blue triangles) and 3 months (MMT +3mo, open blue triangles) after RYGB surgery. Data are mean geometric mean ± 95% CI for skewed distributions.

Figure 5. Expression level of *GCG* and *PCSK2*, and concentrations of glucagon, proglucagon 1-61 and GLP-1 in gut biopsies. Gene expression level of the glucagon gene (*GCG*) (**A**) and the gene encoding prohormone convertase (*PCSK2*) (**B**) and concentrations (pmol/g mucosal tissue) of glucagon (**C**), proglucagon 1-61 (**D**) and glucagon-like peptide 1 (GLP-1) measured with ELISAs (**E**), in biopsies sampled before (Pre G1, Pre X) and after (Post G2, Post A, Post B, Post C) Roux-en Y gastric bypass (RYGB) surgery in individuals with (n = 3) and without type 2 diabetes (n = 5). Data are shown as univariate scatter plots with median (horizontal line). Significant differences (false discovery rate-adjusted $p < 0.1$) between biopsy sites are indicated by letters above data plots. **F** illustrates the gastrointestinal anatomy before (left) and after (right) RYGB with black circles indicating biopsy sites (A, alimentary limb; B, biliopancreatic limb; C, common channel; G1, proximally at the lesser curvature of the stomach; G2, remnant gastric pouch; X, the expected site of entero-entero anastomosis (black line)). RPKM, reads per kilobase million

Figure 6. Co-localization of glucagon and GLP-1 in the small intestine. Immunohistochemical staining of two small intestinal biopsies after RYGB surgery showing glucagon-like peptide 1 (GLP-1) (green) (**A**, **D**) and glucagon (red) (**B**, **E**) and co-localization of glucagon and GLP-1 (yellow) (**C**, **F**). Arrows indicate GLP-1-positive cells (not glucagon-positive).

Figure 7. Differential processing of proglucagon and antibodies for analyses. Differential processing of proglucagon by prohormone convertase (PC) 2 and PC1/3, respectively, and antibodies (AB) used in specific analyses. The numbers refer to amino acid positions in the 160-amino acid proglucagon sequence. GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2; GRPP, glicentin-related pancreatic polypeptide; IP-1, intervening peptide 1; IP-2, intervening peptide 2

Table 1. Plasma acetaminophen, glucose and hormonal responses

		Diet-induced weight loss period (before RYGB)		***Surgery-induced weight loss period (after RYGB)***	
		MMT -3mo (1)	**MMT -1wk** (2)	**MMT +1wk** (3)	**MMT +3mo** (4)
Acetaminophen					
	Basal (mmol/L)	4.00 ± 2.60	5.86 ± 0.27	7.25 ± 9.38	4.57 ± 2.13
	C_{max} (mmol/L)	67.9 ± 11.6^{3,4}	74.0 ± 18.6^{3,4}	136.0 ± 38.8^{1,2,4}	146 ± 33.5^{1,2,3}
	T_{max} (min)	97.5 ± 29.0^{3,4}	94.3 ± 29.7^{3,4}	30.0 ± 0.00^{1,2}	30.0 ± 0.00^{1,2}
	AUC (mmol/L × min)	10.6 ± 2.07^{3,4}	11.9 ± 2.67^{1,3,4}	15.0 ± 3.93^{1,2,4}	17.3 ± 4.53^{1,2,3}
	bsAUC (mmol/L × min)	9.57 ± 1.93^{3,4}	10.5 ± 2.48^{3,4}	13.3 ± 4.60^{1,2,4}	16.2 ± 4.41^{1,2,3}
Glucose					
	Basal (mmol/L)	7.06 ± 2.58	5.98 ± 1.16	5.66 ± 0.76	5.25 ± 0.68
	120-min' (mmol/L)	8.78 ± 3.00^{3,4}	7.35 ± 1.59^{3,4}	5.46 ± 1.29^{1,2,4}	5.03 ± 1.13^{1,2,3}
	C_{max} (mmol/L)	9.61 ± 2.89	8.54 ± 2.76	9.31 ± 2.04	9.40 ± 1.62
	T_{max} (min)	97.5 ± 29.0^{3,4}	77.1 ± 27.1^{3,4}	41.3 ± 14.5^{1,2}	37.5 ± 13.0^{1,2}
	AUC (mmol/L × min)	1900 ± 597	1660 ± 392	1536 ± 272	1464 ± 232
	bsAUC (mmol/L × min)	205 ± 125	225 ± 133	178 ± 155	204 ± 77
Insulin					
	Basal (pmol/L)	209 ± 134^{4}	115 ± 47	90.5 ± 18.7^{4}	62.9 ± 19.6^{1,3}
	C_{max} (pmol/L)	580 ± 236^{4}	572 ± 278^{3,4}	1194 ± 620^{2}	1039 ± 360^{1,2}
	T_{max} (min)	60.0 ± 26.0	64.3 ± 25.0^{4}	41.3 ± 14.5	45.0 ± 15.0^{2}
	AUC (nmol/L × min)	75.8 ± 19.9	71.1 ± 22.3	87.6 ± 44.6	69.6 ± 27.3
	bsAUC (nmol/L × min)	25.6 ± 32.4	43.6 ± 19.4	65.9 ± 42.8	55.3 ± 26.2
C-peptide					

	Basal (pmol/L)	1114 ± 536[4]	797 ± 236[4]	805 ± 269[4]	578 ± 228[1,2,3]
	C_{max} (pmol/L)	2171 (1919;2517)[3]	1841 (1592;2489)[3]	3343 (2210;4461)[1,2]	2590 (2008;3943)
	T_{max} (min)	90 (60;120)[3,4]	60 (60;90)[3,4]	60 (53;60)[1,2]	60 (30;60)[1,2]
	AUC (nmol/L × min)	373 ± 49	349 ± 90	436 ± 163[4]	362 ± 133[3]
	bsAUC (nmol/L × min)	106 ± 107	157 ± 52	243 ± 117	223 ±85
Glucagon					
	Basal (pmol/L)	6.25 ± 2.28	6.14 ± 2.17	5.13 ± 1.05	5.75 ± 0.97
	C_{max} (pmol/L)	7.88 ± 2.37[3]	8.00 ± 1.31[3]	10.5 ± 2.1[1,2]	9.25 ± 1.64
	T_{max} (min)	26.3 ± 18.0	34.3 ± 19.2	45.0 ± 15.0	37.5 ± 13.0
	AUC (pmol/L × min)	1284 ± 404	1378 ± 334	1414 ± 343	1399 ± 257
	bsAUC (pmol/L × min)	-216 ± 223	-96.4 ± 435	184 ± 302	18.8 ± 194
	$bsAUC_{0\text{-}120min}$ (pmol/L x min)	9.38 ± 141[3]	57.9 ± 174	319 ± 147[1,4]	161 ± 118[3]
Proglucagon 1-61					
	Basal (pmol/L)	3.14 ± 0.60[3,4]	3.29 ± 0.70[4]	4.75 ± 1.79[1]	5.63 ± 1.32[1,2]
	C_{max} (pmol/L)	5.29 ± 1.16[3,4]	4.86 ± 0.64[3,4]	8.13 ± 2.09[1,2]	9.00 ± 2.18[1,2]
	T_{max} (min)	71.3 ± 29.8	42.9 ± 21.9[3]	120 ± 73[2]	90.0 ± 65.4
	AUC (pmol/L × min)	1009 ± 258[4]	930 ± 193[3,4]	1530 ± 411[2]	1641 ± 346[1,2]
	bsAUC (pmol/L × min)	255 ± 208	141 ± 226	390 ± 235	291 ± 287
GLP-1					
	Basal (pmol/L)	0.49 ± 0.18	0.53 ± 0.10	0.60 ± 0.26	0.55 ± 0.16
	C_{max} (pmol/L)	1.84 ± 0.85[3,4]	1.86 ± 0.66[3,4]	17.8 ± 8.8[1,2]	20.9 ± 9.0[1,2]
	T_{max} (min)	52.5 ± 29.0[3]	42.9 ± 14.8	45.0 ± 15.0[1,4]	30.0 ± 0.0[3]
	AUC (pmol/L × min)	219 (168;300)[3,4]	207 (186;259)[3,4]	1393 (830;1852)[1,2]	1264 (891;1449)[1,2]
	bsAUC (pmol/L × min)	107 (66;188)[3,4]	87.8 (53.3;121.4)[3,4]	1227 (594;1727)[1,2]	1096 (730;1335)[1,2]
Glicentin					
	Basal (pmol/L)	36.6 ± 10.1[3]	37.6 ± 6.90[3]	60.5 ± 16.7[2,4]	44.8 ± 14.6[3]
	C_{max} (pmol/L)	65.8 ± 10.4[3,4]	61.7 ± 10.0[3,4]	232 ± 44.0[1,2,4]	188 ± 54.0[1,2,3]
	T_{max} (min)	97.5 ± 29.0[4]	107 ± 64.0	67.5 ± 19.8	56.3 ± 9.90[1]
	AUC (nmol/L × min)	13.3 ± 2.40[3,4]	12.5 ± 2.10[3,4]	34.2 ± 9.10[1,2,4]	24.9 ± 7.30[1,2,3]
	bsAUC (nmol/L × min)	4.66 (3.62;5.09)[3,4]	2.88 (2.50;3.77)[3,4]	15.3 (13.2;20.8)[1,2,4]	10.0 (9.00;15.5)[1,2,3]
Oxyntomodulin					
	Basal (pmol/L)	14.5 ± 4.10[3,4]	15.3 ± 3.00[3]	23.3 ± 8.00[1,2,4]	19.4 ± 6.40[1,3]
	C_{max} (pmol/L)	25.1 ± 4.70[3,4]	24.4 ± 3.50[3,4]	89.9 ± 23.8[1,2,4]	69.8 ± 17.4[1,2,3]
	T_{max} (min)	97.5 ± 29.0	81.4 ± 34.8	67.5 ± 19.8	60.0 ± 0.00
	AUC (nmol/L × min)	5.13 ± 0.96[3,4]	4.99 ± 0.75[3,4]	12.7 ± 3.50[1,2,4]	10.1 ± 2.90[1,2,3]
	bsAUC (nmol/L × min)	1.55 (1.16;1.91)	1.26 (0.81;1.78)	5.72 (4.19;8.25)	3.65 (3.43;6.29)
PYY					
	Basal (pmol/L)	7.31 (5.26;9.92)	5.13 (4.87;10.57)	5.80 (5.11;8.86)	6.58 (5.57;10.45)
	C_{max} (pmol/L)	11.1 (10.2;2019.8)[3,4]	12.1 (11.5;27.4)[3,4]	45.3 (35.8;74.1)[1,2]	47 (35;55)[1,2]
	T_{max} (min)	90 (60;120)	60 (45;60)	60 (60;60)	60 (60;60)
	AUC (pmol/L × min)	3182 ± 1900[3,4]	3396 ± 1689	7811 ± 4169[1]	7958 ± 4965[1]
	bsAUC (pmol/L × min)	1085 ± 1340[3,4]	1087 ± 1464[3,4]	6009 ± 3810[1,2]	5672 ± 3868[1,2]
GIP					
	Basal (pmol/L)	15 (12;23)	13 (10;15)	11 (9;11)	14 (13;16)
	C_{max} (pmol/L)	107 (86;164)	151 (101;221)	148 (132;229)	147 (97;320)
	T_{max} (min)	60 (60;75)[3,4]	60 (45;60)[3,4]	45 (30;60)[1,2]	30 (30;30)[1,2]
	AUC (pmol/L × min)	68 (61;96)[4]	87 (77;107)	69 (59;103)	63 (48;110)[1]
	bsAUC (pmol/L × min)	55 (37;75)	75 (62;89)	57 (47;89)	46 (34;91)

Fasting and postprandial responses of glucose, insulin, C-peptide, glucagon, proglucagon 1-61, active glucagon-like peptide 1 (GLP-1), glicentin, oxyntomodulin and total peptide YY (PYY) during mixed meal test (MMT) in Roux-en-Y gastric bypass (RYGB)-operated participants (n = 8) before a diet-induced weight loss at ~3 months (MMT -3mo) and ~1 week (MMT -1wk) before surgery and ~1 week (MMT +1wk) and ~3 months (MMT +3mo) after surgery. Data are mean ± SD for normally distributed variables and medians with inter-quartile ranges in brackets for skewed distributions. Significant difference (false discovery rate-adjusted $P < 0.1$) between MMTs indicated by numerals in superscript. AUC, area under curve; bsAUC, baseline-subtracted AUC; C_{max}, max concentration during MMT; T_{max}, time-to-peak