Glucagon-like peptide-2 and mouse intestinal adaptation to a high-fat diet

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Abstract

Endogenous glucagon-like peptide-2 (GLP2) is a key mediator of refeeding-induced and resection-induced intestinal adaptive growth. This study investigated the potential role of GLP2 in mediating the mucosal responses to a chronic high-fat diet (HFD). In this view, the murine small intestine adaptive response to a HFD was analyzed and a possible involvement of endogenous GLP2 was verified using GLP2 (3–33) as GLP2 receptor (GLP2R) antagonist. In comparison with animals fed a standard diet, mice fed a HFD for 14 weeks exhibited an increase in crypt–villus mean height (duodenum, 27.5 ± 3.0%; jejunum, 36.5 ± 2.9%; P < 0.01), in the cell number per villus (duodenum, 28.4 ± 2.2%; jejunum, 32.0 ± 2.9%; P < 0.01), and in Ki67-positive cell number per crypt. No change in the percent of caspase-3-positive cell in the villus–crypt was observed. The chronic exposure to a HFD also caused a significant increase in GLP2 plasma levels and in GLP2R intestinal expression. Daily administration of GLP2 (3–33) (30–60 ng) for 4 weeks did not modify the crypt–villus height in control mice. In HFD-fed mice, chronic treatment with GLP2 (3–33) reduced the increase in crypt–villus height and in the cell number per villus through reduction of cell proliferation and increase in apoptosis. This study provides the first experimental evidence for a role of endogenous GLP2 in the intestinal adaptation to HFD in obese mice and for a dysregulation of the GLP2/GLP2R system after a prolonged HFD.

Introduction

The presence of nutrients in the gastrointestinal tract is the primary physiological stimulus for enterocyte proliferation. In fact, fasting induces mucosal atrophy and enteral refeeding causes marked intestinal adaptative growth (Altmann 1972, Aldewachi et al. 1975). Luminal or enteral nutrients stimulate intestinal growth both directly by providing energy and protein to the enterocytes and indirectly through hormonal secretion. Several mediators are nutrient regulated, including the proglucagon-derived peptides, glucagon-like peptide-1 (GLP1), and GLP2, and there is evidence for a link among GLP2, intestinal growth, and increased energy intake (Xiao et al. 1999, Shin et al. 2005, Nelson et al. 2008).

GLP2 is a 33-amino acid peptide derived from tissue-specific posttranslational processing of proglucagon in the enteroendocrine L-cells of the ileum and colon (Sinclair & Drucker 2005, Zhou et al. 2006). Endogenous GLP2 is a key mediator of mucosal adaptive growth induced by

Effects of GLP2 occur via the GLP2 receptor (GLP2R), a G-protein-coupled receptor expressed in a tissue- and species-specific manner, but mainly present in the gastrointestinal tract (Munroe et al. 1999, Yusta et al. 2000, Angelone et al. 2012). Expression of Glp2r mRNA in the gastrointestinal tract is the greatest in the proximal small intestine, particularly the jejunum (Ørskov et al. 2005), and our recent work provides evidence for the presence of GLP2R protein throughout the gastrointestinal tract in mice (Amato et al. 2010). Most GLP2 actions appear to be indirect, because GLP2R has not been identified on absorptive enterocytes; rather, it has been localized in enteric neurons, intestinal myofibroblasts, and human or pig rare subsets of enteroendocrine cells (Yusta et al. 2000, Bjerknes & Cheng 2001, Ørskov et al. 2005, Guan et al. 2006).

Reports by several laboratories indicate that high-fat diet (HFD) maintenance promotes the capacity of the gastrointestinal tract to digest and absorb fats, through pronounced effects on the intestinal transcriptoma (de Wit et al. 2011). Moreover, morphological alterations of rat small intestine including hypertrophy, increase in villus height, and increase in the number of enterocytes per villus have been observed in response to dietary fat adaptation (Sagher et al. 1991, Thomson et al. 1996). However, a potential role of GLP2 in mediating the mucosal responses to a HFD has not been considered yet. Recently, we have demonstrated that the HFD is able to upregulate the GLP2R gene and protein expression in murine fundus, with implications for GLP2 gastric relaxant efficacy (Rotondo et al. 2011), but the degree to which the HFD affects the regulation of Glp2r expression in the intestine has not been assessed.

Therefore, the purpose of this study was to analyze the murine small intestine mucosal changes induced by a HFD and to verify a possible involvement of endogenous GLP2 in mediating the response, using GLP2 (3–33) as GLP2R antagonist. In addition, the relationship among plasma GLP2 concentrations, small intestinal Glp2r expression levels, and intestinal morphometric parameters was determined in mice fed a HFD.

**Materials and methods**

**Animals**

The animal studies were approved by Ministero della Sanità (Rome, Italy) and were in compliance with the guidelines of the European Communities Council Directive of 24 November 1986. Male C57BL/6j (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy) at 4 weeks of age, were housed under standard conditions of light (12 h light:12 h darkness cycle) and temperature (22–24 °C), with free access to water and food. After acclimatization (1 week), the animals were weighed and divided in two groups, which were fed a standard diet (STD) (4RF25, Mucedola, Milan, Italy), or fed for 14 weeks a HFD (PF4051/D, Mucedola) consisting of 23% protein, 38% carbohydrates, and 34% fat (60% caloric fat content).

Animals were weighed and killed by cervical dislocation. The abdomen was immediately opened and the duodenum and jejunum were excised, cleaned of luminal contents, weighed, and processed for subsequent analysis.

**Peptides**

Synthetic GLP2 (3–33) was provided by Caslo Laboratory (Lyngby, Denmark). Purity (>95%) and correctness of structure were confirmed by mass, sequence, and HPLC analysis.

**Histological sections and morphometric estimates**

Segments of duodenum and jejunum were removed gently from the mesenteric border and processed for light microscopy. For comparative purposes, histological analyses were consistently performed on intestinal segments taken from identical anatomic positions. Each segment was routinely formalin fixed and paraffin embedded. Sections with a thickness of 5 μm were obtained and stained using hematoxylin and eosin. Crypt–villus height and number of cells per villus were measured by examining at least 22 well-oriented villi from different sections from each intestinal segment and animal. The thickness of the muscularis layer was assessed by quantitative histomorphometry in the sections. Some
slides were processed using Periodic acid-Schiff stain kit (Sigma) and the number of goblet cells was counted in at least 22 villi per mouse and normalized to the number of positive cells per 100 μm. The sections were examined using an automated Leica DMS5000 B microscope (Leica, Milan, Italy) connected to a high-resolution camera, Leica DC300 F (Leica). The examination and the computer analysis of the histological sections were performed without knowledge of the origin of the tissue samples.

**Immunohistochemistry**

Immunohistochemistry for the proliferative marker, Ki67, or for caspase-3, a key protein of apoptosis, was performed by a streptavidin–biotin complex method using Histostain-Plus Bulk Kit (Invitrogen Corporation, Cat no. 85-8943). The used primary antibodies were anti-Ki67 antigen (clone MIB-1, DAKO; dilution 1:200) and caspase-3 (Cell Signaling Technology, Danvers, MA, USA, dilution 1:200, Cat no. 9662). After deparaffinization and rehydration, the tissue sections were incubated for 10 min with protein blocking agent (DAKO). Subsequently, the primary antibodies were added to the sections. Nonimmune rabbit serum was used for negative controls, and appropriate positive controls were done simultaneously. Diaminobenzidine was used as chromogen for caspase-3, a key protein of apoptosis, was performed by a blinded observer and the number of Ki67 staining cells per crypt was determined. Percent of apoptotic cells were assessed as number of caspase-3 immunopositive cells divided by total number of cells in the crypt–villus region.

**Real-time RT-PCR**

Total RNA was isolated from full thickness samples of duodenum and jejunum of mice fed a STD or HFD using the PureLink RNA Mini Kit (Invitrogen) and cDNA was synthesized using High Capacity cDNA RT Kit (Applied Biosystems).

**Glp2r** expression was determined using the primers, Fwd: 5’-TCATCTCCCTCTTGGCCTCTAC-3’; Rev: 5’-TGACTGAGATGATACCCATTCAA-3’, to generate a 196 bp product. Another set of primers, Fwd: 5’-CGGGATCCTCGGCTGGCTGGACTC-3’; Rev: 5’-GAATTCTCGGCTGGCTGGACTC-3’, were used to generate a 286 bp product from mouse β-actin. The primers were verified on gene sequence by public databases (mouse Glp2r cDNA: GenBank accession no. AV605231; mouse β-actin cDNA: GenBank accession no. NM007393) and then span on. Two microlitres (100 ng) of cDNA, produced from RT reaction, was added to 12.5 μl SYBR Green 2× PCR Master Mix (Applied Biosystems), 1.88 μl 2 mM specific primer pair, and dH₂O up to 25 μl final volume. No-reverse transcriptase control and no-template control reactions were done with every assay to ensure the specificity of the reaction and the absence of any contamination. Quantitative real-time PCR was performed on ABI PRISM 7300 sequence detection system (Applied Biosystems). Relative mRNA expression was quantified with the 2⁻ΔΔC_T method, using SDS Software (Applied Biosystems) and β-actin-mouse RNA was analyzed as an endogenous control. β-Actin was selected as reference gene because it was stably expressed in the different nutritional states.

**Western blotting**

Tissue segments, excised by mice fed standard or HFD, were homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40) containing protease inhibitors (2 mM phenylmethylsulphonyl fluoride, NaVO₃). Proteins (50 μg) were separated by NuPAGE 10% Bis–Tris Gel 1.0 mm (Invitrogen) and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science), as described previously (Rotondo et al. 2011). Membranes were incubated overnight with antibody to GLP2R (Santa Cruz Biotechnology) (1:100 dilution) and then incubated with secondary antibody conjugated to HRP recommended for GLP2R detection (Santa Cruz Biotechnology) (diluted 1:2000) followed by ECL detection (Pierce, Rockford, IL, USA). β-Actin (Santa Cruz Biotechnology) was used as a loading control. Mouse intestine mucosal cells, which do not express the GLP2R (Bjerknes & Cheng 2001), obtained by gently scraping the tissue, were chosen as negative controls. Densitometric analysis of blots was performed using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD, USA).

**Plasma GLP2 quantification**

In mice fed a STD or HFD, blood draws were performed in the morning after 2 h of fasting in order to avoid variation in the plasma GLP2 quantification due to free food access. The blood was drawn by cardiac puncture, immediately transferred into chilled tubes containing a final concentration of 1 mg/ml EDTA, and centrifuged at 825 g at 4 °C for 10 min to obtain plasma, which was stored at −80 °C until analysis. Quantification of plasma GLP2 was carried out with the RSCYK142R enzyme immunoassay kit for...
mouse GLP2 (Biovendor, Heidelberg, Germany) according to the manufacturer’s instructions. The experimental detection limit of the assay was 0.412 ng/ml.

**GLP2 (3–33) treatment**

In another set of experiments, mice after 6 weeks on the HFD and the age-matched control animals were injected once a day i.p. with 100 μl GLP2 (3–33) (30 or 60 ng) or PBS (vehicle control) for 4 weeks, in accordance with the protocol previously reported (Iakoubov et al. 2009). The sixth week was chosen as time of onset of the chronic treatment because our preliminary studies showed that the change in weight gain in HFD-fed mice was evident by as early as 6 weeks. The day after the last injection, body weight was measured and the mice were killed for assessment of morphometric analyses.

**Statistical analysis**

Results are shown as means±S.E.M. The letter n indicates the number of experimental animals. The comparison between the groups was performed by ANOVA followed by Bonferroni’s post-test using Prism Version 4.0 Software (Graph Pad Software, Inc., San Diego, CA, USA). A P value <0.05 was considered to be statistically significant.

**Results**

**Effects of a chronic HFD**

After 14 weeks on a HFD, mice exhibited increased body weight and hepatic steatosis with respect to mice fed a STD for the same period (data not shown) and they were considered to have diet-induced obesity (DIO). The body weight of age-matched HFD-fed mice was significantly higher than STD-fed (lean) animals (38.6±0.8 vs 27.3±0.6 g; P<0.001; n=20 for each group).

Also the small intestinal wet weight in DIO mice was increased significantly by 26.5±7.8% (n=6 for each group; P<0.01) in comparison with lean animals. To assess whether these changes in intestinal weight were independent of the changes in body weight, small intestinal weights were also normalized to body weights. Duodenal and jejunal crypt–villus height as well as the cell number per villus were increased significantly (Fig. 1).

In addition, we found that the Ki67-positive cell number in the crypt region was enhanced in HFD-fed animals compared with STD-fed mice, but we did not observe any difference in the percent of caspase-3-positive cells in the villus–crypt (Fig. 2). No difference was seen in the number of goblet cells per villus and in the thickness of the external muscular layer, although the latter showed a tendency towards an increase (18.5±3.5% in duodenum and 13±4.7% in jejunum) in HFD-fed animals compared with STD-fed mice.

**Peptide plasma concentrations**

In order to determine whether changes in GLP2 circulating concentrations were associated with increases in the morphometric parameters observed in DIO mice, we measured plasma GLP2 levels. GLP2 concentrations ranged from 0.5 to 1.2 ng/ml and these concentrations were affected by the HFD. As reported in Fig. 3, chronic exposure to a HFD significantly increased the plasma GLP2 concentrations by about 15%. On the contrary, GLP2 plasma levels progressively decreased in lean mice after fasting (24 h), but returned to pre-fasting (basal) levels in 6-h re-fed mice.
GLP2R expression

Expression of the GLP2R mRNA was detected in all samples. The chronic exposure to a HFD caused an increase in GLP2R mRNA intestinal expression, which was greater in the duodenum than in the jejunum. Also, western blotting experiments showed that GLP2R protein levels in the duodenum and jejunum increased compared with animals on a STD. GLP2R protein was not detectable in the mucosal cells (Fig. 4).

Effects of GLP2 (3–33) on intestinal growth induced by HFD

To investigate the role of endogenous GLP2 in the response of the small intestine to a HFD, mice of both groups were treated daily for 4 weeks with GLP2 (3–33). As shown in Fig. 5, chronic treatment with GLP2 (3–33) (30–60 ng) did not significantly modify the crypt–villus height in STD-fed mice. On the contrary, in DIO mice, administration of GLP2 (3–33) (30–60 ng) significantly reduced the crypt–villus height, the number of cells per villus as well as the number of Ki67-positive cells, and it increased the percent of caspase-3-positive cells in both the duodenum and the jejunum (Fig. 6). In addition, mice on a HFD chronically treated with GLP2 (3–33) gained less weight than PBS-treated mice (+5.3 ± 1.1 vs +17.8 ± 2.9 g% respectively), although food intake/die (4.3 ± 0.2 vs 4.2 ± 0.3 g respectively) and small intestinal weight relative to body weight were not affected.

Discussion

The responsiveness of mouse or human small intestine to exogenous GLP2 has been well demonstrated (Drucker et al. 1996, Hartmann et al. 2000, Jeppesen et al. 2001, 2005, Estall & Drucker 2006). Furthermore, endogenous GLP2 is a physiological regulator of refeeding-induced and resection-induced intestinal adaptative growth in rodents (Dahly et al. 2003, Shin et al. 2005, Bahrami et al. 2010); it may play a role in the growth of the small intestine around birth in pigs (Petersen et al. 2003), but it is not yet known whether endogenous GLP2 may be implicated in the regulation of the gut morphological changes following
a chronic HFD. This is an interesting issue in view of the acquired evidence that GLP2 is a nutrient-dependent signal that facilitates the absorption of dietary fat (Meier et al. 2006, Hsieh et al. 2009) and there may be a link between GLP2 function and dyslipidemia. In this study, we provide evidence for a role of endogenous GLP2 in the intestinal adaptation to a chronic HFD and for a dysregulation of the peptide plasma levels and of Glp2r expression in the small intestine of DIO mice.

Initially, to analyze whether small intestinal morphology was affected by the HFD in mice, we took into account some morphometric parameters, which could be likely influenced by GLP2, according to the literature (Hartmann et al. 2000, Shin et al. 2005, McDonagh et al. 2007). Duodenal and jejunal segments were examined because fat absorption commonly takes place in the proximal and middle part of the small intestine and it has been already reported that dietary fat has the strongest effects on gene expression in these regions of the small intestine (de Wit et al. 2011). In addition, the major effects of exogenous GLP2 treatment were noted in the mucosa of the upper and middle small intestine, with lesser effects in the distal small intestine (Hartmann et al. 2000).

In our experimental model, mice chronically fed a HFD showed changes in some morphometric parameters, consistent with the results of the previous studies (Sagher et al. 1991, Thomson et al. 1996, Scoaris et al. 2010, De Barros Alencar et al. 2012), which were similar in duodenal and jejunal segments. Indeed, we cannot establish whether the effects observed are specifically induced by the fat or by the different caloric intake. However, other reports described significant trophic effects of long-chain fatty acids or a HFD on small intestinal mucosa of normal animals (Singh et al. 1972, Williamson & Chir 1978, Grey et al. 1984, Little et al. 2007). Early exposure to a HFD augments and accelerates intestinal adaptation in a rat model with short bowel syndrome (Sukhotnik et al. 2004).
We found an increase in the crypt–villus height, which results from variation in the number of enterocytes per villus. This might be functional to extend the capacity of fat absorption. These observations corroborate the hypothesis that in obese mice, increased surface of absorption contributes to the weight gain of these animals, as previously suggested (de Wit et al. 2008, Scoaris et al. 2010). Additionally, we observed a number of Ki67-positive cells more pronounced in the crypt of HFD-fed mice than in those fed a STD, but we failed to find differences in the apoptotic cell number. Together, these data suggest that only the increased proliferation rate is responsible for the raise of the small intestinal villi induced by dietary fat.

However, although on the basis of our experiments, it remains to be determined whether effects of a HFD on intestinal architecture depend on caloric content or on the dietary fat itself, the main objective of this study was to verify a possible involvement of GLP2 in the intestinal response. To elucidate the endogenous physiological action of GLP2, it is necessary to eliminate the impact of the peptide. So far, three different approaches have been used: attenuation of circulating GLP2 using immunoneutralizing antibodies (Hartmann et al. 2002), administration of GLP2 (3–33), GLP2R antagonist (Shin et al. 2005, Nelson et al. 2008, Iakoubov et al. 2009, Trivedi et al. 2012), or use of Glp2r−/− mice (Bahrami et al. 2010, Lee et al. 2012). The GLP2R antagonist has enabled experiments delineating the importance of endogenous GLP2 action in vivo (Shin et al. 2005, Nelson et al. 2008), while gene knockout studies are often confounded by the development of compensatory mechanisms that reduce and blunt the consequences of the elimination. Therefore, we verified our working hypothesis using GLP2 (3–33) as GLP2R antagonist.

In our study, treatment over 4 weeks with GLP2 (3–33), which was well tolerated by the mice and did not induce any observable intestinal dysfunction (e.g. diarrhea), did not modify the crypt–villus height in control animals as previously suggested (Iakoubov et al. 2009). In mice fed a HFD, it did not modify the food intake or small intestine-normalized weight but reduced the crypt–villus height in a dose-dependent manner. Moreover, in DIO small intestine, GLP2 (3–33) decreased the number of cells per villus and the crypt Ki67-positive cells, and it increased the percent of caspase-3-positive cells, suggesting that changes in crypt–villus height are due to decreased proliferation rate and increased cell apoptosis, likely through reduction of the actions of endogenous GLP2. Indeed, GLP2 (3–33) has been shown to be biologically inactive with respect to
the stimulation of intestinal growth (Munroe et al. 1999), but it has been shown to function both as a weak partial agonist and as an antagonist of the GLP2R in rodents (Thulesen et al. 2002, Shin et al. 2005, Nelson et al. 2008, Baldassano et al. 2009). Doses of GLP2 (3–33) similar to those of our experiments have been administered to antagonize the effects of endogenous GLP2 (Shin et al. 2005), while GLP2 (3–33) induces responses as agonist at markedly higher amount of peptide (Hartmann et al. 2000, Thulesen et al. 2002). Furthermore, administration of 30 or 60 ng GLP2 (3–33) for 4 weeks is effective at preventing the development as well as the progression of colonic dysplastic growth in mice (Iakovoubov et al. 2009). Our evidence for a role of the GLP2R in the intestinal adaptive response to a HFD is in line with previous report that GLP2 immunoneutralization reduces intestinal growth associated with streptozotocin-induced, insulin-deficient, hyperphagic diabetes in the rat (Hartmann et al. 2002).

Mucosal changes due to the luminal presence of high fat were positively associated with changes in the plasma concentration of bioactive GLP2. In fact, increased GLP2 levels, in DIO animals, were consistent with the associated increases in crypt–villus height that occur following a chronic HFD. This finding supports the hypothesis that a HFD stimulates GLP2 release to a greater extent than a STD. Indeed, a nutrient-dependent hypothesis that a HFD stimulates GLP2 release to a greater extent than a STD. It is interesting to note that we observed increased gene and protein expression of intestinal GLP2R in DIO mice despite greater plasma GLP2 concentrations. Although receptor downregulation after exposure to the receptor ligand is a common phenomenon and it has been reported to occur for GLP2R in pigs during development (Petersen et al. 2003), our observations are similar to GLP2R upregulation described in rats given exogenous GLP2 (Koopmann et al. 2008). Moreover, consistent with our results, increased nutrient intake increases plasma GLP2 and GLP2R mRNA in ruminant ileum (Taylor-Edwards et al. 2010). This positive correlation of GLP2 plasma levels and GLP2R mRNA and protein abundance in murine small intestine suggest that Glp2r expression is not negatively affected by increased plasma GLP2 concentrations. This is the first study to report changes in Glp2r expression at an intestinal level after a HFD. In fact, regulation of Glp2r expression has not been well described, especially in response to changes in nutrient intake. In mice, jejunal Glp2r mRNA expression was not affected by 24-h fasting and refeeding periods (Shin et al. 2005), but the nutritional state greatly influences the expression in the stomach (Rotondo et al. 2011).

Although we do not know the mechanism by which receptor expression in the small intestine is changed in response to a HFD, the upregulation of the GLP2/GLP2R system could further promote fat absorption in the intestine, as GLP2 does in physiological conditions (Hsieh et al. 2009).

In conclusion, taken together, these findings suggest that GLP2 may play a role in the murine intestinal adaptation to a HFD, leading to increasing energy intake, although its contribution relative to that of other growth factors remains to be determined. GLP2 (3–33) may represent a useful tool as mucosal anti-proliferative agent.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
S B was involved in study conception and design, chronic treatments of animals, statistical analysis, and interpretation of data; A A performed biomolecular experiments; F C was involved in the morphological analysis; F R performed the morphological analysis; and F M designed the study and wrote the paper.

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