

Inulin-type fructans modulate gastrointestinal peptides involved in appetite regulation (glucagon-like peptide-1 and ghrelin) in rats*

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(Received 20 February 2004 – Revised 29 April 2004 – Accepted 17 May 2004)

The hypothesis tested in the present study is that dietary fructans are able to modulate gastrointestinal peptides involved in the control of food intake, namely glucagon-like peptide (GLP)-1 (7-36) amide and ghrelin. After 3 weeks of treatment with a standard diet (control) or the same diet enriched with 100 g fructans varying in their degrees of polymerization (oligofructose (OFS), Synergy 1 (Syn) or long chain inulin)/kg, male Wistar rats were deprived of food for 8 h before sample collection. Dietary energy intake throughout the experiment was significantly lower ($P < 0.05$) in fructans-fed rats than in control rats, leading to a significant decrease ($P < 0.01$) in epididymal fat mass at the end of the treatment in OFS- and Syn-treated rats. GLP-1 (7-36) amide concentration in portal vein serum was higher in OFS- and Syn-fed than in control rats. Both GLP-1 (7-36) amide concentration and proglucagon mRNA concentrations were significantly greater ($P < 0.05$) in the proximal colonic mucosa of fructans-fed rats *v.* controls. Normally active ghrelin concentration in plasma increases during food deprivation and rapidly falls during a meal. In the present study, after 8 h of food deprivation, active ghrelin in the plasma remained significantly lower ($P < 0.05$) in OFS and Syn-fed than in control rats. These results are in accordance with the modifications of dietary intake and fat-mass development in short-chain fructans-treated rats and demonstrate the potential modulation of GLP-1 (7-36) amide and ghrelin by fermentable fibres such as fructans, which are rapidly and extensively fermented in the proximal part of the colon.

Glucagon-like peptide-1 (7-36) amide: Ghrelin: Inulin-type fructans: Oligofructose: Orexigenic: Anorexigenic

Our understanding of the molecular mechanisms regulating body weight provides potential opportunities for therapeutic development and renewed hope for potential dietary intervention. Current recommendations for the management of obesity and diabetes mellitus include an increase in dietary fibre intake, which may contribute to lower fasting and postprandial plasma glucose concentrations and improvement of glycaemic control (Vinik & Jenkins, 1988; American Diabetes Association, 2000). Dietary fibre, which may help to control food intake, is interesting in the context of nutritional management of obesity. Our previous studies have demonstrated that fructans obtained from chicory (*Cichorium intybus*) root inulin (Inu; a dietary fibre largely fermented in the caeco-colon) may be helpful in the control of plurimetabolic syndrome associated with obesity: when added to the diet, they lessen steatosis in obese Zucker rats, and may reduce triacylglycerolaemia in rats and in human subjects (Daubioul *et al.* 2000; Delzenne & Williams, 2002; Delzenne, 2003). Moreover, oligofructose (OFS), a short-chain fructan, given at the dose of 100 g/kg diet to Wistar rats, reduces postprandial glycaemia and partially restores insulin secretion (Kok *et al.*

1998; C Daubioul, unpublished results). A significant reduction in daily food ingestion in OFS-fed rats contributes to the improvement of steatosis and glycaemia in obese Zucker *falfa* rats or diabetic rats respectively (Kok *et al.* 1998; Daubioul *et al.* 2002; C Daubioul, unpublished results). We have previously shown that feeding OFS leads to an increase in total caecal glucagon-like peptide (GLP)-1 (7-36) amide concentration in rats (Kok *et al.* 1998). GLP-1 (7-36) amide is a thirty amino-acid peptide secreted from the L cells, which are mainly located in the jejunum, ileum and colon (Orskov *et al.* 1989). There are several papers showing that peripheral injection of GLP-1 (7-36) amide decreases food intake and consequently body weight in rats and human subjects (Flint *et al.* 1998; Naslund *et al.* 1999; Meier *et al.* 2002; Zander *et al.* 2002). Therefore, we propose that portal GLP-1 (7-36) amide could be a key hormone mediating the effect of OFS, and other fermentable fibres, on food intake, and glucose and lipid metabolism. The mechanism and relevance of endogenous modulation of GLP-1 (7-36) amide production by dietary fibre is poorly documented, but some results suggest that this peptide could constitute a link

Abbreviations: CT, control; GLP, glucagon-like peptide; Inu, inulin; OFS, oligofructose; Syn, Synergy 1.

*Results presented (oral communication) in part at the 9th European Nutrition Conference, 1–4 October 2003, Rome, Italy, and at the American Diabetes Association Research Symposium, 9–12 December 2003, Long Beach, LA, USA.

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between the outcome of fermentation in the lower part of the gut and the systemic consequences of the intake of 'colonic food' (such as fructans). Other gastrointestinal peptides are implicated in the regulation of body weight and food intake, such as a newly discovered gastric-derived hormone ghrelin. It is a circulating twenty-eight amino-acid peptide, primarily identified as the endogenous ligand of the pituitary growth hormone secretagogue receptor (Kojima *et al.* 1999). It emerges as one of the most powerful physiological orexigenic and adipogenic agents (Tschop *et al.* 2000) able to 'orchestrate' hunger and food-seeking through its fluctuating plasma concentrations (Cowley *et al.* 2003). GLP-1 (7-36) amide production occurs in different parts of the distal intestine (Orskov *et al.* 1989), and the site of production might influence the systemic distribution of GLP-1 (7-36) amide through the portal vein. Therefore, we have analysed the modulation of both portal GLP-1 (7-36) amide and peripheral ghrelin concentrations in the serum of rats fed three types of fructans, differing through their preferential site and extent of fermentation. We have also clarified the major intestinal site of proglucagon expression and GLP-1 (7-36) amide synthesis after fructans feeding, and established a relationship between peptide modulation and fructans effect on energy intake and fat-mass development.

Material and methods

Animals and diets

Male Wistar rats (Harlan, Horst, The Netherlands; six rats per group), weighing 145–160 g, were housed in individual cages in a temperature- and humidity-controlled room with a 12 h light–dark cycle. After an acclimatization period of 5 d before the experiment, control (CT) rats were fed a powdered A04 standard diet (UAR, Villemoisson-sur-Orge, France), whereas OFS-, Synergy 1 (Syn)- and Inu-treated rats received a diet prepared by mixing 90 g A04 standard diet with 10 g corresponding fructan (Raftilose P₉₅, Synergy 1 and Raftiline HP respectively (Orafti, Tienen, Belgium)). The A04 standard diet contained the following (g/kg dry diet): protein 193 (consisting of equivalent mix of soyabean and fish proteins); total carbohydrate obtained from maize, wheat, barley and bran (including starch 380, saccharose 30, cellulose 50, non-digestible carbohydrate 80) 704; lipid 30; mineral mixture 60; vitamin mixture 13 (Daubioul *et al.* 2000). Food intake, taking into account spillage, was assessed three times per week. The mean daily energy intake (kJ/d) was calculated as follows: food intake (g) × energy value of diet (kJ/g). The energy value for the control diet was 13.86 kJ/g and for OFS, Syn and Inu diets it was 13.08 kJ/g.

All rat experiments were approved by the local committee and the housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230315).

Chemicals

Raftilose P₉₅, Syn 1 and Raftiline HP (Orafti) are a mixture of glucosyl-(fructosyl)_n-fructose and (fructosyl)_m-fructose, but with an average degree of polymerization of 4.5

for Raftilose P₉₅ and 25.0 for Raftiline HP. Syn 1 consisted of Raftilose P₉₅–Raftiline HP (1:1, w/w). Other chemicals used were of the purest grade available and were purchased from Sigma (St Louis, MO, USA) and Merck (Darmstadt, Germany).

Blood samples

On day 21, food was withheld and 8 h later rats were anaesthetized by intra-peritoneal injection of sodium pentobarbital solution (60 mg/kg body weight; Nembutal[®], Sanofi Santé Animale, Benelux, Brussels). Portal vein blood samples were collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) containing dipeptidyl peptidase IV inhibitor (Linco Research, St Charles, MO, USA); after centrifugation, plasma was stored at –80°C. Concentrations of GLP-1 (7-36) amide were measured using an ELISA kit specific for GLP-1 (7-36) amide without cross-reactivity towards GLP-1 (9-36) amide, GLP-2 and glucagon (GLP-1 active ELISA kit; Linco Research). Cava vein blood samples were collected in EDTA tubes and plasma was stored at –30°C. Concentrations of active ghrelin were measured using a RIA kit specific for active ghrelin without cross-reactivity towards des-octanoylghrelin (Ghrelin Active RIA kit; Linco Research).

Tissue samples

Segments of the caecum and colon (proximal, medial, distal colon, corresponding to 20 mm segments taken just after the caecal junction, in the middle of the colon and just before the rectum respectively) were immediately excised, flushed with ice-cold saline (9 g NaCl/l), immersed in liquid N₂ and stored at –80°C for further mRNA and peptides analysis. Full and empty caecum, and epididymal fat pads, were weighed.

Intestinal glucagon-like peptide-1 (7-36) amide extraction

Extraction of GLP-1 (7-36) amide from intestinal segments (ileum, caecum and colon) was carried out with ethanol–acid solution (5 ml/g tissue). Samples were homogenized at maximum speed and placed at 4°C for 24 h. The homogenate was centrifuged (2000 g) and the supernatant fraction was decanted and diluted 200-, 500- and 500-fold in saline (9 g NaCl/l) for caecum, ileon and colon respectively. Concentrations of intestinal GLP-1 (7-36) amide were measured as previously described for blood samples.

Isolation of total RNA

Total RNA was isolated from each intestinal segment according to Chomczynski & Sacchi (1987) using the RNeasy Total RNA Isolation System (Promega, Leiden, The Netherlands). Approximately 250 mg intestinal tissue was used to extract total RNA. The quantity and the purity of RNA were determined by UV spectrophotometry at 260 nm and 280 nm. Total RNA (20 µg) was loaded onto agarose gel containing formaldehyde and visualized by ethidium bromide UV light-staining to check ribosomal RNA 18S and 28S integrity.

Proglucagon messenger and actin mRNA RT-PCR

RT-PCR was performed with an input of 1 µg RNA using the kit for RT-PCR (Acces RT-PCR system; Promega). Primers used for the amplification of cDNA of interest were for the sequences of the forward and reverse primers respectively: 5'-GTAATGCTGGTACAAGGCAG-3' and 5'-TTGATGAAGTCTCTGGTGGCA-3' for proglucagon gene, and 5'-CTGACCGAGCGTGGCTACAG-3' and 5'-GGTGCTAGGAGCCAGGGCAG-3' for actin gene. The twenty-three cycles used for the detection of the proglucagon and actin transcripts correspond to the linear portion of the amplification curve (results not shown). PCR products (3 µl from each) were separated on an agarose (18 g/l) gel in tris-acetate EDTA buffer and visualized by ethidium bromide UV light-staining. Quantification of the PCR products was performed using the fluorimetric method: Pico-green® dsDNA Quantitation Reagent and Kit (Molecular Probes, Leiden, The Netherlands).

Statistical analysis

Results are expressed as mean values with their standard errors. Statistical differences between groups were evaluated by ANOVA followed by Tukey honestly significant difference *post hoc* test using SPSS 9.0.0 for Windows (SPSS, Chicago IL, USA). The level of significance was set at $P < 0.05$.

Results

Food energy consumption and growth

Energy intake during the experimental period was significantly different between groups (Fig. 1). Total energy consumption was significantly lower in OFS, Syn and Inu groups than in the control group ($P < 0.05$). Weight gain throughout the study was not significantly different between groups (Fig. 2). However, body weight was not significantly different between groups at the end of the treatment.

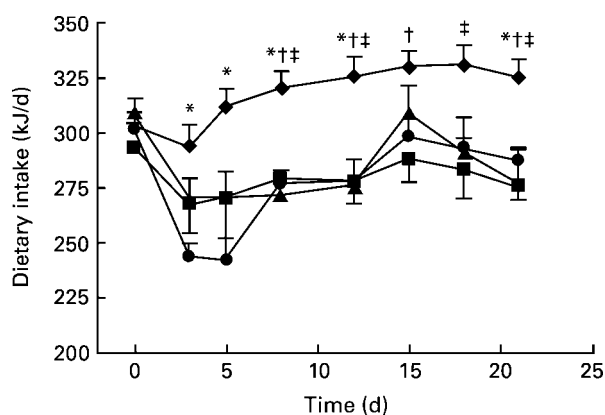


Fig. 1. Dietary intake (kJ/d) of rats fed a control diet (◆) or a diet supplemented with oligofructose (OFS, ●), Synergy 1 (Syn, ▲) or inulin (Inu, ■). For details of diets and procedures, see p. 522. Values are means with their standard errors shown by vertical bars (six rats per group). Mean values were significantly different from those of the control rats: * $P < 0.05$ OFS, † $P < 0.05$ Syn, ‡ $P < 0.05$ Inu.

Organ weights

The epididymal adipose tissue weight was about 30 % lower in OFS and Syn rats than in CT rats (g/kg body weight: CT 14.9 (SEM 102.0), OFS 10.7 (SEM 0.8), Syn 9.6 (SEM 0.6), Inu 13.7 (SEM 1.5); OFS and Syn *v.* CT $P < 0.05$, OFS and Syn *v.* Inu $P > 0.05$). Macroscopic analysis of the organs revealed caecum enlargement in OFS and Syn rats. The total caecum weight was significantly higher in OFS and Syn than in controls (CT 5.5 (SEM 0.3) g, OFS 12.0 (SEM 1.2) g, Syn 10.8 (SEM 1.6) g, Inu 8.3 (SEM 0.7) g; OFS and Syn *v.* CT $P < 0.05$). The caecal tissue weight was significantly higher in OFS, Syn and Inu than in control rats (g: CT 1.2 (SEM 0.1), OFS 2.0 (SEM 0.2), Syn 1.8 (SEM 0.1), Inu 1.5 (SEM 0.1); OFS, Syn and Inu *v.* CT $P < 0.05$, OFS *v.* Inu $P < 0.05$).

Intestinal proglucagon mRNA and glucagon-like peptide-1 (7-36) amide concentrations

Proximal colon proglucagon mRNA level was significantly greater in samples taken from OFS and Syn rats compared with controls ($P < 0.05$), whereas median colon proglucagon mRNA level was raised in OFS rats only ($P < 0.05$) (Table 1). Proglucagon mRNA levels did not differ between groups in caecum and distal colon.

Proximal colon GLP-1 (7-36) amide concentration was almost doubled in OFS and Syn rats compared with controls (Fig. 3). A positive correlation exists between proglucagon mRNA and GLP-1 (7-36) amide concentration in the proximal colon of all rats ($R 0.620$; $P < 0.001$, Pearson's correlation). Due to the important caecal tissue enlargement in OFS rats, and despite a similar peptide concentration/g tissue, GLP-1 (7-36) amide content expressed as pmol/total caecum was 3-fold the control value in OFS than in CT rats ($P < 0.05$) (Fig. 3).

Plasma glucagon-like peptide-1 (7-36) amide and ghrelin concentrations

Portal vein GLP-1 (7-36) amide concentrations were raised by 45 and 34 % in OFS and Syn rats respectively compared

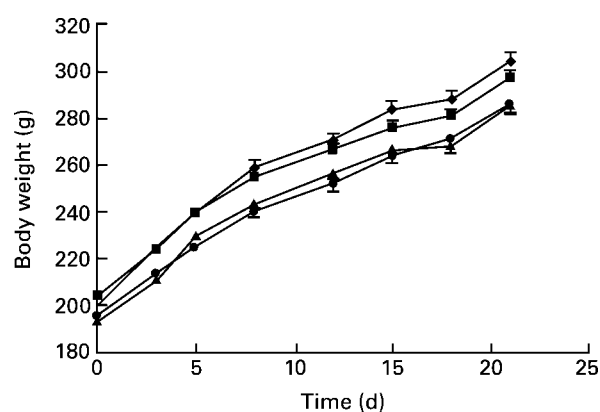


Fig. 2. Change in body weight (g) in rats fed a control diet (◆) or a diet supplemented with oligofructose (●), Synergy 1 (■) or inulin (▲). For details of diets and procedures, see p. 522. Values are means with their standard errors shown by vertical bars (six rats per group).

Table 1. Effects of a control diet and diets containing oligofructose, synergy 1 or inulin on intestinal proglucagon mRNA concentration*
(Mean values with their standard errors for six rats per group)

Diet	Caecum (RFU)		Proximal colon (RFU)		Medial colon (RFU)		Distal colon (RFU)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CT	0.57 ^a	0.02	0.83 ^a	0.07	0.88 ^a	0.06	0.73 ^a	0.06
OFS	0.64 ^a	0.06	1.29 ^b	0.07	1.07 ^b	0.06	0.84 ^a	0.05
Syn	0.61 ^a	0.04	1.22 ^b	0.11	0.93 ^{ab}	0.06	0.70 ^a	0.05
Inu	0.69 ^a	0.20	1.08 ^a	0.13	0.88 ^{ab}	0.07	0.73 ^a	0.05

RFU, relative fluorescence units (proglucagon mRNA/B-actin mRNA); CT, control; OFS, oligofructose; Syn, synergy 1; Inu, inulin.

^{a,b} Mean values for each organ with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see p. 522.

with CT rats (pmol/l: CT 7.82 (SEM 0.68), OFS 11.35 (SEM 1.18), Syn 10.49 (SEM 1.69), Inu 8.39 (SEM 1.25); $P > 0.05$).

Active ghrelin concentration measured in the cava vein at the time the rats were killed, i.e. 8 h after the last meal, remained significantly lower in OFS and Syn rats than in CT rats ($P < 0.05$) (Fig. 4).

Discussion

A few studies suggest that dietary fibre (rhubarb fibre, fermentable dietary fibre) may promote proglucagon expression and GLP-1 (7-36) amide production in rats and dogs (Reimer & McBurney, 1996; Reimer *et al.* 1997; Massimino *et al.* 1998). We have postulated that dietary fructans could modify the intestinal production of proglucagon-derived peptide, namely GLP-1 (7-36) amide. Therefore, we decided to compare the effect of three Inu-derived fructans, which differed in their degree of polymerization. OFS has a mean degree of polymerization 4.5 and is fermented mainly in the caecum and in the upper part of the colon

(Van Loo *et al.* 1999). Inu has a mean degree of polymerization 25.0 and is thus fermented in the lower colon segment; Syn contains short- and long-chain fructo-oligosaccharides. In rats pre-treated with those fructans for 3 weeks, the caecal enlargement and caecal content accumulation persisted 8 h after the last meal, and was much more pronounced in OFS and to a lesser extent in Syn rats. Another fermentable compound, lactitol, added at the concentration of 100 g/kg diet also produced a 2-fold increase in caecal tissue weight (Gee *et al.* 1996). The phenomenon was accompanied by an increase in plasma enteroglucagon concentration, another peptide synthesized from intestinal proglucagon.

Is the caecum the main organ responsible for proglucagon-GLP-1 (7-36) amide production after fructans feeding? By analysing mRNA proglucagon and GLP-1 (7-36) amide tissue concentration, we found that GLP-1 (7-36) amide content, even in CT rats, is mainly produced in the proximal and medial colon. Fructans containing short-chain oligosaccharides (OFS and Syn) preferentially increased both mRNA proglucagon and GLP-1 (7-36) amide concentration in the proximal and, to a lesser extent, in the median colon. However, even if GLP-1 (7-36) amide concentration/g caecal tissue remained unchanged after fructans feeding, caecum enlargement, observed mainly in rats fed short-chain fructans (OFS) caused a 3-fold increase in GLP-1

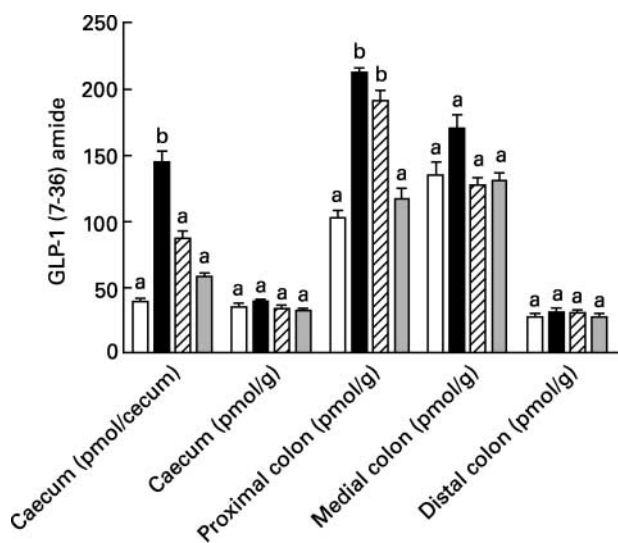


Fig. 3. Intestinal glucagon-like peptide (GLP)-1 (7-36) amide concentration of rats fed a control diet (□) or a diet supplemented with oligofructose (■), Synergy 1 (▨) or inulin (▩). For details of diets and procedures, see p. 522. Values are means with their standard errors shown by vertical bars (six rats per group). ^{a,b} Mean values with unlike superscript letters were significantly different (Tukey test separately for each organ; $P < 0.05$).

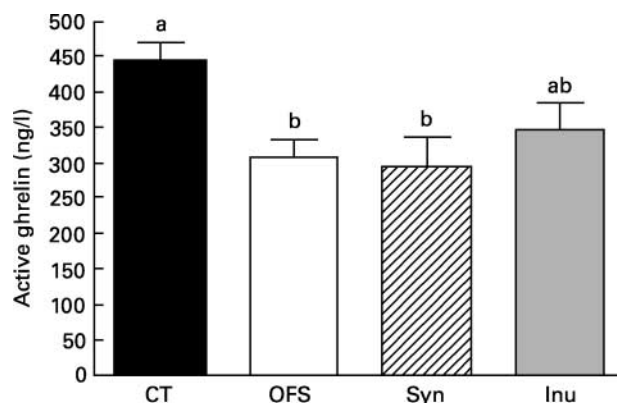


Fig. 4. Plasma active ghrelin (ng/l) in rats fed control diet (CT) or a diet supplemented with oligofructose (OFS), Synergy 1 (Syn) or inulin (Inu). For details of diets and procedures, see p. 522. ^{a,b} Mean values with unlike superscript letters were significantly different (Tukey test separately for each organ; $P < 0.05$).

(7-36) amide content in the whole organ compared with controls. An increase in proglucagon mRNA concentration has already been shown in dogs fed fermentable dietary fibre (100 g/kg diet) for 14 d (Massimino *et al.* 1998); this was accompanied by higher GLP-1 (7-36) amide incremental area under the curve after a glucose load. Here, we showed that both fructans, which cause a significant increase in proximal colon GLP-1 (7-36) amide, also produce an increase in GLP-1 (7-36) amide concentration in the portal vein. In mice, a high-fibre diet (300 g/kg diet) increased serum GLP-1 (7-36) amide, a phenomenon linked by the author to the increased proglucagon mRNA content in the colon, but also in ileum and jejunum (Nian *et al.* 2002). Reimer *et al.* (1997) have also shown that the addition of 50 g rhubarb fibre/kg diet to rats for 14 d increases proglucagon mRNA in the ileum, but not in the colon; the authors attributed the lack of changes in the colon to the diurnal variation in colonic production of SCFA (Reimer *et al.* 1997).

In fact, SCFA, and mainly butyrate, seem to be the best candidates to explain an effect on intestinal proglucagon expression (Tappenden *et al.* 1998). We did not investigate whether the increase in proglucagon mRNA concentration was due to a greater gene expression or to an increase in mRNA stability (Philippe *et al.* 1987). When fructans is present in the diet of rats (90 g/kg), intestinal butyrate concentration is doubled, but there is also an increase in acetate and propionate (Le Blay *et al.* 1999). Moreover, the profile of SCFA (the relative proportion of acetate, propionate, butyrate) in the caecal content differs following the degree of polymerization of fructans ingested by rats (Nyman, 2002); the SCFA profile is not similar in the caecal and colonic content of rats treated with OFS (Le Blay *et al.* 1999).

Could those modifications of SCFA be involved in the differential modulation of proglucagon mRNA content by dietary fructans? This question is important, and the relevance of qualitative and quantitative modifications of SCFA observed in fructans-fed rats in the modulation of proglucagon gene expression and GLP-1 (7-36) amide secretion will require an adequate *in vitro* intestinal model (Cao *et al.* 2003).

Is the colonic and portal intestinal GLP-1 (7-36) amide concentration observed in our present study in OFS and Syn rats a prerequisite to generate a decrease in food consumption? We have observed that energy intake was similarly decreased in the three groups of rats receiving fructans compared with the control, whereas Inu had no major effects on GLP-1 (7-36) amide. Due to its capacity to form a particle gel network, Inu might be involved in a reduction in gastric emptying, which could contribute to a reduced food intake, by a mechanism that may be independent of gastrointestinal peptides modulation (Franck, 2002). The food efficiency coefficient (weight gain (g/d):food intake (g/d)) in the previous 24 h was slightly lower in Inu-treated rats than in controls on day 4 of the treatment, but not later on during the treatment (results not shown).

Could other peptides be implicated in the satietogenic effect of fructans? Glucose-dependent insulinotropic polypeptide concentration was also promoted through

OFS feeding in rats in a similar protocol (Kok *et al.* 1998); glucose-dependent insulinotropic polypeptide is a hormone involved in GLP-1 (7-36) amide production and secretion (Rocca & Brubaker, 1999).

However, GLP-1 (7-36) amide and ghrelin concentrations are inversely correlated after glucose ingestion (Djurhuus *et al.* 2002). Moreover, Lippl *et al.* (2004) have demonstrated that GLP-1 (7-36) amide contributes to the inhibition of ghrelin secretion in an isolated rat stomach model. Therefore, we measured serum ghrelin concentrations, which remained lower in short-chain fructans-fed rats than in CT rats. Interestingly, we may find a parallel between GLP-1 (7-36) amide increase, ghrelin decrease, epididymal fat mass development, since the latter variable is significantly decreased in OFS and Syn rats. Few experimental arguments have been published to explain how ghrelin and/or GLP-1 (7-36) amide play a role in metabolic regulation of adipose tissue.

Conclusion

In conclusion, we report that short-chain fructans (present in OFS, and to a lesser amount in Syn), which are fermented in the caecum and in the proximal colon, lead to an increase in proglucagon–GLP-1 (7-36) amide synthesis, with consequences on the portal concentration of GLP-1 (7-36) amide (increase) and on peripheral concentration of ghrelin (decrease). It is thus able to modulate endogenous production of incretins and anorexigenic–orexigenic peptides through the ingestion of dietary fibre in rats, the effect being dependent on the site of fermentation. The increase in colonic GLP-1 (7-36) amide occurs together with a correlated increase in GLP-2 content (results not shown). This later peptide could be involved in the trophic effect of fructans on distal intestine (Tappenden *et al.* 2003). GLP receptor knock-out animals would be a useful model to assess the relevance of the modulation of GLP in the physiological effects related to feeding fructans (Burcelin *et al.* 2001; Preitner *et al.* 2004).

Acknowledgements

Supported by a FSR grant from the Université Catholique de Louvain. P. D. C. is financed by Patrimoine de la Faculté de Médecine, Université Catholique de Louvain, Brussels, Belgium. OFS, Syn and Inu were kindly provided by Orafiti (Tienen, Belgium).

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