Involvement of endogenous glucagon-like peptide-1(7–36) amide on glycaemia-lowering effect of oligofructose in streptozotocin-treated rats

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Abstract

We have evaluated the influence of oligofructose (OFS), a fermentable dietary fibre, on glucose homeostasis, insulin production and intestinal glucagon-like peptide-1 (GLP-1) in streptozotocin-treated diabetic rats.

Male Wistar rats received either i.v. streptozotocin (STZ; 40 mg/kg) or vehicle (CT); one week later, they were fed for 6 weeks with either the standard diet (STZ-CT), or with a diet containing 10% oligofructose (STZ-OFS); both diets were available ad libitum. In a second set of experiments (duration 4 weeks), a supplemental group of food-restricted rats (STZ-Res) receiving a similar intake as CT rats, was added.

OFS improved glucose tolerance and reduced food intake as compared with STZ-CT rats in both the post-prandial state and after an oral glucose tolerance test. After 6 weeks, portal and pancreatic insulin concentrations were doubled in STZ-OFS rats. Food restriction improved these parameters when compared with STZ-CT rats, but to a lesser extent than in the STZ-OFS group. We have shown that OFS treatment increased portal and colonic GLP-1(7–36) amide levels and doubled colonic proglucagon and prohormone convertase 1 mRNA levels; both OFS and food restriction lowered ileal GLP-1(7–36) amide levels as compared with levels in STZ-CT rats.

We propose that OFS, through its fermentation in the colon, promotes the expression and secretion of colonic peptides, namely GLP-1(7–36) amide, with beneficial consequences on glycaemia, insulin secretion and hyperphagia in diabetic rats.

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Introduction

Dietary fibres are often cited as food components susceptible to lower fasting and post-prandial plasma glucose concentrations, and are proposed as key nutrients in the control of glycaemia in diabetic patients (Roberfroid & Delzenne 1998, Jenkins et al. 1999). Soluble dietary fibres, such as guar gum, pectin, or mucilages, reduce post-prandial glycaemia by delaying gastric emptying through their gel-forming effect (Nuttall 1993). But there are other dietary fibres which do not exhibit gel forming properties which seem to be promising in the control of metabolic disorders associated with glucose intolerance and obesity. This is the case with dietary fructans, which are commonly found in several vegetables and cereals (onion, garlic, wheat etc.) and in food products in which they are added for their nutritional or organoleptic properties (fat or sugar replacer) (Roberfroid & Delzenne 1998). Fructans are mainly known as prebiotics, meaning that, through their fermentation in the caeco-colon, they selectively stimulate lactic acid-producing bacteria. These bacteria produce short chain fatty acids (SCFA) involved in the modulation of gut physiological functions (immunity, cell proliferation, mineral absorption etc.) (Delzenne 2003, Delzenne et al. 2003). We have shown in previous studies that oligofructose – a short chain fructan obtained from inulin decreases steatosis by reducing spontaneous food intake in obese Zucker Fa/Fa rats (Daubioul et al. 2002). Oligofructose also decreases the glucose/insulin ratio in high fructose-fed rats, suggesting an improved beta-cell glucose sensitivity (Busserolles et al. 2003). We have also demonstrated that a lower post-prandial glycaemia in oligofructose-fed rats could participate in decreased lipogenesis and triacylglycerol–very low density lipoprotein (TG-VLDL) secretion (Kok et al. 1998a, Delzenne & Williams 2002). The fermentation products of dietary fibres have been shown to increase proglucagon mRNA in the colon (Reimer & McBurney 1996, Cani et al. 2004).
In the intestine, the post-translational modification of the proglucagon gene by prohormone convertase 1 (PC1) leads to the production of glucagon-like peptide-1(7–36) amide (GLP-1(7–36) amide) which, among its pleiotrophic effects, participates in pancreatic beta cell proliferation and neogenesis and is also involved in the control of food intake (Orskov et al. 1989, Domon-Dell et al. 2002, Drucker 2002). The modulation of glucose tolerance by oligofructose in hyperglycemia/hypoinsulinemia has never been related to its efficiency to modify endogenous GLP-1(7–36) amide production. Therefore, the aims were to study (1) the effect of oligofructose on glucose tolerance and insulin production and (2) to evaluate GLP-1(7–36) amide production in streptozotocin-treated diabetic rats.

Materials and Methods

Animals and diets

Rats received care in compliance with the institution’s guidelines from the National Academy of Sciences (NIH publication 86–23; http://www.nih.gov). All animal experiments were approved by the local committee and the housing conditions were as specified by the Belgian Law of November 14 1993 on the protection of laboratory animals (agreement no. LA 1230315).

In all experiments, 8-week-old male Wistar rats weighing 220–250 g were obtained from Harlan (Horst, The Netherlands) and were housed in a temperature- and humidity-controlled room with a 12 h light/12 h darkness cycle. Animals were housed and acclimated in individual wire mesh-bottomed cages. Rats were acclimated for 7 days, and were given free access to a powdered A04 standard diet (A04, UAR, Villemoisson-sur-Orge, France) and water before the experimental period. The diet was removed at 0900 h and streptozotocin (STZ) (Sigma, St Louis, MI, USA) (40 mg/kg body weight) or the same volume of saline (citrate buffer 0·1 M pH 4·5) was injected into the caudal vein of rats (n = 10 per group) during the afternoon.

Rats then received the standard diet and tap water ad libitum for one week. One week after STZ injection, only rats with post-prandial glycaemia between 15 mM and 35 mM were kept for the experiment (remaining animals n = 5 per group), and these were randomised in order to get the same mean glycaemia in all groups before nutritional treatment.

In a first experiment, rats were fed either the standard diet (STZ–CT) or the oligofructose diet (STZ–OFS), prepared by mixing 900 g standard diet with 100 g Raftilose P95 (ORAFI, Tienen, Belgium; Raftilose P95 consisted of a mix of fructooligosaccharides with a number of fructosyl moieties from 2 to 7). Both diets were available ad libitum. All animals were killed after 6 weeks (n = 5 per group).

In a second experiment, an additional group was added (STZ–Res), consisting of streptozotocin-treated diabetic animals food-restricted in order to receive the same amount of diet as the non-diabetic rats (CT). All the animals (n = 5 per group) were killed after 4 weeks. In all experiments, food intake and body weight were monitored twice a week.

Blood and tissue samples

An oral glucose tolerance test (gavage with 2 g glucose/kg body weight) was performed after 4 weeks of treatment (experiment 1) in rats previously fasted for at least 18 h, and blood was sampled at 0, 15, 30, 120 and 180 min for glucose and insulin measurement.

During the treatment, blood samples were taken from the tail vein in EDTA tubes (Sarstedt, Nümbrecht, Germany), and the plasma obtained by centrifugation was stored at −20 °C. At the end of the experiment, rats were anaesthetised by intra-peritoneal injection of pentobarbital (60 mg/kg body weight, Nembutal, Sanofi Santé Animale Benelux, Brussels, Belgium), and portal vein blood samples were collected in EDTA tubes containing dipeptidyl peptidase IV inhibitor (10 µl/ml) (DPPIV inhibitor, Linco Research, St Charles, MI, USA); after centrifugation, plasma was stored at −80 °C.

Segments of distal ileon (4 cm of intestine taken above the junction with the caecum), and colon corresponding to segments taken between the caecal junction and the rectum, were immediately excised, flushed with ice-cold saline, immersed in liquid nitrogen, and stored at −80 °C for further mRNA and peptides analysis.

Pancreatic insulin content and morphometry

Pancreata were removed, weighed and processed either for measurement of pancreatic insulin content or for morphometric analysis. Insulin was extracted with ethanol/acid (0·15 mol/l HCl in 75% v/v ethanol in water). Pancreata were placed in ice-cold fixative (20 g/l paraformaldehyde and 2 g/l glutaraldehyde in 70 mmol/l phosphate buffered saline (PBS), pH 7·4) overnight at 4 °C, followed by 4 washes at 4 °C in PBS over a 48-h period, dehydrated in ethanol and embedded in paraffin. Pancreatic beta cell mass was calculated following immunohistochemistry for insulin on sections obtained throughout the pancreas of known weight. The area of tissue was measured in three to six different sections per animal (five animals per group: non-diabetic control (CT), streptozotocin control (STZ–CT), streptozotocin + oligofructose diet (STZ–OFS) and streptozotocin + restricted diet (STZ–Res)) and beta cell volume density was estimated by calculating the proportion of cells immunoreactive for insulin per sectional area of total pancreas. The beta cell volume density corresponds to the ratio of beta cell area to pancreatic parenchymal area. The beta cell mass was calculated by multiplying the beta cell volume density by the weight of the pancreas (Arany et al. 2004).
**Peptides and glucose measurement in biological samples**

Glucose concentration was measured using an enzymatic procedure (Elitech Diagnostics, Brussels, Belgium). Plasma insulin was measured by ELISA (ultrasensitive rat insulin-high range rat insulin ELISA kit, Mercodia, Uppsala, Sweden). Plasma C-peptide was measured by RIA (rat C-peptide RIA kit, Linco Research).

Extraction of GLP-1(7–36) amide from intestinal segments (ileum, caecum and colon) was carried out with ethanol/acid (5:1 v/v) solution (5 ml/g tissue). Samples were homogenised at top speed (24 000 r.p.m.) and kept for 24 h at 4 °C. Homogenates were centrifuged (2000 g) and the supernatant was decanted and diluted in saline 200-fold for caecum and 500-fold for ileon and colon. Concentrations of GLP-1(7–36) amide were measured either in the plasma or in tissue extracts, using an ELISA method (GLP-1 active ELISA kit, Linco Research).

**mRNA measurement**

Total RNA was isolated from each intestinal segment according to the protocol provided with the RNAGents total RNA isolation system (Promega, Leiden, The Netherlands). Briefly, approximately 250 mg intestine were added to denaturing solution (guanidium isothiocyanate 4 M, sodium citrate 26 mM, N-lauryl sarcosine 0·5%, beta-mercaptoethanol 0·125 M) and homogenised 2 times at top speed (24 000 r.p.m.) for 15 s. Sodium acetate 250 µl 2 M pH 4, and 2·5 ml phenol:chloroform:iso-amyl alcohol (99:24:1, pH 4·7) were added to each sample. Tubes were kept for 15 min at 4 °C and then centrifuged at 10 000 g for 20 min at 4 °C. About 2·5 ml of the RNA containing upper phase were recovered to a new centrifuge tube. An equal volume of isopropanol was added to precipitate RNA within 3 h 30 min at −20 °C. Tubes were centrifuged at 10 000 g for 15 min at 4 °C; the supernatant was decanted and the RNA pellet was washed by adding of 2·5 ml ice-cold 75% ethanol, and centrifuged at 10 000 g for 15 min at 4 °C (2 times). The ethanol was drawn off and the pellet was diluted in 400 µl RNase free water. RNA quantity and purity were determined by UV spectrophotometry at 260 nm and 280 nm. Total RNA (20 µg) was loaded onto agarose gel containing formaldehyde and was visualised by ethidium bromide UV light staining to check ribosomal RNA 18S and 28S integrity.

**Proglucagon, prohormone convertase 1 (PC1) and actin mRNA RT-PCR conditions**

RT-PCR was performed with an input of 1 µg RNA using the kit for RT-PCR (Access RT-PCR system, Promega). Primers used for the amplification of the cDNAs of interest were: forward 5’-GTATATCGTGGTACAGGCAG-3’ and reverse 5’-TTGATGAAGTCTCTTGTTGGCA-3’ for the proglucagon gene; forward 5’-GGTACCCAAAACCTCCAGCA-3’ and reverse 5’-GGCTTGAGCCTTTCCAGC-3’ for PC1; and forward 5’-CTGACCCGACCGTGCTCACAG-3’ and reverse 5’-GGTGCTAGAGCCAGGCGACG-3’ for the actin gene. The 23 cycles used for the detection of the proglucagon and actin and the 35 cycles for PC1 transcripts correspond to the linear portion of the amplification curve (data not shown). PCR products (3 µl from each) were separated on a 1·8% agarose gel in Tris-acetate EDTA and were visualised by ethidium bromide UV light staining. Quantification of the PCR products was performed using the fluorimetric method using the Picogreen dsDNA quantitation reagent and kit (Molecular Probes, Leiden, The Netherlands).

**Results**

**Effects of oligofructose on plasma glucose and insulin levels during oral glucose tolerance test (OGTT) in streptozotocin-treated diabetic rats**

As shown in Fig. 1, oligofructose improved glucose tolerance and increased insulin levels in streptozotocin-treated diabetic rats. The glucose area under the curve (AUC) was significantly lower in STZ-OFS than in STZ-CT rats, but remained higher than that measured in CT rats. Plasma insulin response to glucose during OGTT was markedly increased in STZ-OFS as compared with STZ-CT rats. A similar insulin AUC was measured in CT and STZ-OFS rats.

**Effects of oligofructose on plasma insulin, C-peptide, pancreatic insulin content and beta cell mass in streptozotocin-treated diabetic rats**

Since plasma insulin was significantly improved in STZ-OFS rats during the OGTT, we investigated the potential modulation of insulin production by oligofructose by measuring C-peptide, pancreatic insulin content and beta cell mass at the end of the experiment. As shown in Fig. 2A, post-prandial glycaemia of STZ-OFS rats was significantly lower than in STZ-CT rats, but remained 20% higher than in CT rats. Plasma insulin content (Fig. 2B) of STZ-OFS rats was significantly higher than STZ-CT rats, but slightly lower than the value in CT rats.
Plasma C-peptide content exhibited a similar profile to that of plasma insulin (Fig. 2B insert). Pancreatic insulin content (Fig. 3A) was drastically reduced in STZ-CT rats as compared with CT rats. However, both pancreatic insulin level and beta cell mass were about two times higher in STZ-OFS rats than in STZ-CT rats (Fig. 3B).

Effects of oligofructose on body weight gain and food consumption in streptozotocin-treated diabetic rats

At the end of the treatment, the body weight gain was 60% lower in STZ-CT than in CT and STZ-OFS rats (Fig. 4A). From the first week following STZ treatment, the STZ-CT group presented hyperphagia which
remained significantly higher than in the CT group until the end of the experiment (Fig. 4B). The rats in the STZ-OFS group normalised their food consumption so as to eat the same amount as the rats in the CT group from the first week until the end of the treatment (Fig. 4B).

Effects of oligofructose on plasma portal concentrations of GLP-1(7–36) amide in streptozotocin-treated diabetic rats

Our previous studies revealed that oligofructose feeding increased plasma portal GLP-1(7–36) amide concentrations in normal rats (Cani et al. 2004). This peptide is known to improve glucose tolerance by modulating insulin secretion, beta cell mass and food intake (Drucker 2003). Since, in the present study, all these parameters were improved by oligofructose, we measured plasma portal GLP-1(7–36) amide. As shown in Fig. 5, STZ treatment induced a significant increase in portal GLP-1(7–36) amide levels: the level doubled in STZ-CT as compared with CT rats. Nevertheless, STZ-OFS rats exhibited a more marked increase in portal GLP-1(7–36) amide levels, reaching four times the CT value. Thus, we confirmed that oligofructose feeding increases plasma portal GLP-1(7–36) amide.

Effect of oligofructose and food restriction on beta cell mass

We performed a second set of experiments in order to identify the influence of the specific effect of oligofructose on the modulation of GLP-1(7–36) amide production versus food restriction in streptozotocin-treated diabetic rats.

We confirmed that beta cell mass was about two times higher in STZ-OFS rats than in STZ-CT rats and that food restriction did not influence beta cell mass.
(mg/pancreas: CT $7.97 \pm 1.37^a$, STZ-CT $0.59 \pm 0.16^b$, STZ-OFS $1.46 \pm 0.57^b$, STZ-Res $0.55 \pm 0.21^b$, % of beta cell mass: CT $0.85 \pm 0.14^a$, STZ-CT $0.08 \pm 0.03^b$, STZ-OFS $0.17 \pm 0.06^b$, STZ-Res $0.06 \pm 0.02^b$; mean values with different superscript letters were significantly different, $P<0.05$). Histological analysis confirmed these results. STZ-CT and STZ-Res rats exhibited a lower number and size of Langerhans islets than CT rats, and also included a lot of necrotic cells. In the pancreatic tissue of STZ-OFS rats, the alterations were more discrete, the number and the size of Langerhans islets being higher than those observed in STZ-CT rats (data not shown).

**Effect of oligofructose and food restriction on portal and intestinal GLP-1(7–36) amide content in streptozotocin-treated diabetic rats**

Figure 6A shows that STZ–Res rats exhibited a similar plasma portal GLP–1(7–36) amide concentration as CT rats. Levels of plasma portal GLP–1(7–36) amide were two times higher in STZ–CT than in CT rats, and four times higher in STZ–OFS than in CT rats. GLP–1(7–36) amide is produced by L–cells (mainly located in the ileon and colon) from posttranslational processing of proglucagon by the PC1 enzyme. Therefore, GLP–1(7–36) amide, proglucagon and PC1 mRNA levels were measured in these intestinal segments.

As shown in Fig. 6B, ileal GLP–1(7–36) amide concentration was slightly increased (30%) in STZ-CT rats compared with CT rats; this phenomenon did not occur in STZ-OFS and in STZ-Res rats. A major and significant increase (by about threefold) in GLP–1(7–36) amide concentration was observed in the colonic tissue of STZ-OFS rats. Proglucagon mRNA in the different intestinal segments was similar in CT and STZ-CT rats, whereas

**Figure 5** Effect of oligofructose on plasma portal vein GLP-1(7–36) amide content in streptozotocin-diabetic rats after 6 weeks of treatment. CT and STZ-CT rats were fed a standard diet and STZ-OFS rats were fed a standard diet with 10% oligofructose. Data are means ± s.e.m., $n=5$. Mean values with different superscript letters are significantly different, $P<0.05$.

**Figure 6** Effect of oligofructose and food restriction on the levels of GLP-1(7–36) amide in the portal vein (A) and the intestinal segment (B), and on the levels of proglucagon (C) and PC1 mRNA (D). CT and STZ-CT rats were fed a standard diet and STZ-OFS rats were fed a standard diet with 10% oligofructose. STZ-Res rats were food restricted so as to receive the same amount of food as the CT rats. RFU ratio, relative fluorescence units ratio of proglucagon or PC1 mRNA/beta actin mRNA. Data are means ± s.e.m., $n=5$. Mean values with different superscript letters are significantly different, $P<0.05$. Open bars, non-diabetic CT group; solid bars, STZ-CT group; hatched bars, STZ-OFS group; stippled bars, STZ-Res group.
PC1 mRNA was increased twofold in the colonic tissue of STZ-CT compared with CT rats. This effect was not observed in food-restricted rats. OFS treatment increased colonic proglucagon mRNA and PC1 mRNA by about 2- and 2.5-fold respectively compared with CT rats (Fig. 6C,D). Food restriction abolished all the effects of STZ treatment on intestinal GLP-1 synthesis.

Discussion

In the present paper, we report an interesting effect of dietary oligofructose on glucose homeostasis in STZ-treated diabetic rats: the addition of oligofructose at a dose of 10% for 4 weeks in the diet of rats improves glycaemia and plasma insulin, both in the post-prandial state and after an oral glucose load. Moreover, the treatment with oligofructose allows an improvement of pancreatic insulin and beta cell mass which was observed after 6 weeks of treatment. The influence of OFS on glucose metabolism and satiety might not be attributed to a gel-forming or viscous effect, as suggested for other dietary fibres (Nuttall 1993). Another mechanism can be proposed: an increasing number of papers demonstrates a key role of GLP-1(7–36) amide, secreted by the L-cells of intestinal tissue, in the control of glucose homeostasis and pancreatic beta cell function (increased insulin secretion, beta cell neogenesis, ...). The ileal GLP-1(7–36) amide concentration observed in STZ-CT rats may not be attributed to proglucagon or PC1 overexpression. The ileal overproduction of GLP-1(7–36) amide in STZ-treated diabetic rats is clearly linked to hyperphagia since it is prevented in both food-restricted and STZ-OFS animals. In this protocol, we demonstrate that OFS treatment has the same effect in diabetic rats as that previously observed in normal rats: it produces an increase in GLP-1(7–36) amide in the colon with consequences on its level in the portal vein (Cani et al. 2003, 2004). But what is the mechanism? Although data are scarce, there are a few papers that suggest that dietary fibres (rhubarb fibre, fermentable dietary fibres) may promote colon proglucagon expression and GLP-1(7–36) amide production in rats and dogs (Reimer & McBurney 1996, Reimer et al. 1997, Massimino et al. 1998). The increase in colonic GLP-1(7–36) amide after OFS treatment occurs together with higher levels of proglucagon and PC1 mRNA. The link between OFS feeding and proglucagon and PC1 gene expression in L cells is plausible, since the SCFA (such as acetate, propionate, butyrate) produced in the caeco-colon through the fermentation of fructans are involved in GLP-1(7–36) amide synthesis. The direct colonic instillation or systemic administration of a mix of SCFA increases proglucagon mRNA (Tappenden et al. 1996, 1998, Tappenden & McBurney 1998). Butyrate, which is overproduced in the caeco-colon of oligofructose-treated rats (Le Blay et al. 1999), seems to be the more potent among SCFA in the modulation of proglucagon gene expression; the molecular mechanism would involve an increase in caudal family homeobox protein cdx-2 expression, a transcription factor known to activate the rat proglucagon gene promoter (Jin & Drucker 1996, Domon-Dell et al. 2002).

The results obtained in the STZ-Res group, which had a 'more severe' food restriction than the STZ-OFS group, allows us to draw two conclusions: (1) the higher GLP-1 synthesis in STZ-CT rats is clearly linked to hyperphagia, since it is avoided by a drastic caloric restriction and (2) the beneficial effect of OFS is not due to food restriction only, since the improvement of pancreatic beta cell mass is observed in the STZ-OFS group and not in the STZ-Res group.

The modulation of colonic GLP-1(7–36) amide production by oligofructose in diabetic rats allows a fourfold increase in the portal concentration of GLP-1(7–36) amide, the portal vein being considered as the key biological compartment in which this peptide acts to regulate glucose homeostasis (Burcelin et al. 2001). Several studies report the interest in oligofructose as a means of lowering glycaemia in various animal models (Kok et al. 1998b, Daubioul et al. 2000). However, Perrin et al. (2003) have recently reported that oligofructose, given at a dose of 5% in diabetes-prone BioBreeding (BB) rats, was unable to modulate colonic mucosa and, consequently, could not counteract protein-induced hyperglycaemia in this model.
The authors suggested that the effects of fermentable fibres on the intestine are important in allowing them to play a regulatory role. We propose that the capacity of oligofructose to modulate L-cells-derived GLP-1 synthesis is an important factor which must be taken into account in the interpretation of the physiological effect on satiety and glucose metabolism. Peptides other than GLP-1, such as peptide YY, are also increased in intestinal tissue and portal veins of oligofructose-fed non-diabetic animals, this phenomenon being accompanied by lower serum ghrelin levels (Cani et al. 2004).

The relevance of the effect of fructans on levels of GLP-1(7–36) amide, glycaemia and satiety in humans is only poorly documented. An increase in serum GLP-1 (7–36) amide was already shown in patients presenting with gastro-intestinal reflux, who received 3 × 6·6 g/day oligofructose in their diet for 7 days, thus suggesting that the promotion of GLP-1(7–36) amide secretion may occur in humans despite the much lower intake of oligofructose/day as compared with rat studies (Piche et al. 2003). On the other hand, a decrease in glycaemia appears during fructans treatment in moderately hyperglycaemic patients (Yamashita et al. 1984).

In conclusion, dietary oligofructose, through their fermentation in the colon, promote the secretion of intestinal peptides, a phenomenon contributing to improved glucose homeostasis and a normalisation of the high food intake which is characteristic of streptozotocin-treated diabetic rats. Our results suggest that dietary fibre, which exhibits a high fermentation in the gut, might be proposed as a nutritional adjuvant in the management of diabetes, based on the endogenous promotion of incretin peptides secretion by the colon.

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References


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