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Abbreviations used: Glucose-6 phosphatase (G6Pase), Catalytic subunit of G6Pase (G6PC), starch-enriched diet (SED), protein-enriched diet (PED)

Research highlights

- New model of mice with specific G6Pase gene knockout in the intestine
- Molecular evidence of the regulatory role of intestinal gluconeogenesis in the control of food intake
- Intestinal gluconeogenesis is a causal link in protein-induced satiety

Keywords

Glucose-6 phosphatase, gluconeogenesis, intestine, protein-enriched diet, food intake,

Abstract

Protein-enriched diets are well known to initiate satiety effects in animals and humans. It has been recently suggested that this might be dependent on the induction of gluconeogenesis in the intestine. The resulting intestinal glucose release, detected by a "so-called" glucose sensor located within the walls of the portal vein and connected to peripheral afferents, activates hypothalamic nuclei involved in the regulation of food intake, in turn initiating a decrease in hunger. To definitively demonstrate the role of intestinal gluconeogenesis in this mechanism, we tested the food intake response to a protein-enriched diet in mice with an intestine-specific deletion (using an inducible Cre/loxP strategy) of the glucose-6 phosphatase gene (I-*G6pc*$^{-/-}$ mice) encoding the mandatory enzyme for glucose production. There was no effect on food intake in I-*G6pc*$^{-/-}$ mice fed on a standard rodent diet compared to their wild-type counterparts. After switching to a protein-enriched diet, the food intake of wild-type mice decreased significantly (by about 20% of daily calorie intake), subsequently leading to a decrease of 12 ± 2% of initial body weight after 8 days. On the contrary, I-*G6pc*$^{-/-}$ mice were insensitive to the satiety effect induced by a protein-enriched diet and preserved their body weight. These results provide molecular evidence of the causal role of intestinal gluconeogenesis in the satiety phenomenon initiated by protein-enriched diets.

1-Introduction

The massive development of obesity in western countries makes it increasingly crucial to better understand its underlying mechanisms. These include food intake disorders which are assumed to play a major role. In this context, the well-known satiety effect induced by protein-enriched diets in animals and humans has long been used to help obese subjects to decrease their sensation of hunger and lose weight [1-3]. Despite the number of studies dealing with the decrease of hunger induced by food proteins, the mechanisms by which the latter produce their satiety effects remain poorly understood [4-6]. Recently, we strongly suggested from studies in rats that the induction of intestinal gluconeogenesis provides a physiological explanation for the satiety effects induced by protein-enriched diets [7]. The existence of intestinal gluconeogenesis was demonstrated 10 years ago [8-10] and has been confirmed in humans [11, 12] and other species [13]. The three key regulatory enzymes of gluconeogenesis, namely glucose-6-phosphatase (G6Pase), cytosolic phosphoenolpyruvate carboxykinase and glutaminase, appeared to be strongly induced in the small intestine of rats fed on a protein-enriched diet (PED) [7]. This induction of gluconeogenic genes in the small intestine resulted in a portal release of glucose, lasting during the post-absorptive period in rats fed on the PED. The reduction of food intake consecutive to portal infusions of glucose has been reported in several previous studies on rats [14-16]. Moreover, portal infusions of glucose modify the electrical activity of portal afferents and neurons in the central area involved in the control of food intake [17-19]. In the particular case of PED in rats, the amount of glucose released by the gut accounted for no more than about 20% of total glucose occurrence in the whole body [7]. This portal glucose flux, however, was sufficient to be sensed by a “so-called” portal glucose sensor connected to afferents surrounding the portal vein and which activate the hypothalamic nuclei involved in the regulation of food intake,

thereby causing a decrease in subsequent food consumption [7, see 20-21 for recent reviews]. Interestingly, intestinal gluconeogenesis has also provided a mechanistic explanation for the hunger-curbing effects known to take place in another particular nutritional situation. Hence morbidly obese patients having undergone gastric bypass surgical procedures, rapidly report a decrease of their hunger sensations [22]. The induction of intestinal gluconeogenesis has been strongly suggested to account for the satiety effects and decreased food intake in a gastric bypass model in mice [23, 24 for recent review].

To definitively demonstrate the role of intestinal gluconeogenesis in controlling food intake, we developed a transgenic mouse model with time-dependent inactivation of this function specifically in the small intestine, by using a Cre/loxP strategy. To this end, we targeted the gene encoding the glucose-6 phosphatase catalytic subunit (G6PC), the key enzyme of gluconeogenesis, which governs the last biochemical reaction preceding the release of glucose by the organ. In this work, we tested whether the potency of PED to decrease food intake might be blunted after suppression of the capacity of the intestine to perform gluconeogenesis.

2-Material and methods

2-1 Generation of intestine-specific *G6pc*-null mice

B6.g6pc$^{lox/w}$ mice, in which exon 3 of *G6pc* gene was surrounded by two *loxP* sites (Figure 1A) [25], were crossed with transgenic B6.vill$^{creERT2/w}$ mice, expressing inducible Cre^{ERT2} recombinase under the control of the villin promoter [26]. The litters were genotyped at ten days old from tail genomic DNA analysis by PCR with specific primers as described previously [25]. Heterozygous B6.g6pc$^{lox/w}$.vill$^{creERT2/w}$ mice were crossed to generate

homozygous mice. To induce the excision of the *G6pc* exon 3, male adult (7-8 week-old) B6.g6pc$^{lox/lox}$.vill$^{creERT2/w}$ mice were injected intraperitoneally once daily with 100µl tamoxifen (10mg/ml, Sigma-Aldrich) on five consecutive days to obtain I-*G6pc*$^{-/-}$ mice. Wild-type C57Bl/6J (WT) mice were treated similarly to obtain control mice. All the mice were housed in the animal facility of Lyon 1 University (Animaleries Lyon Est Conventionnelle et SPF) under controlled temperature (22°C) conditions with a 12-hour light-12-hour dark cycle. The mice had free access to water and to a standard rodent starch-enriched diet (SED, Harlan).

All the procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. The regional animal care committee (CREEA, CNRS, Rhône-Alpes Auvergne, France) approved all the experiments.

2-2 Food intake and body weight measurements

Mice were individually housed with food and water *ad libitum*. Food consumption and body weight were monitored every day for two weeks. The composition of the diets was essentially based on that of a standard rodent diet with modifications in the starch-glucose/protein ratio, which were 65%/20% in SED and 20%/65% in protein-enriched diet (PED, Safe) (energy basis). The PED was isocaloric for SED (3.3 kcal/g). Proteins were a mixture of soya protein and casein (50/50).

2-3 Gene expression analyses

Mice were killed by cervical dislocation in the post-absorptive state (6h after food removal). Tissues were rapidly removed and frozen using tongs previously chilled in liquid N_2. The frozen tissues were kept at -80°C until use. Total RNAs were isolated from tissues with

TRIzol reagent (Invitrogen). Reverse transcription and real-time PCR were performed as described previously [27], by using sequence-specific primers (Table 1). The mouse ribosomal protein mL19 transcript (*Rpl19*) was used as a reference. G6Pase activity was assayed under conditions of maximal velocity, according to a procedure reported previously [10].

2-4 Statistical analyses

Results are reported as means ± SEM. The different groups were compared by Mann-Whitney tests.

3-Results and discussion

3-1 Initiation of intestine-specific deficiency of G6Pase in mice

We disrupted G6Pase specifically in the intestine, by a time-dependent and tissue-specific knockout of the *G6pc* gene, based on the same Cre/lox strategy as described previously by Mutel et al [25]. The transgenic B6.g6pc$^{lox/w}$ mice were crossed with transgenic B6.vill$^{creERT2/w}$ mice to generate heterozygous B6.g6pc$^{lox/w}$.vill$^{creERT2/w}$ mice. These mice express the inducible Cre^{ERT2} recombinase under the control of the villin promoter, conferring them with intestine-specific expression of the recombinase [26]. The mice were genotyped by PCR analysis of tail genomic DNA (Figure 1B). Treatment of male homozygous B6.g6pc$^{lox/lox}$.vill$^{creERT2/w}$ mice with tamoxifen at 7-8 weeks old resulted in the specific excision of exon 3 from *G6pc* alleles in the small intestine, generating a 595 bp PCR fragment from the I-*G6pc*$^{-/-}$ mice (Figure 1C). In the liver and kidney, the two other gluconeogenic tissues expressing *G6pc*, the same PCR amplification generated a 1189 bp fragment from the I-*G6pc*$^{-/-}$ mice, confirming the specific deletion in the small intestine (Figure 1C). Comparable

data were obtained at one week and up to fourteen weeks after the tamoxifen injections, confirming the persistence of the specific *G6pc* gene knockout in the small intestine. The truncated *G6pc* mRNA appeared to be strongly up-regulated, with 2.5 times more *G6pc* mRNA in the small intestine of I-*G6pc*$^{-/-}$ mice than in controls (Figure 2A). This could be accounted for by a compensatory increase in gene expression in the absence of G6PC protein, as was also observed in the liver when the specific *G6pc* knockout was done in this organ [25]. To obviate the absence of G6PC protein, we assayed G6Pase activity in the proximal jejunum of WT and I-*G6pc*$^{-/-}$ mice. In WT mice, the jejunum G6Pase activity was doubled after 3 days of feeding on PED (Figure 2B). This was comparable to what has been reported in the rat [7]. Residual non specific G6Pase activity was present in I-*G6pc*$^{-/-}$ mice, comparable to that which could be measured in non-gluconeogenic organs [28, 29]. This might be accounted for by the presence of G6PC3 (Figure 2D), a ubiquitous glucose-6-phosphatase-related protein exhibiting weak G6Pase activity *in vitro*, but having no gluconeogenic role *in vivo* [30 for review]. This activity was not increased by PED feeding (Figure 2B) in agreement with the assumption that this low activity was not representative of "gluconeogenic" specific G6Pase activity. The expression of *G6pt (Slc37a4)* and *G6pc3* genes, encoding the glucose-6 phosphate translocase and the G6PC3, respectively, was unaffected, as was observed in the liver-specific *G6pc* knockout mice (Figure 2C and D) [25].

3-2 Absence of reduced food intake by protein-enriched diet in I-*G6pc*$^{-/-}$ mice

Male I-*G6pc*$^{-/-}$ mice exhibited a growth rate similar to that of WT mice, when fed on a standard rodent starch-enriched diet (SED). One week after intestinal *G6pc* knockout, food intake and body weight were monitored over 13 successive days. The I-*G6pc*$^{-/-}$ and WT mice were matched for age at the beginning of the experiment. The body weight of I-*G6pc*$^{-/-}$ was similar to that of the control mice (Figure 3C). During the first five days, the I-*G6pc*$^{-/-}$ mice

fed on SED ate like the WT mice (12.4 ± 0.5 kcal/day/mouse in the I-*G6pc*$^{-/-}$ mice versus 11.6 ± 0.5 kcal/day/mouse in the control mice) and their body weight was constant, as was that of WT mice (Figure 3A and C). Then, the SED (20% energy intake from protein) was replaced by a PED (65% energy intake from protein) and the mouse food intake was monitored for another week. As with the WT mice, the I-*G6pc*$^{-/-}$ mice fed on PED ate slightly less the first day it was given to them (reduction of 22% and 15% of food intake, respectively). This lower food intake in PED-fed mice was comparable to that reported previously in rats [7]. As described previously in rats, the WT mice ate about 20% less with the PED than with the SED and this lower food intake was maintained throughout the experiment (Figure 3A). In contrast, after an initial decrease in food intake, consecutive to the change in diet, the food intake of I-*G6pc*$^{-/-}$ mice fed on PED rapidly increased to reach the same level as that previously observed when they were fed on SED (Figure 3A). Moreover, the accumulated food intake, which was exactly the same in both I-*G6pc*$^{-/-}$ and WT animals fed on SED, became significantly different from the first day after switching to PED (Figure 3B). In parallel, the body weight of I-*G6pc*$^{-/-}$ mice was stable throughout the PED (25.4 ± 0.4 g/mouse versus 25.8 ± 0.3 g/mouse at day 6 and at day 13, respectively; Figure 3C), whereas the body weight of WT mice decreased significantly after 5 days on PED (Figure 3C). After 8 days on PED, the WT mice lost around 3g/mouse, i.e. a decrease of 12 ± 2 % of their initial body weight (Figure 3C). These results therefore demonstrate that mice with specific knockout of the *G6pc* gene in the intestine are no longer sensitive to the satiety effect induced by food proteins. The G6Pase catalytic subunit is the mandatory enzyme for endogenous glucose production from the three gluconeogenic organs of the body. In agreement with this assumption, mice with total *G6pc* knockout die shortly after weaning [31] and patients with inherited deficiency in *G6PC* (glycogen storage disease type 1a) suffer from severe hypoglycaemic episodes if not frequently fed with oral carbohydrates [30 for review].

Moreover, mice with liver-specific *G6pc* knockout rapidly develop the hepatic features of the GSD1a pathology [25]. It can therefore be inferred that mice with specific deletion of *G6pc* in the intestine are unable to release glucose from intestinal biochemical pathways. Therefore, this novel I-*G6pc*$^{-/-}$ mouse model allowed us to definitively strengthen the paradigm that the satiety signal induced by food proteins can be accounted for by a portal glucose release from intestinal gluconeogenesis. What is particularly noteworthy about this phenomenon is that there are long-term changes in body weight that occur if PED-feeding is continued (for at least one week, see Figure 3C). On the contrary, treatments that suppress food intake in otherwise normal animals (e.g., such as administering cholecystokinin at the start of every meal) result in the expected reduction of meal size, but with a concomitant increase in meal frequency and little or no change in body weight [32]. A likely explanation for this difference may rely on the fact that intestinal gluconeogenesis, depending on the induction of gluconeogenic enzyme expression, takes place on a lasting basis, decreasing hunger, i.e. through satiety, during the inter-meal period and for the forthcoming meal [7]. On the contrary, cholecystokinin is a satiation hormone, reducing hunger for the associated meal but with no lasting action thereafter. This may explain the compensation of its action by increased meal frequency [32].

To conclude, it should be mentioned here that intestinal gluconeogenesis and its portal-brain sensing might also be causal in the improvement in insulin sensitivity after feeding on protein-enriched diet in rats and after gastric bypass surgery in mice [23, 27]. This is in line with the improved glucose control previously reported in obese diabetic patients fed on a protein-enriched diet [33], or undergoing gastric bypass surgery [22, 34]. Moreover, it has been suggested, from studies on mice [35] and one GSD1 patient [36], that intestinal G6Pase may be implicated in the absorption of glucose from the intestinal lumen, which may provide an alternative route to the direct transport pathway mediated by glucose transporters

only. Thus, I-*G6pc*$^{-/-}$ mice will certainly be very useful in the near future to gain better understanding of the role of intestinal gluconeogenesis in the central control of insulin sensitivity and of intestinal G6Pase in the assimilation of glucose from food.

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Figure Captions

Figure 1. PCR genotype analysis and intestine-specific excision of *G6pc* exon 3.

(A) Diagram of the floxed *G6pc* allele (*G6pc*^{lox}) and the exon 3 deleted *G6pc* allele (*G6pc*^{del}). Black rectangles represent exons and the 5'and 3' untranslated sequences are indicated by white rectangles. Lox P sequences are represented as triangles while flags indicate frt sequences. Exon 3 of *G6pc* was specifically excised by CRE recombinase (*G6pc*^{del}).

(B) Genomic DNA, extracted from tail of ten day-old mice, was amplified using specific *Lox-G6pc* primers (p2 sense: 5'-GCTCATTCTCACACCTACAGTTGG-3' and p3 antisense: 5'-TGTTCCTAACTACTGAGCCATTGCTCC-3'), which yielded products of 486 and 385 bp for the floxed *G6pc* (*G6pc*^{lox}) and the WT (*G6pc*^{w}) alleles, respectively. Specific *Villin-Cre* primers (Sense: 5'-CAAGCCTGGCTCGACGGCC-3' and antisense: 5'-CGCGAACATCTTCAGGTTCT-3') were used to amplify a 300 bp *Cre* fragment. The expected sizes are shown on the left of the panel.

(C) Genomic DNA extracted from intestine, liver and kidney of I-*G6pc*$^{-/-}$ (-/-) and WT C57Bl/6J (+/+) mice was amplified using specific *G6pc* primers (p1 sense: 5'-AGGGAGTTGACCAGAGGAACTTTGG-3' and p3 antisense). Fragments of 1189, 1029 and 595 bp correspond to the *G6pc*^{lox}, *G6pc*^{w} and *G6pc*^{del} alleles, respectively. The expected sizes are shown on the left of the panel.

Figure 2. Analysis of G6Pase gene expression in the intestine.

Levels of *G6pc* (A), *G6pt (Slc37a4)* (C) and *G6pc3* (D) mRNA were measured by RT-qPCR in the intestine of I-*G6pc*$^{-/-}$ (black bars) and wild-type (open bars) mice. The results are expressed as a ratio relative to *Rpl19* expression. Data were obtained fourteen weeks after tamoxifen injections from mice fasting for 6 hours and are expressed as means ± SEM (n=8

mice per group). *Values significantly different from WT (P<0.01). G6Pase activity (B) was assayed in the proximal jejunum of I-*G6pc*$^{-/-}$ (-/-) and WT (+/+) mice, fed on SED (filled bars) or fed for 3 days on PED (hatched bars). Data were obtained two weeks after tamoxifen injections from mice fasting for 6 hours and are expressed as means ± SEM (n=5 mice per group). *Values significantly different (P<0.01).

Figure 3. Evolution of food intake and body weight of I-*G6pc*$^{-/-}$ mice fed on PED versus SED. Daily food intake expressed in % of the mean food intake calculated from the consumption of I-*G6pc*$^{-/-}$ mice over the three preceding days (A), accumulated food intake expressed in kcal (B) and body weight expressed in g (C) of I-*G6pc*$^{-/-}$ (filled squares) and WT (open squares) mice. Mice were fed on SED during the first five days and then on PED for one more week. Data were obtained one week after gene deletion and are expressed as means ± SEM (n=9 mice per group). Values significantly different from WT (P<0.05*) are indicated.

Table 1

Oligodeoxyribonucleotide primer sequences for qPCR **(**S=sense and AS=antisense).

Name	Sequence
Exon1 G6pc S	5’-TTACCAAGACTCCCAGGACTG-3’
Exon2 G6pc AS	5’-GAGCTGTTGCTGTAGTAGTCG-3’
G6pt S	5’-TGGTTGGTCTGGTCAACGTA-3’
G6pt AS	5’-TGCCAAGATAGGTCCCAAAC-3’
G6pc3 S	5’-GCACATTTCCCTCACCAAGT-3’
G6pc3 AS	5’-GGTTGATGGACCAGGAAAGA-3’
Rpl 19 S	5’-AGAAGATTGACCGCCATAT-3’
Rpl 19 AS	5’-TTCGTGCTTCCTTGGTCTTAGA-3’

Figure 1

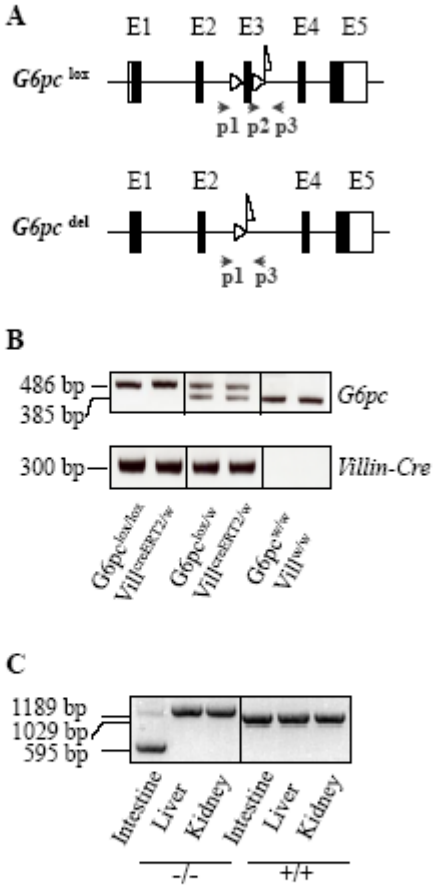

A
E1
E2
E3
E4
E5
G6pc lox
p1 p2 p3
E1
E2
E4
E5
G6pc del
p1 p3
B
486 bp
385 bp
G6pc
300 bp
Villin-Cre
G6pc lox/lox Villcre ERT2/w
G6pc lox/w Villcre ERT2/w
G6pc w/w Villw/w
C
1189 bp
1029 bp
595 bp
Intestine
Liver
Kidney
Intestine
Liver
Kidney
-/-
+/+

Figure 2

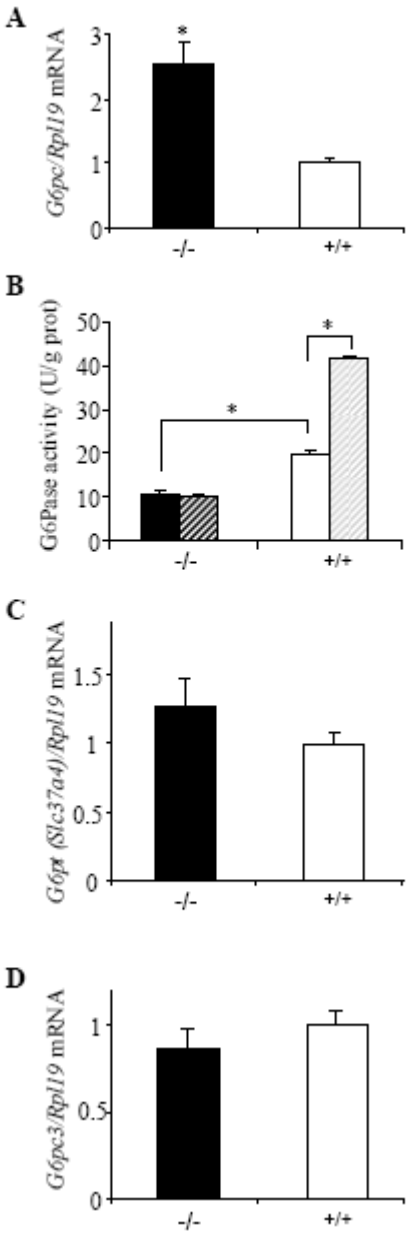
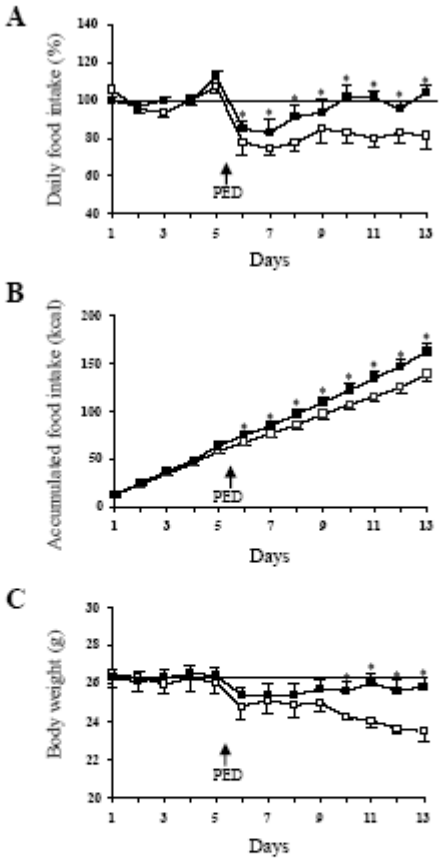

A
G6pc/Rpl19 mRNA
3
2
1
0
*
-/-
+/+
B
G6Pase activity (U/g prot)
50
40
30
20
10
0
*
*
-/-
+/+
C
G6pt (Slc37a4)/Rpl19 mRNA
1.5
1
0.5
0
-/-
+/+
D
G6pc3/Rpl19 mRNA
1
0.5
0
-/-
+/+

Figure 3


A
Daily food intake (%)
PED
Days
B
Accumulated food intake (kcal)
PED
Days
C
Body weight (g)
PED
Days