Portal sensing of intestinal gluconeogenesis is a mechanistic link in the diminution of food intake induced by diet protein

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Summary

Protein feeding is known to decrease hunger and subsequent food intake in animals and humans. It has also been suggested that glucose appearance into portal vein, as occurring during meal assimilation, may induce comparable effects. Here, we connect these previous observations by reporting that intestinal gluconeogenesis (i.e., de novo synthesis of glucose) is induced during the postabsorptive time (following food digestion) in rats specifically fed on protein-enriched diet. This results in glucose release into portal blood, counterbalancing the lowering of glycemia resulting from intestinal glucose utilization. Comparable infusions into the portal vein of control postabsorptive rats (fed on starch-enriched diet) decrease food consumption and activate the hypothalamic nuclei regulating food intake. Similar hypothalamic activation occurs on protein feeding. All these effects are absent after denervation of the portal vein. Thus, portal sensing of intestinal gluconeogenesis may be a novel mechanism connecting the macronutrient composition of diet to food intake.

Introduction

The disorders of food intake have a major impact on the worldwide expansion of obesity and type 2 diabetes mellitus (Flier, 2004; Friedman, 2000). This urges the scientific community to seek novel mechanisms of control of the sensations of hunger and satiety. The brain, particularly specific hypothalamic nuclei, plays a crucial role in this control. It ceaselessly senses nutrients and hormones provided by the periphery, and adapts energy intake and expenditure to the needs of the body (Obici et al., 2003; Schwartz and Morton, 2002).

In this context, it is well known that protein feeding decreases hunger sensation and subsequent food intake in animals and human (Barkeling et al., 1990; Booth et al., 1970; Jean et al., 2001; Rolls et al., 1988). However, the mechanism by which proteins induce their specific hypophagia effects is still unsolved (Barkeling et al., 1990; Booth et al., 1970; Jean et al., 2001; Rolls et al., 1988). Actually, the major hormones decreasing food intake produced by the body are either unaffected (leptin) or even slightly decreased (insulin) if the proportion of protein in the diet is increased (Jean et al., 2002). Similarly, the participation of the two main gut-derived hormones possibly involved in food intake i.e., cholescystokinin (Moran, 2000) and PYY_{3-36} (Batterham et al., 2002; Halatchev and Cone, 2005) seems excluded. In fact, PYY_{3-36} is unspecifically secreted in response to food in proportion to the calories ingested (Pedersen-Bjergaard et al., 1996; Schwartz and Morton, 2002) and the cholecystokinin release is stimulated by proteins as well as by lipids in animals (Backus et al., 1995; Douglas et al., 1990) and humans (Hopman et al., 1985), whereas lipids have a much lower suppressing effect on food intake than proteins (Rolls et al., 1988).

Besides hormones, glucose per se is a key signaling molecule able to suppress food intake (Thorens and Larsen, 2004). Contrasting with most of the circulating hormonal signals (for instance, leptin or insulin), which are supposed to be directly sensed by the brain (hypothalamus) through the median eminence (Flier, 2004; Schwartz and Morton, 2002), the sensing of glucose-induced effects takes place in the portal vein (Langhans et al., 2001; Thorens and Larsen, 2004; Tordoff and Friedman, 1986; Tordoff et al., 1989). It is dependent on glucose-sensitive cells present in the wall of the portal vein, the signal being transmitted to the brain via afferences of the vagus nerve (Adachi et al., 1984; Schmitt, 1973; Shimizu et al., 1983; Thorens and Larsen, 2004). As a related point, we previously reported that gluconeogenesis genes are expressed in the small intestine (SI) in rats and humans (Rajas et al., 1999, 2000). This confers on this tissue the capacity to form glucose and release it into the portal blood, this capacity actually taking place only when these genes are induced, such as in fasting and diabetes (Croset et al., 2001; Mithieux et al., 2004a). Because portal glucose is able to induce hypophagia, this raised the attractive hypothesis that intestinal gluconeogenesis might have the capacity to decrease hunger sensation and food intake. Moreover, it as been previously reported that rats fed on a high-protein and carbohydrate-free diet can adapt very rapidly by inducing hepatic gluconeogenesis, the induction of the main liver gluconeogenesis enzymes is even more rapid than during the adaptation to fasting (Boisjoyeux et al., 1986). Thence, we tested the hypothesis that protein feeding might induce gluco-

neogenesis in the SI, which gives the possibility of detecting glucose release by the portal glucose sensor, and thus of decreasing food intake.

Results and discussion

Protein feeding induces hypophagia, SI gluconeogenesis, and portal glucose release during the postabsorptive period

In agreement with previous results (Jean et al., 2001), rats fed on a diet moderately enriched in protein (50% by weight as protein) ate and grew less than rats fed on a control starch enriched diet (SED) (17% as protein). Rats ate slightly less the first day they were given the PED, but this was rapidly compensated within a couple days, as previously reported (Jean et al., 2001). Food intake, monitored over 6 successive days from the third day on PED feeding, was 17.4 ± 0.6 g/day/rat in PED-fed rats versus 20.5 ± 0.7g/day/rat in SED-fed rats (mean ± SEM, n = 6 per group, $p < 0.05$, Student's t test for unpaired value). This represented a 15% lower food intake in PED-fed rats, very comparable to that previously reported (Jean et al., 2001). This resulted in a weight gain of 49.8 ± 2.6 g in rats fed on the protein-enriched diet (PED) versus 60.6 ± 3.1 g in SED-fed rats (mean ± SEM, n = 8 per group) after 7 days, and 84.7 ± 3.4 g versus 102.4 ± 2.9 g, respectively, after 15 days (differences were significant at $p < 0.01$, Student's t test for unpaired value), whereas rats were perfectly matched for weight at the beginning of the experiment: 201.0 ± 1.4 g for SED-fed and 200.7 ± 1.6 g for PED-fed rats. Because rats fed on PED weighed progressively less, we also expressed the results as kcal ingested per kg per day during the monitoring period of 6 days. PED-fed rats ingested 278.0 ± 2.2 kcal/kg/day whereas SED-fed rats ingested 319.2 ± 2.6 kcal/kg/day. The difference was slightly lower compared to the expression of results as g of food ingested/day/rat, but still significant ($p < 0.05$, Student's t test for unpaired values). It is interesting to mention that the composition of the protein diet given herein was chosen on the basis that rats allowed to self-select the macronutrient composition of their diet choose a very comparable protein-enriched diet (Jean et al., 2002). It seems therefore unlikely that the protein-induced hypophagia might be due to lesser palatability of the diet or malaise. Also in disagreement with the latter hypothesis, it has been previously reported that protein-feeding decreases food intake by inducing satiety phenomena and not malaise or taste aversion (L'Heureux-Bouron et al., 2004; Westerterp-Plantenga, 2003). However, because the role of palatability was not addressed here, it could not be formally excluded that differences in palatabilities of SED and PED may account at least in part for the effects observed.

In line with our hypothesis, the expression of the two major regulatory genes of gluconeogenesis in the SI, i.e., glucose-6 phosphatase (Glc6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Mithieux et al., 2004b) was dramatically induced in rats fed on the PED compared to those fed on the SED. A double induction was observed on the amount of protein detected by immunoblotting (Figures 1A and 1B) and on the enzyme activity determined under conditions of maximal velocity (Figure 1C). Moreover, there was a marked induction by about 80% of the expression of the glutaminase gene in rats fed on the PED (Figure 1). The latter gene was strongly suggested to have a crucial potentiating role in SI gluconeogenesis (Mithieux et al., 2004a). The experiments shown in Figure 1 were performed on day 7, but separate experiments clearly indicated that induction occurred from the first 24 hr of PED feeding (data not shown). Moreover, results from the proximal jejunum are shown in Figure 1, but comparable induction occurred in all parts of the SI, from duodenum up to ileum. It is of note that in previous studies in which carbohydrates were totally replaced by protein, induction of gluconeogenesis enzymes was reported in the liver (Boisjoyeux et al., 1986). In contrast, no induction of either gluconeogenesis genes occurred in the liver with the diet moderately enriched in protein used herein (data not shown). In the liver, this was consistent with the fact that no change in plasma insulin occurred (Table 1) and, in the SI, with our previous observation that more than the insulin status was crucial to determine Glc6Pase gene induction in this tissue (Mithieux et al., 2004a, 2004b). It must be mentioned that the induction of gene expression by protein diets in the SI is not unique to genes of gluconeogenesis. The gene of the intestinal oligopeptide transporter PepT1 for example was shown to be markedly induced under such nutritional conditions (Adibi, 2003). In line with the induction of intestine specific genes by proteins in vivo, protein hydrolysates were shown to activate in vitro the transcription of cholecystokinin and proglucagon genes, via cAMP-dependent activation of the nuclear factor CREB (cAMP response element binding protein) (Gevrey et al., 2002, 2004). Interestingly, the latter factor was also shown to be essential in the transcriptional control of Glc6Pase and PEPCK genes by cAMP (Gautier-Stein et al., 2005; Quinn et al., 1988).

We then tested whether gluconeogenic gene induction re-

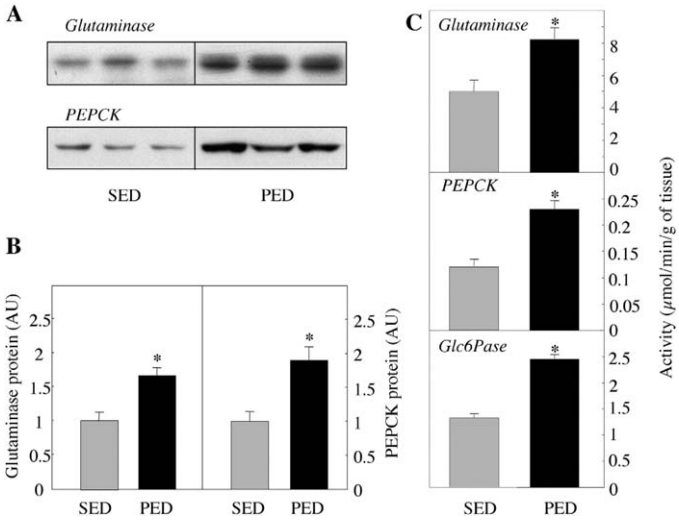

Figure 1. Induction of gluconeogenesis genes in the rat SI upon PED feeding

A) Representative Western blot analysis of glutaminase and PEPCK in the proximal jejunum for three rats fed on the SED (left) and 3 rats fed on the PED (right) for 7 days.

B) Densitometric analysis performed from six rats per group. The results are expressed as arbitrary units (AU), *, $p < 0.05$, Student's t test for unpaired samples.

C) Enzymatic activities assayed under conditions of maximal velocity (n = 6 per group) (Mithieux et al., 2004a; Rajas et al., 1999, 2000). The results are expressed as μmol/min/g of wet tissue, *, $p < 0.05$, Student's t test for unpaired values.

Table 1. Plasma glucidic parameters in SED- and PED-fed rats

Feeding pattern	[3-^{3}H] glucose specific activity (dpm/μmol)		Plasma glucose (mmol/l)		Fractional extraction	Plasma insulin (pmol/l)
	artery	vein	artery	vein		
SED	10,539 ± 681	10,738 ± 610	9.3 ± 0.6	8.6 ± 0.5[a]	0.06 ± 0.01	304 ± 42
PED 1 days	12,298 ± 556	11,874 ± 616[a]	9.5 ± 0.8	9.2 ± 0.9	0.06 ± 0.02	268 ± 182
PED 2 days	10,394 ± 555	9,777 ± 492[b]	9.4 ± 0.5	9.6 ± 0.5	0.04 ± 0.01	294 ± 99
PED 3 days	10,802 ± 484	10,273 ± 442[b]	10.5 ± 0.4	10.6 ± 0.5	0.04 ± 0.01	352 ± 136
PED 7 days	11,143 ± 297	10,401 ± 209[a]	9.4 ± 0.3	9.5 ± 0.2	0.05 ± 0.01	334 ± 55
PED 15 days	9,659 ± 477	9,011 ± 482[a]	10.3 ± 0.4	10.1 ± 0.4	0.07 ± 0.02	312 ± 80
PED 2 days + SED 1 day	10,601 ± 800	10,353 ± 609	8.8 ± 0.8	8.3 ± 0.7[a]	0.07 ± 0.02	329 ± 91

Rats (n = 8 per group) were either fed on the control SED or fed on SED and then on the PED for 1, 2, 3, 7, and 15 days or the PED for 2 days and the SED for 1 more day. The results are expressed as mean ± SEM. [a,b]Value in vein different from that in artery, $p < 0.05$ and $p < 0.01$, respectively (Student's t test for paired values).

sulted in the release of glucose into the portal vein, as demonstrated in fasting or diabetes (Croset et al., 2001; Mithieux et al., 2004a). Because the SI is also a strong glucose-utilizing organ, we used the previous combination of arterio-venous balance determination and glucose tracer dilution analysis to separate the uptake and release of glucose by the SI (Croset et al., 2001; Mithieux et al., 2004a). Though rats ate slightly less the first day they were given the PED (see above), this was not sufficient to significantly alter plasma glucose and insulin concentrations in comparison with rats fed on the SED (Table 1). The SI blood flow in rats fed on the PED for one day (6.2 ± 0.7 ml/min) was not different from that in SED-fed rats (5.9 ± 0.9 ml/min), and it was not further modified during the following days. Rats were deprived of food for 6 hr, anesthetized, and fitted with catheters. This was a time when the SI was completely cleared up to the very distal ileum in both SED-fed and PED-fed rats. This prevented glucose absorbed from the intestinal lumen from giving ambiguous results. It should be noted that the plasma concentration of cholecystokinin, i.e., the main candidate hormone putatively affected by protein ingestion (Jean et al., 2002), was identical in PED-fed rats (for 7 days) (2.85 ± 0.8 pg/ml, mean ± SEM, n = 6) and in SED-fed rats (3.82 ± 1.14 pg/ml). Blood was then sampled after 90 min of [3-^{3}H]-glucose infusion (Croset et al., 2001). The [3-^{3}H]-glucose specific activity (SA) was the same in portal and arterial blood in SED-fed rats (Table 1). This was an indication that there was no release of glucose into the portal blood in these rats. This also confirmed that food digestion was completed (see above). There was a significant decrease in plasma glucose concentration in the portal vein as compared to the artery (Table 1), reflecting glucose utilization by the tissue. The intestinal glucose balance (IGB), calculated from these glucose concentrations, was in the same order range as that of the intestinal glucose uptake (IGU), which was deduced from the arterial glucose concentration and the [3-^{3}H]-glucose removal by the SI (i.e., fractional extraction or FX) (Tables 1 and 2). Consequently, the calculated intestinal glucose release (IGR) was not different from nought (–3.4 ± 2.7 μmol/kg/min, mean ± SEM., n = 8) (Table 2). These data were in agreement with our previous data in postabsorptive and/or short-term fasted rats previously fed a starch-based diet (Croset et al., 2001; Mithieux et al., 2004a). In contrast, in rats fed on the PED, the glucose SA was significantly lower (–3.4%) in the portal compared to arterial blood from the first day of PED feeding (Table 1). This revealed a significant release of unlabeled glucose into the portal blood. Consequently, the decrease in glycemia observed in the portal blood was almost negligible as compared to that in SED-fed rats. From the second day and up to 15 days of PED feeding, the decrease in glucose SA between the portal vein and the artery was deeper (around –6%), and there was no more difference in plasma glycemia between vein and artery (Table 1). Accordingly, the calculated intestinal glucose balance (IGB) was lower than that in SED-fed rats from one day of PED feeding onward and then approached zero between 2–15 days (Table 2). The intestinal glucose uptake (IGU) was the same in PED- and SED-fed rats (Table 2) with no alteration throughout the feeding period. These data revealed a significant intestine glucose release (IGR) of 6.9 ± 2.1 μmol/kg/min, representing 12% of the endogenous glucose production (EGP) the first day of PED feeding. The glucose release increased twice (13.1 ± 2.8 μmol/kg/min or 19% of PEG) from day 2 and underwent no further change thereafter (Table 2). In PED-fed rats infused with [U-^{14}C] glutamine, there was a significant ($p < 0.05$) increase (+3.6%) in the ^{14}C-glucose SA in the portal vein compared to the artery. This indicated that glucose molecules released by the SI had incorporated ^{14}C-glutamine. This was in agreement with the increased expression of the glutaminase gene (Figure 1) and with the fact that glutamine is the main, if not the only, substrate of SI gluconeogenesis (Croset et al., 2001; Mithieux, 2001). It was noticeable that the total endogenous glucose production (EGP) was not altered in PED-fed compared to SED-fed rats, while an intestinal component appeared with PED feeding. This was in agreement with the observation that glucose production by the liver is proportionally inhibited by portal glucose appearance (Sindelar et al., 1997). Interestingly, intestine glucose production was highly characteristic of PED. It was in fact equal to nought in rats fed on the PED for 2 days and refed on the SED for 1 day (Tables 1 and 2), or in rats fed on a high-fat diet as previously described (Mithieux et al., 2002) (data not shown). These data strongly suggested that the induction of gluconeogenic genes in the SI resulted in the portal release of glucose during the postabsorptive period in rats specifically fed on the PED.

Portal glucose appearance induces hypophagia per se

Next, the question arose of whether such intestinal glucose fluxes delivered to the portal vein, which were quantitatively low but continuous, were capable of decreasing food intake in

Table 2. Intestinal glucose fluxes in SED- and PED-fed rats

Feeding pattern	IGU	IGB	IGR (% of EGP)	EGP
SED	12.9 ± 5.5	16.3 ± 6.0	–3.4 ± 2.7	72.9 ± 4.5
PED 1 days	14.7 ± 5.6	7.9 ± 3.9	6.9 ± 2.1 (12%)	61.9 ± 2.7
PED 2 days	9.0 ± 2.2	–4.1 ± 4.2	13.1 ± 2.8 (19%)	70.7 ± 3.6
PED 3 days	9.7 ± 2.9	–2.8 ± 2.7	12.5 ± 1.9 (17%)	74.5 ± 1.9
PED 7 days	10.4 ± 4.0	–3.9 ± 1.2	14.3 ± 4.1 (21%)	68.9 ± 1.8
PED 15 days	16.5 ± 4.8	4.0 ± 4.4	12.3 ± 2.8 (17%)	73.7 ± 2.4
PED 2 days + SED 1 day	11.7 ± 3.9	8.9 ± 4.3	2.9 ± 3.7	63.6 ± 5.0

Rats were either fed on the control SED or fed on the SED and then on the PED for 1, 2, 3, 7, and 15 days or the PED for 2 days and the SED for 1 more day. All procedures required for the determination of IGU, IGB, IGR, and EGP are fully described in Experimental Procedures. The results are expressed as μmol/kg/min and are the mean ± SEM (n = 8).

a way similar to the previously studied glucose fluxes, which were quantitatively higher but more transient (the latter being merely designed to match postprandial glucose absorption) (Langhans et al., 2001; Tordoff and Friedman, 1986; Tordoff et al., 1989). Rats were fitted with indwelling catheters into the portal vein. After one week, allowing complete recovery after surgery, rats were infused with glucose at 12 and 25 μmol/kg/min, i.e., matching the mean and maximal values of IGR determined individually in PED-fed, rats respectively. It must be noted that this rather high scatter in IGR values (Table 2) may be either intrinsically inherent in IGR or at least partly attributable to the lack of precision of the tracer approach (this point is discussed in Croset et al., 2001). In separate experiments (data not shown), we checked that there was no modification of either plasma glucose or insulin in peripheral blood induced by such low portal glucose infusions. This was in keeping with the coordinate suppression of hepatic glucose production (see above). In a first approach, we studied rats fed on the control SED (i.e., they exhibited none of the alterations putatively induced by PED-feeding). This allowed us to rule out that any other parameter altered on PED feeding could hide the effects induced by portal glucose appearance. Glucose infusion with both the above fluxes significantly decreased the rats food intake compared to saline infusion (Figure 2). The decrease amounted to about 16% and 22% at 12 and 25 μmol/kg/min, respectively, at the beginning of the dark period, i.e., the time of preferential feeding in rodents (Figure 2A). The suppression of intake was even more pronounced at the beginning of the light period: –30% and –38% at 12 and 25 μmol/kg/min, respectively (Figure 2A). This was a specific effect of glucose because there was no decrease in food intake caused by portal infusions of other nutrient metabolites such as lactate (25 μmol/kg/min) or alanine (25 μmol/kg/min) (data not shown). These data strongly suggested that portal glucose appearance, as occurring on PED feeding during the postabsorptive period, was able per se to account for the decreased food intake induced by the diet.

In a second approach, we studied rats fed on the PED (i.e., they exhibited all the alterations putatively induced by PED feeding including endogenous IGR). In line with the results of continuous food consumption monitored over several successive days (see above), food intakes tended to be lower than those in the previous protocol involving the SED diet under the three conditions of infusion (Figure 2A). However, the differences were not significant ($p > 0.1$) in this short-term fasting-refeeding transition study. This might possibly be due to subtle differences in the food behavior adaptation to this type of transition, which were out of the scope of the present study. It was of note that portal glucose infusion at both rates was still able to further decrease food intake compared to saline infusion (Figure 2A). The decreases in regard to the control saline infusion value were in the same order range as those in the protocol involving the SED diet. These data strongly suggested that among the various parameters putatively altered by PED feeding during the postabsorptive period, portal glucose appearance has a dominant effect in relation to the control of food intake.

Because the role of the portal glucose sensor in deriving either the central response to hypoglycemia (Hevener et al., 2000; Thorens and Larsen, 2004) or the activation of the central areas involved in the response to portal glucose is established (Adachi et al., 1984; Schmitt, 1973; Shimizu et al., 1983), we further focused on the role of the portal nervous system in the decreased food intake induced by glucose infusion. Rats were

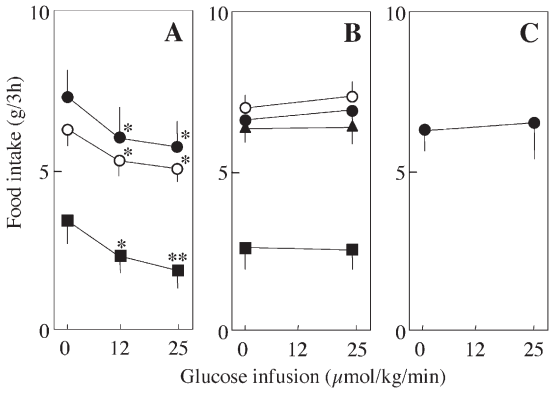

Figure 2. Effect of glucose infusions on food intake in SED-fed (filled symbols) and PED-fed (open symbols) rats

A) Effect of infusions of saline (0) or glucose (12 or 25 μml/kg/min) into the portal vein in SED-fed (data from six rats, filled circles) and PED-fed rats (data from 6 rats, open circles) at the beginning of the dark phase; and in SED-fed rats at the beginning of the light phase (data from eight rats, filled squares). The results are the mean ± SEM: *, **, different from respective saline infusion $p < 0.05$ and $p < 0.01$, respectively (Student's t test for paired values).

B) Effect of similar portal infusions in rats with capsaicin-treated portal vein (data from five rats under each condition, symbols as above). Filled triangles refer to an experiment of mechanical denervation of the portal vein (data from four rats).

C) Effect of infusions into peripheral blood (data from four rats, symbol as above).

treated by local application of capsaicin around the portal vein at the time when portal catheters were inserted into the portal vein (Jancso et al., 1980). Capsaicin is a neurotoxic agent inducing the selective inactivation of weakly myelinated or unmyelinated autonomic afferent fibers (Blackshaw et al., 2000; Holzer, 1991). After recovery, rats were studied for their food intake in response to portal infusions as above. There was no effect of portal glucose infusion on food intake in capsaicin-treated rats, either during the dark or the light phase. This absence of effect was noted in SED-fed as in PED-fed rats (Figure 2B). It is known that, even if extreme care is taken during the application, capsaicin might impact neighboring tissues and/or nerve fibers (Zafra et al., 2004). Thus, portal vein denervation was also mechanically performed (by gentle scrapping). The results were identical to those in capsaicin-treated animals (Figure 2B). However, one must bear in mind that nerve fibers from the liver travel along the same way than those from the portal vein. Therefore, it cannot be formally excluded that they might also be impacted by the denervation treatment and that a part of the glucose signal may be detected in the liver. Also, because we did not check the neuroanatomical result of denervation, it is possible that not all nerve fibers were impacted by the treatment. However, it seems obvious that at least those involved in the glucose signal transmission were inactivated. At last, rats were fitted with indwelling catheters placed into the right jugular vein instead of the portal vein. They were also studied for food intake in response to saline or glucose infusions. Again in line with the key role of portal vagal afferences in the central control of food intake by portal glucose, there was no alteration of food intake induced by glucose infusions into this peripheral vein (Figure 2C).

We further ascertained that the hypophagia effect induced by PED (see above) was dependent on the presence of a functional portal glucose sensor. With this aim, rats were either denervated around the portal vein as above (without catheter insertion) or sham-operated (i.e., treated by local application of saline instead of capsaicin around the portal vein). After recovery for 7 days under SED feeding, they were further given either the SED or the PED. After two days for familiarization with the PED (see above), the growing rate of rats was studied during 5 more days. In line with former data, sham-operated rats fed on the PED ate and grew less during the study period than their counterparts fed on the SED. In contrast, in rats with inactivated portal glucose sensor, there was no effect of PED on food intake and the animals ate and grew as sham-operated rats fed on the SED (Figure 3). This strongly suggested that the PED-induced hypophagia was dependent on a functional portal glucose sensor. It is interesting to note that the reversal of PED effect by capsaicin disagreed with the hypothesis that a lower palatability might account for the induced decrease in food intake (see above). It was noteworthy that there was no significant effect of portal denervation of the growing rate of rats fed on the SED (Figure 3). The latter had the possibility to sense portal glucose deriving from food assimilation and not glucose deriving from gluconeogenesis (see Figure 1 and Table 2). This strongly suggested that the decreasing effect on food intake promoted by portal glucose might not evidenced during the periods of active food assimilation, possibly because of redundant potent mechanisms of control of food intake concomitantly taking place (e.g., mechanical distension). Conversely, these results also emphasized that the control of food

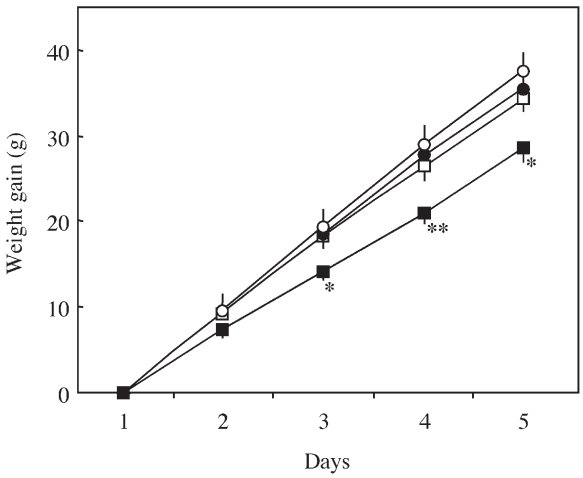

Figure 3. Dependency of the sensitivity to PED feeding on the portal vein innervation

The growing rate of sham-operated rats fed on the PED (filled squares) was compared to rats with denervated portal vein fed on the same diet (open squares) and to sham-operated rats (filled circles) and denervated rats (open circles) fed on the SED. The results are the mean ± SEM from six rats per group. *, **, different from value in counterparts with denervated portal vein, $p < 0.05$ and $p < 0.01$, respectively (Student's t test for unpaired values).

intake depending on portal glucose sensing should be a mechanism essential in the periods of low portal appearance and specific of glucose from intestinal gluconeogenic origin (a situation taking place in the postabsorptive period in rats fed on the PED and/or when food assimilation is close to ending). Interestingly, our data are in keeping with previous results suggesting that portal glucose infusion is important in determining future food intake rather than in terminating ongoing meal (Baird et al., 1997). It is likely that, when available, animals with selective blockade of intestinal gluconeogenesis (e.g., mice with targeted inactivation of Glc6Pase gene in the SI) will allow not only to confirm our data, but also to address more in depth these questions.

Activation of hypothalamic nuclei induced by portal glucose appearance matches that induced by protein feeding

Finally, we studied whether the hypophagia effects evidenced in postabsorptive rats fed on PED, or in postabsorptive rats fed on SED and infused with glucose into the portal vein, were both consistent with the occurrence of central events taking place in the main hypothalamic nuclei known to be involved in the regulation of food intake. Because c-*fos* expression is a well-recognized marker of neuronal activation (Halatchev and Cone, 2005; Pocai et al., 2005), we studied c-*fos* expression in the arcuate nucleus (ARC), the ventromedian nucleus (VMN), the dorsomedian/paraventricular nucleus (DM/PVN), and lateral hypothalamus (LH). The results shown in Figure 4 were from the PVN, but it was noteworthy that infusions of glucose into the portal vein or protein feeding elicited very similar c-*fos* expressions in all aforementioned hypothalamic areas. The selectivity of c-*fos* activation in these hypothalamic areas was ascertained from photomicrographs showing larger brain areas

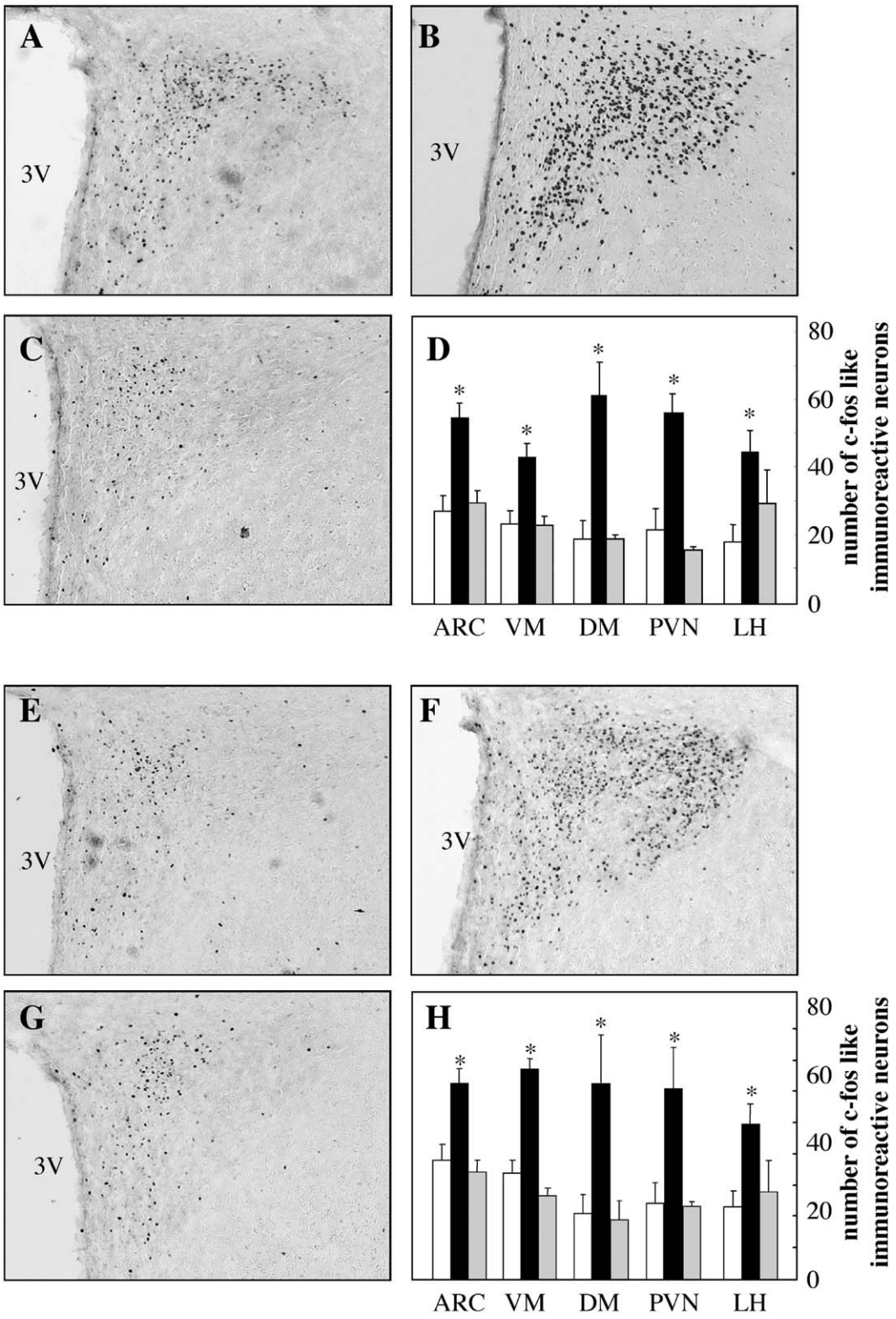

Figure 4. Induction of c-*fos* expression by portal glucose infusion or PED-feeding in rat hypothalamus

A–C) Photomicrographs showing c-*fos*-like immunoreactive neurons in PVN of normal rats infused into the portal vein with saline (**A**) or glucose at 25 μmol/kg/min (**B**), and of denervated rats infused with glucose (**C**). Magnification = 100×. 3V refers to third ventricle.

D) Number of c-*fos*-like immunoreactive neurons in arcuate nucleus (ARC), ventromedian hypothalamus (VM), dorsomedian hypothalamus (DM), paraventricular nucleus (PVN), and lateral hypothalamus (LH). Saline and glucose infusions in normal rats: open and filled columns, respectively; Glucose infusion in denervated rats: gray column. The results are expressed as the mean ± SEM of three rats per group; * different from saline, $p < 0.05$ (Student's t test for unpaired values).

E–G) Photomicrographs showing c-*fos*-like immunoreactive neurons in PVN of normal rats fed on SED (**E**) or PED (**F**) and of denervated rats fed on PED (**G**).

H) Number of c-*fos*-like immunoreactive neurons in ARC, VM, DM, PVN, and LH. SED-fed and PED-fed normal rats: open and filled columns, respectively; PED-fed denervated rats: gray columns. Magnification and expression of results as above.

(Figure S1 in the Supplemental Data available with this article online). The number of c-*fos* immunoreactive neurons was increased by a factor of 2 to 3 in all areas in postabsorptive rats infused with glucose compared to control rats infused with saline (Figures 4A, 4B, and 4D). The increases were quantitatively comparable in postabsorptive rats fed on PED in regard to control rats fed on SED (Figures 4E, 4F, and 4H). In contrast, there was no increase in c-*fos* labeling in any of these areas in rats in which the portal vein had been denervated (Figure 4C, 4D, 4G, and 4H). It may appear paradoxical that activation took place in the LH, especially because the latter has been classically considered as a feeding center (global destruction of the LH leading to hypophagia) and because reciprocal relationships between activities of the LH and VMH (conversely assumed to be a satiety center) were previously reported (Anand, 1961; Oomura et al., 1964). However, changes in the neuronal activity in the LH promoted by portal glucose were already reported (Schmitt, 1973; Shimizu et al., 1983). Moreover, one cannot predict from c-*fos* activation the final effect of those neurons that were selectively activated by PED or portal glucose in the LH. Further identifications of the neuromediators expressed in the c-*fos*-activated neurons by double labeling experiments would allow to specify the mechanisms induced by glucose and/or diet proteins. However that may be, our data strongly suggested that the central hypothalamic events elicited either by portal glucose infusion or PED-feeding were similar, and as a whole resulted in decreasing food intake.

In conclusion, we report in this study that protein feeding markedly increases the expression of the regulatory genes of gluconeogenesis in the SI in rats. This promotes endogenous glucose synthesis and release into the portal blood, a phenomenon lasting during the postabsorptive time, i.e., after the period of assimilation of food glucose. This portal glucose flux, sensed by the portal glucose sensor, is able per se to activate the hypothalamic nuclei involved in the regulation of food intake and to cause a decrease in subsequent food consumption. As such, these results provide an answer to the question of how protein-enriched meals decrease hunger and induce hypophagia, unsolved up to now (Barkeling et al., 1990; Booth et al., 1970; Jean et al., 2001; Rolls et al., 1988; Westerterp-Plantenga, 2003). Together with other recent results (Abbott et al., 2005; Gutierrez-Juarez et al., 2004; Peters et al., 2005; Pocai et al., 2005), our data further point out the connection between the central nervous system and the integrated digestive system, and the crucial role of the vagal transmission in the central control of energy homeostasis. Moreover, they bring to light a novel concept of control of food intake, involving the SI glucose metabolism as a key relay from the macronutrient composition of the diet to the amount of food ingested. Because gluconeogenesis is present in the human intestine (Battezzati et al., 2004; Mithieux et al., 2004b; Rajas et al., 1999, 2000), and protein-induced diminution of hunger concerns human beings (Barkeling et al., 1990; Booth et al., 1970; Rolls et al., 1988), the SI glucose metabolism may be a new target in the treatment of food intake disorders.

Experimental procedures

Animals and tissues sampling

Rats weighing 260–280 g were acclimated to our animal house under controlled temperature (22°C) and light conditions (light/dark, 12 hr/12 hr) with free access to water and SED diet. The composition of the diets was essentially based on that of a standard chow diet, as previously described (Mithieux et al., 2002), with modifications in the ratio starch-glucose/protein, that was 50%/17% in SED and 17%/50% in the PED (weight basis), respectively. Protein was a mixture of soya protein and casein (50/50). All experiments intended for tissue sampling were performed in the postabsorptive state, i.e., 6 hr after food removal, a time when food absorption was completed. The intestine was sampled essentially as described (Mithieux et al., 2004a). All procedures for gene expression analyses were previously described in detail (Mithieux et al., 2004a; Rajas et al., 1999, 2000).

Glucose flux measurements

Rats in the postabsorptive state were anaesthetized with pentobarbital (70 mg/kg), fitted with catheters and infused with [3-^{3}H] glucose as previously described (Croset et al., 2001; Mithieux et al., 2004a). After infusion for 10 min, a laparotomy was performed, the inferior mesenteric circulation was stopped by ligatures to exclude the blood flow coming from the cecum and the colon. After 90 min, a time where a steady state of glucose SA was obtained (Croset et al., 2001), blood was gently sampled simultaneously in the carotid artery and in the portal vein, using a catheter inserted into a superior mesenteric vein and pushed up to the junction with pancreaticoduodenal vein. Total EGP was obtained from the [3-^{3}H] glucose infusion rate and the SA of arterial glucose (Croset et al., 2001; Mithieux et al., 2002). The FX represents the fraction of [3-^{3}H] glucose removed by the SI. It was calculated as [([3-^{3}H] glucose SA_{artery} × glucose $concentration_{artery}$) − ([3-^{3}H] glucose SA_{vein} × glucose $concentration_{vein}$)]/[([3-^{3}H] glucose SA_{artery} × glucose $concentration_{artery}$). The intestinal glucose uptake (IGU) was deduced from this FX, the arterial plasma glucose and the intestinal blood flow (IBF): IGU = IBF × plasma glucose $concentration_{artery}$ × FX. The determination of IBF using radiolabeled microsphere technique was previously described (Croset et al., 2001). The intestinal glucose balance (IGB) is the net result of both the uptake and release of glucose by the SI. It was calculated from the difference between the arterial and venous glucose concentrations and IBF, as IGB = IBF × (glucose $concentration_{artery}$ − glucose $concentration_{vein}$). Finally, the intestinal glucose release (IGR) was derived from IGB and IGU by resolving: IGB = IGU − IGR.

All procedures used for determinations of plasma glucose, insulin, and glucose SA were described in detail previously (Croset et al., 2001; Mithieux et al., 2004a). Plasma CCK concentration was determined by radioimmunoassay after ethanol extraction, as previously described (Miazza et al., 1985). The crossreactivity of the antibody used with gastrin was less than 1%.

Studies in conscious animals

To equip them with indwelling catheters, rats were anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg). In rats intended for infusions into portal blood, a laparotomy was performed. A polyethylene catheter was inserted into a superior mesenteric vein, pushed up to the junction with pancreaticoduodenal vein, and secured with biological glue. A passage under the skin up to the basis of the neck was done to recover the other end of the catheter, which was secured with a dacron mesh button tether (Harvard apparatus). In rats intended for inactivation of portal vein innervation, a gauze compress moistened with 80 μl of a solution of capsaicin (10 mg/ml) in water:ethanol:tween 20 (8:1:1, vol/vol), was applied around the portal vein for 15 min at the time of surgery. In rats intended for infusions in peripheral blood, the indwelling catheter was inserted into the right jugular vein, and the other end recovered at the basis of the neck as above. After sewing up the incisions, rats were allowed to recover from surgery for one week with free access to SED and water. A solution of polyvinylpyrrolidon (7% (w/w) in saline, 200 μl) was infused each day to prevent coagulation.

In food intake experiments, 4 hr before the food consumption period, rats were placed into individual cages without food, with free access to water, and were infused with either saline or glucose (12 or 25 μmol/kg/min). At the time when the conditions of illumination changed (beginning of dark or light), they were given SED (or PED) for 3 hr while infusion was maintained. The latter time was supposed to match the transition from postprandial to postabsorptive period. The amount of food ingested was monitored at the end of this 3 hr time. Each rat was studied at least two to three times with either solution, infused in a random order. Mean values were first calculated for each solution in each rat taken individually. These values were then used

to calculate a mean value for each solution in each group, and to carry out statistical analysis.

To c-*fos* immunochemistry experiments, rats were infused under the same conditions as above, except that they were not given food when illumination changed. Infusions were prolonged for 2 additional hr before anesthesia and further brain postfixation (see below).

All protocols used in this work were performed according to the recommendations of our local animal ethics committee for animal experimentation.

c-fos immunochemistry

To allow valuable comparison with rats with portal infusions (see above), animals fed either on SED or PED were anesthetized 6 hr after removal of food. When portal vein denervation was involved, it was performed 7 days before the experiments as in the study relative to Figure 3. After anesthesia, rats were transcardiacaly infused with ice-cold 0.9% saline for 10 min, followed by 4% paraformaldehyde in PBS (phosphate-buffer saline) infusion for 20 min. The brains were removed, postfixed in ice cold 4% paraformaldehyde for 2 hr and cryoprotected in 30% sucrose in PBS for 2–3 days, at 4°C. They were then frozen and cut into 40 μm coronal sections using a freezing cryostat. The sections were exposed to 0.3% hydrogen peroxide for 30 min. They were then preincubated in PBS containing 3% normal goat serum and 0.25% Triton (blocking solution) for 2 hr, and further incubated for 48 hr with rabbit polyclonal c-*fos* antiserum (1/20,000 dilution; Ab-5, Oncogene Sciences, San Diego, California) in blocking solution. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG diluted at 1/600 (Vector Laboratories, Burlingame, California) for 1 hr, and with streptavidin horseradish peroxidase for 30 min, both in blocking solution. C-fos expression was visualized for fos-like immunoreactivity (FLI) using diaminobenzidine and hydrogen peroxide. A BH2 microscope (Olympus Corp, Melville, New York) connected via a color video camera to a PC computer using the Imagenia 2000 software (Biocom, Les Ullis, France) was used for cell counting. Each stained section was screened for visualization of FLI in neurons. In the regions of interest, fos-positive nuclei were counted following the area outlined, on numerous sections and on both sides, according to The Rat Brain in Stereotactic Coordinates (Paxinos and Watson, 1998).

Supplemental data

Supplemental Data include one figure and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/5/321/DC1/.

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