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Presence of leptin receptors in rat small intestine and leptin effect on sugar absorption

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Abstract Leptin is involved in food intake and thermogenesis regulation. Since leptin receptor expression has been found in several tissues including small intestine, a possible role of leptin in sugar absorption by the intestine was investigated. Leptin inhibited D-galactose uptake by rat small intestinal rings 33% after 5 min of incubation. The inhibition increased to 56% after 30 min. However, neither at 5 min nor at 30 min did leptin prevent intracellular galactose accumulation. This leptin effect was accompanied by a decrease of the active sugar transport apparent V_{max} (20 vs. 4.8 μmol/g wet weight 5 min) and apparent K_m (15.8 vs. 5.3 mM) without any change in the phlorizin-resistant component. On the other hand, immunohistochemical experiments using anti-leptin monoclonal antibodies recognized leptin receptors in the plasma membrane of immune cells located in the lamina propria. These results indicate for the first time that leptin has a rapid inhibitory effect on sugar absorption and demonstrate the presence of leptin receptors in the intestinal mucosa.

Key words: Leptin; Sugar absorption; Rat

1. Introduction

Leptin is a 16 kDa hormone, product of the *ob* gene, secreted from placenta and fat cells [1], that reaches the hypothalamus through systemic circulation where it is thought to act as a satiety factor. As a result, it appears to regulate body weight and fat deposition through effects on appetite as well as on metabolism and thermogenesis [2,3].

Since the cloning of the leptin receptor, the *ob-R* gene, from mouse choroid plexus [4] and the identification of five different isoforms [5,6], many studies have reported the expression of receptors in most peripheral tissues as well as in hematopoietic and lymphoid cell lines [6–8]. These results suggest that leptin may have a more complex role besides regulating food intake and energy expenditure than initially thought, raising the question of additional physiological functions of leptin in different tissues. For example, in isolated pancreatic islet, where expression of mRNA encoding Ob-Rb receptor has been found, leptin inhibits basal insulin secretion and glucose-stimulated insulin secretion in normal and *ob/ob* mice (obese mice with total leptin deficiency due to a mutation in the *ob* gene), but not in *db/db* mice, which are leptin-resistant due to a mutation in the receptor gene [9]. Furthermore, in rat kidney, autoradiography experiments with ^{125}I-leptin indicate the presence of leptin receptors in the internal medulla, and in vivo administration of leptin in normally hydrated rats shows a diuretic effect [10].

Chronic leptin treatment restores fertility to *ob/ob* mice, suggesting that leptin is also involved in the reproductive function linking adiposity and reproduction [11].

Localization of the leptin receptor (Ob-Ra form) on the epinephrine-secreting cells in the adrenal medulla suggests that epinephrine may mediate body weight homeostasis [12]. Furthermore, in isolated fat cells, leptin has a lipolytic action suggesting an autocrine/paracrine mechanism [13], and decreases insulin-stimulated glucose uptake when cells are incubated with leptin for 16 h [14]. In contrast, short-term exposure of rat skeletal muscle or adipocytes to leptin does not affect basal or insulin-stimulated glucose uptake [15].

Likewise, expression of leptin receptor, ob-Rb and ob-Ra isoforms, has been found in intestine using RT-PCR, Northern blot and RNase protection analysis [6,7], indicating that leptin may have a role in the regulation of gastrointestinal function. However, to our knowledge, nobody has yet reported any data concerning a leptin effect in the gut. Since the intestine is the site where glucose and other nutrients are absorbed after food intake and digestion and it is therefore one of the pathways which ultimately affect appetite and general metabolism, we set out to investigate the potential action of leptin in sugar absorption and also the possible location of leptin receptors in rat small intestine using immunohistochemical techniques.

2. Materials and methods

2.1. Uptake experiments

Incorporation of D-galactose into everted jejunal rings was determined as originally described by Crane and Mandelstam [16]. Briefly, rats were anesthetized with sodium pentothal (s.c., 60 mg/kg) and a segment (20 cm) of jejunum was quickly excised and rinsed with ice-cold saline solution (140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM KH_2PO_4, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$, pH 7.4), everted and cut into ~30 mg pieces. Groups of 4–6 intestinal rings were incubated for 5 or 30 min at 37°C and continuously gassed with O_2 in the named buffer containing 1.0 mM D-galactose and 0.1 μCi/10 ml of D-[^{14}C]galactose (specific activity 50–60 mCi/mmol; Amersham Radiochemical Center, London, England) in the absence (controls) and in the presence of 78 nM recombinant mouse leptin (Peprotech EC Ltd., London, UK). This initial leptin concentration was chosen on the basis of optimal lipolytic effects obtained in in vitro experiments [13]. At the end of the incubation period, tissues were washed in ice-cold saline solution, blotted carefully to remove excess moisture, weighed wet, and extracted by shaking for 24 h in 1 ml of 100 mM HNO_3 (0–4°C). 200 μl duplicate samples were taken and radioactivity was determined by liquid scintillation counting on a Wallac 1409 counter (Pharmacia, Finland). Rings were dried at 110°C for 48 h to obtain the total tissue water (difference between wet and dry weights) and extracellular fluid was determined by incubating the tissue for 20 min with saline solution containing 0.02 μCi/ml ^{3}H-PEG-4000 (polyethylene glycol [1,2-^{3}H], 0.5-2 mCi/g, NEN

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Research Products, Du Pont, UK). 1 mM phlorizin was used to measure passive uptake of galactose in the absence and in the presence of 78 nM leptin. A dose-dependent effect of leptin was obtained measuring 1 mM galactose uptake in the presence of 0.20, 0.39, 0.78, 7.80 and 78 nM leptin at 5 and 30 min. Kinetic experiments were performed at 5 min with galactose concentrations between 0.5 and 20.0 mM in the absence and in the presence of 0.39 nM leptin. Mediated uptake was obtained by the difference between total uptake and the uptake in the presence of 1 mM phlorizin (diffusion) at those sugar concentrations. Values are expressed as μmol D-galactose per g wet weight and as mM D-galactose concentration in the tissue intracellular fluid.

2.2. Immunohistochemistry

Rat jejunal everted rings were fixed for 22 h at room temperature (RT) in Bouin's fixative, dehydrated through graded alcohols and cleared in xylene at RT. Tissues were infiltrated and embedded in paraffin. Sections of 3 μm thickness were cut and mounted on slides. After blocking with 3% normal goat serum in PBS for 30 min, sections were incubated for 4 h at RT with 1 μg/ml leptin PBS. Leptin binding sites were detected with three monoclonal antibodies against mouse recombinant leptin (4B3, 6H11, 7H9) obtained in our laboratory as follows. Adult BALB/c mice (Charles River Breeding Laboratories, France) were subcutaneously administered with 60 μg whole mouse recombinant leptin and 60 μg of a peptide, a T-cell determinant with amino acid sequence FISEAIIHVLHRS named FISEA [17], both dissolved in 100 μl of PBS and homogenized with 100 μl of Freund's complete adjuvant. Mice were boosted twice, at 1 month intervals, with the same immunogen homogenized in Freund's incomplete adjuvant. Fifteen days after the last immunization, one mouse (selected on the basis of ELISA results) received an intravenous injection of 60 μg of leptin in 200 μl of PBS, and 3 days later, hybridomas were produced by fusion of NS-1 cells with spleen cells, as previously described [18]. Three monoclonal anti-leptin antibodies, termed 4B3 (IgG_1), 7H9 (IgG_{2b}) and 5F5 (IgM), produced by hybridoma culture, were obtained from culture harvest. Hybrid clones were characterized by immunoblot of whole mouse recombinant leptin as well as by an indirect ELISA coated with 0.1 μg/well of leptin or 30 different leptin synthetic peptides [3] to characterize the specificity of the antibodies. The indirect ELISA and the immunoglobulin class determination were done essentially as previously described [18]. For immunohistochemistry, crude supernatants from 7H9, 6H11 and 4B3 were mixed 1:1:1 respectively, and added to sections. Mouse monoclonal antibodies localization was performed as previously described [19]. The same method was used for control samples but omitting the leptin incubation step. Sections were counterstained with modified Harris-type staining, dehydrated, cleared in xylene and mounted in DPX for viewing with an optical microscope (Nikon, Optiphot, Tokyo, Japan).

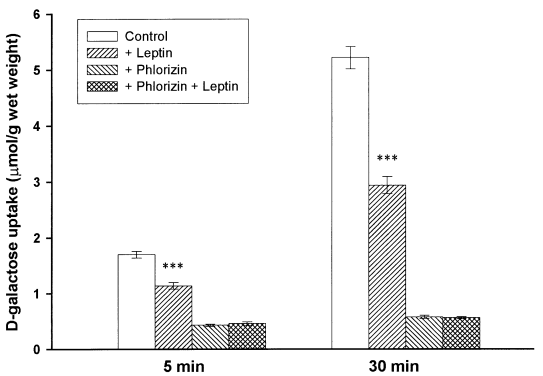

Fig. 1. Effect of leptin on D-galactose uptake. Intestinal rings were incubated in a saline solution containing 1 mM D-galactose ± 1 mM phlorizin with or without (controls) 78.00 nM leptin. Values are the means ± S.E.M. of 40–45 determinations from 12 different experiments. Groups were compared by Mann-Whitney's *U*-test. ***$P < 0.001$ vs. control.

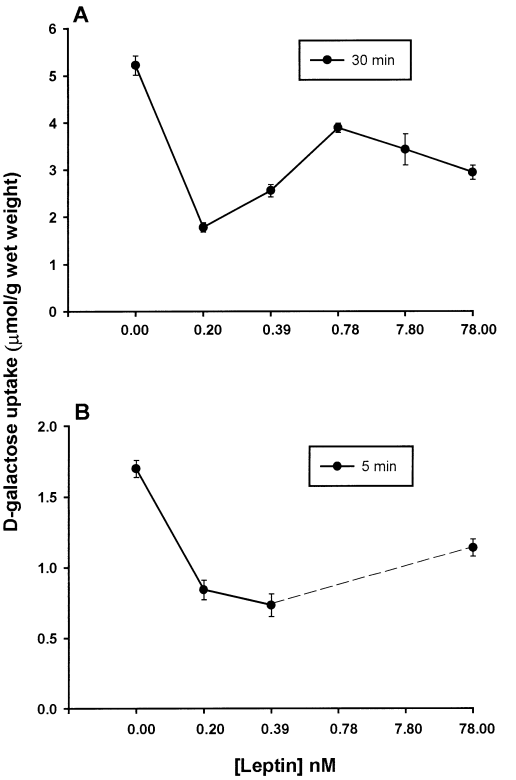

Fig. 2. Dose-dependent effect of leptin on D-galactose uptake by rat intestinal rings. 1 mM D-galactose uptake with or without different leptin concentrations (0.20–78.00 nM) was measured at 30 min (A) and at 5 min (B). Values are the means ± S.E.M. of 10–15 determinations from four different experiments. Groups were compared by Mann-Whitney's *U*-test. For all leptin concentrations assayed $P < 0.001$ vs. control (0.00 nM leptin).

3. Results

Leptin has a clear inhibitory effect on intestinal sugar uptake. After a 5 min incubation period, uptake of 1 mM D-galactose, 1.70 μmol/g w.w., decreases to 1.14 μmol/g w.w. (33% inhibition) when 78 nM leptin is present in the incubation medium, indicating a rapid effect on the initial entry of the sugar into the tissue (Fig. 1). The inhibition due to leptin becomes higher after 30 min (56%). In this case, uptake decreases from 5.22 to 2.94 μmol/g w.w., when sugar entry has already reached the steady state. After both 5 and 30 min incubation time, even in the presence of leptin, galactose accumulates in the intracellular fluid reaching higher concentrations than that of the incubation medium. Thus, sugar concentration in the intracellular fluid is diminished by leptin from 2.31 to 1.55 mM after 5 min incubation and from 7.09 to 3.99 mM at the steady state. To confirm that the galactose Na^+-dependent transport was affected by leptin and not the diffusional pathway, several experiments were performed in the presence of 1 mM phlorizin, which completely blocks the active transport, with and without leptin 78 nM. The phlorizin-resistant component represented 25% of the total uptake at 5 min and 12% at 30 min (Fig. 1) and neither at 5 min not at 30 had leptin any effect on it indicating that the active galactose transporter, SGLT1, was the one affected.

To determine the effect of leptin on galactose kinetics, we first studied the dose-response curve in order to choose the

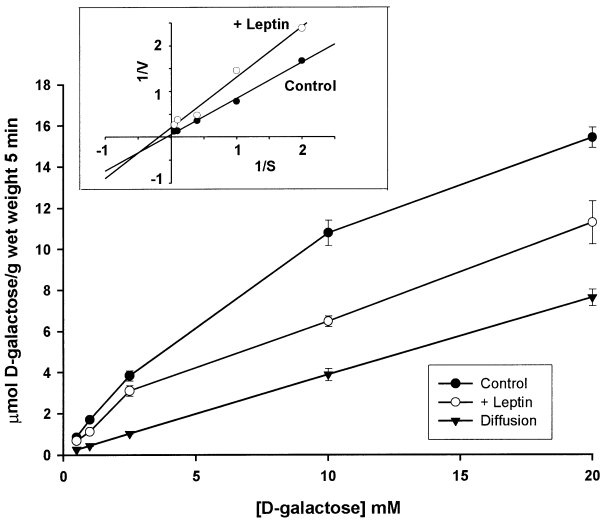

Fig. 3. Concentration dependence of galactose uptake by rat intestinal rings. D-Galactose (0.5–20.0 mM) uptake was measured at 5 min in the absence or in the presence of 0.39 nM leptin. The diffusion component was obtained at the same sugar concentration with 1 mM phlorizin. Values are the means ± S.E.M. of 10–15 determinations from three different experiments. Inset: Lineweaver-Burk plot of galactose active transport.

optimal leptin concentration to be used. Values in the range of the leptin plasma concentration [20] produced maximal inhibition: 0.20 and 0.39 nM at 30 min (Fig. 2A) and at 5 min respectively (Fig. 2B). We then chose 0.39 nM leptin and 5 min incubation time (initial entry of sugar into the rings) for the kinetic studies. Fig. 3 shows the concentration dependence of galactose uptake in the absence and in the presence of leptin, and galactose diffusion. The inset shows the Lineweaver-Burk plot of the sugar active transport. Non-linear regression analysis of the kinetic data revealed a K_m and V_{max} decrease in the presence of leptin: 15.8 vs. 5.3 mM for K_m and 20 vs. 4.8 μmol/g w.w. 5 min for V_{max}.

Leptin inhibition of sugar absorption presumes the presence of leptin receptors in the intestine. To verify this assumption, we performed immunohistochemical studies in order to localize leptin receptors in the small intestine. Receptors were detected within the lamina propria of the mucosa where leptin binds to the plasma membrane of immune cells. These cells, mainly granulocytes and macrophages, are located along the whole lamina propria. Macrophages are seen predominantly at the middle/apical side of the villi, while granulocytes, not stained with hematoxylin-eosin, are diffusely distributed, sometimes tightly associated with enterocytes at their basolateral membrane as can be observed at the top right of Fig. 4A. In the negative control, no immunoreactivity was observed (Fig. 4B).

4. Discussion

The current study demonstrates that leptin inhibits active transport of galactose in rat intestinal rings. Concentration values in the physiological range [20], 0.20 and 0.39 nM, produced maximal effects, diminishing as leptin concentration increased, which agrees with other in vitro studies [21]. Leptin does not modify the passive component of sugar absorption, being the effect due to a decrease of the transport component. Kinetic analysis indicate a mixed type inhibition with decrease of K_m and V_{max}. Using immunohistochemical techniques, the presence of leptin receptors in immune cells of intestinal mucosa is demonstrated here.

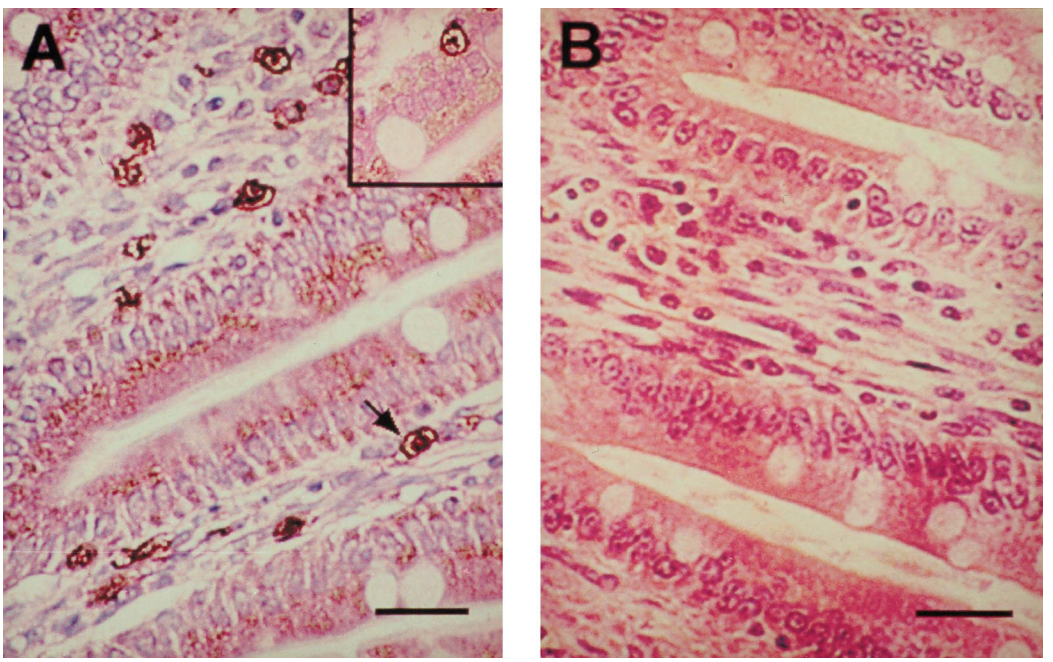

Fig. 4. Immunoperoxidase localization of leptin receptors in rat jejunum. A: Immunostaining is found at the plasma membrane of immune cells, macrophages (arrow) and granulocytes, of the lamina propria. The inset shows a granulocyte in close contact with the basolateral pole of an enterocyte. B: No immunoreactivity was observed in samples not incubated with leptin (controls). The images correspond to jejunum specimens from one of the rats used in the uptake experiments. Bar: 30 μm.

Taking into account the in vitro method used, the inhibitory effect cannot be explained by indirect actions on intestinal motility or blood flow, but are more likely due to an action on the enterocytes. The rapid decrease in galactose uptake induced by leptin after only 5 min incubation seems to confirm this. Neither at 5 nor at 30 min does leptin prevent sugar accumulation into the tissue.

It is well known that intestinal sugar transport is controlled by long-term adaptation of its capacity to metabolic changes. Thus, the small intestine has the ability to adjust its absorptive function under different dietary, developmental, environmental or pathological conditions [22]. However, studies performed in the past years give evidence that a normal activity of the intestinal epithelium results from the integration of multiple regulatory influences including hormones, mediators, neurotransmitters released from endocrine enteric nerve endings, immune effector cells and even mesenchymal elements [23,24]. These intercellular signals initiate intracellular changes, which regulate the transport of nutrients by the enterocytes in a short-term mode. So, glucagon-37, an enteric hormone released during the absorptive state, specifically stimulates cAMP-mediated glucose transport in rat isolated perfused intestine and isolated enterocytes [25]. On the other hand, serotonin, secreted by enterochromaffin cells in the mucosa and in myenteric plexus neurons, induces a short-term reduction in the Na^+-dependent uptake of L-leucine in rat intestinal rings. This effect, accompanied by a reduction of the apparent V_{max}, as found in the present study, is triggered by intracellular processes related to protein kinase C [26]. Furthermore, preliminary experiments performed in our laboratory demonstrate that cholecystokinin also inhibits galactose uptake in rat intestinal rings [27]. Using *Xenopus laevis* oocytes as expression system [28] it has been demonstrated that protein kinases regulate SGLT1 expression by controlling the distribution of transporters between intracellular compartments and the brush border membrane [29].

The binding of leptin to plasma membrane receptors of immune cells of rat intestine is consistent with previous reports that show leptin receptor expression in lymphoid cell lines [6,30]. These immune cells are located in the lamina propria, and some of them are in contact with the enterocytes (Fig. 4A, inset), suggesting that there may exist a mediator between those cells and the enterocytes, which activates the intracellular mechanisms directed to regulate sugar uptake. In fact, high levels of leptin binding have been found in the plasma membrane of peritoneal macrophages in which leptin is able to enhance the production of cytokines [30] that are known to initiate a cascade of actions in intestinal epithelial cells [24]. Therefore, cytokines could be potential candidates for mediating leptin inhibition on galactose intestinal transport. Other immune cells of the lamina propria, such as mast cells, are frequently found close to the basolateral pole of epithelial cells. They regulate chloride secretion by enterocytes through an indirect mechanism, which is apparently mediated by histamine [23]. As the effects of several hormones and peptides on nutrient uptake by brush border transporters are mediated by protein kinases [25,26], it can be suggested that cytokines could act in the same way.

Since leptin decreases appetite and increases basal metabolism [2,3], the inhibitory effect on intestinal sugar uptake reported in this study would be consistent with those two functions in order to play a role in body weight and composition homeostasis.

In summary, it is reported for the first time that leptin induces an inhibitory effect on intestinal sugar uptake and also the immunolocalization of leptin receptors in immune cells in the mucosa.

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