In vivo regulation of intestinal absorption of amino acids by leptin

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Abstract

Leptin is secreted by the gastric mucosa and is able to reach the intestinal lumen and bind to its receptors located in the apical membranes of enterocytes. We have previously demonstrated that apical leptin inhibits uptake of amino acids in rat intestine *in vitro* and in Caco-2 cells. The aim of the present work was to investigate the effect of leptin on absorption of amino acids using *in vivo* techniques, which generate situations closer to physiological conditions. *In vivo* intestinal absorption of amino acids in rats was measured by isolating a jejunal loop and using the single-pass perfusion system. Disappearance of glutamine (Gln), proline (Pro), and β-alanine (β-Ala) from the perfusate, in the absence or presence of leptin, was measured using a radioactivity method. Luminal leptin (25 nM) inhibited the absorption of 2 mM Pro, 5 mM β-Ala, and 5 mM Gln by approximately 45% after 5–15 min; the effect remained constant until the end of the experiment (80 min) and was rapidly and completely reversed when leptin was removed from the perfusion medium. Moreover, leptin was able to regulate the absorption of galactose and Gln in the same animal, indicating a direct action of the hormone on the specific transporters implicated in the uptake of each nutrient. The results of the present work indicate that luminal leptin decreases absorption of amino acids *in vivo* in a short-term manner and in a reversible way. These results, together with our previous findings, make it evident that leptin can be considered as a hormone which provides the intestine with a control mechanism to handle absorption of nutrients.

Key Words

- amino acids transporters
- *in vivo*
- leptin
- rat intestine

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Introduction

Leptin is a 167-amino acid peptide initially identified in adipose tissue (Zhang *et al*. 1994) and described as a hormone involved in the regulation of food intake and energy expenditure through binding to its receptor located in the hypothalamus (Pelleymounter *et al*. 1995, Tartaglia *et al*. 1995). Nevertheless, the results from studies performed since its discovery 20 years ago (Zhang *et al*. 1994) have largely demonstrated that leptin is a multifunctional hormone that can be synthesized and act in many different peripheral tissues (Margetic *et al*. 2002, Gertler 2009). In this regard, shortly after the identification of leptin, we demonstrated that leptin inhibited sugar absorption in rat intestine *in vitro* by short-term regulation of the Na^+/glucose cotransporter SGLT1 (Lostao *et al*. 1998). We later determined that leptin receptors were expressed in both apical and basolateral membranes of human and murine enterocytes (Barrenetxe *et al*. 2002). In the same year, another group reported that the chief cells of the stomach secreted leptin into the gastric lumen after a meal (Bado *et al*. 1998). This study was followed by the demonstration that the chief cells also secreted the leptin soluble receptor (Cammisotto *et al*. 2006), and that leptin remains stable in

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the gastric juices because binding to this receptor protects it from the acidic pH and the proteolytic activity of the stomach, favoring its arrival to the small intestine (Guilmeau *et al*. 2003, 2004). All these results supported our initial discovery about the role of luminal leptin as regulator of intestinal sugar absorption, and were followed by further studies on leptin modulation of sugar transport (Barrenetxe *et al*. 2001, 2004, Iñigo *et al*. 2004, 2007, Ducroc *et al*. 2005, Sakar *et al*. 2009). With regard to amino acid absorption, we have recently shown *in vitro* using rat intestine and Caco-2 cells that leptin also inhibits glutamine (Gln) and phenylalanine transport by the regulation of gene expression and protein activity of the implicated transporters, ASCT2 and B^0AT1 (Ducroc *et al*. 2010, Fanjul *et al*. 2012). Also, we have reported that in Caco-2 cells, leptin inhibits uptake of proline (Pro) and β-alanine (β-Ala) by PAT1 (Fanjul *et al*. 2012). Given that those studies were performed using *in vitro* models, the purpose of this study was to investigate whether leptin modulation of absorption of those amino acids could also be observed using *in vivo* techniques, which more closely resemble real physiological conditions.

The results indicate that leptin inhibits the absorption of Gln, Pro, and β-Ala *in vivo*, after short-duration exposure of the intestinal mucosa to the hormone, and that this effect is rapidly reversible.

Materials and methods

Animals and surgical procedure

The studies were carried out using male Wistar rats (180–250 g), purchased from the Applied Pharmacobiology Research Centre (CIFA) of the University of Navarra (Pamplona, Spain). The animals were housed at room temperature (20–22 °C), with a 12 h light:12 h darkness cycle, and had free access to water and standard chow diet (Harlan Ibérica, Barcelona, Spain). Food was removed 16–18 h before the experiments. The rats were anaesthetized by i.p. injection of a mixture (4:1) of ketamine (Ketolar, Parke-Davis, Barcelona, Spain) and medetomidine chlorhydrate (Domtor, Pfizer Orion Corporation, Espoo, Finland) at a dose of 0.25 ml/100 g of body weight. The rat was then placed in a temperature-controlled cabinet (30–35 °C) for the experimental procedure. The abdomen was opened by a mid-line incision, and a 20–30 cm jejunal loop (5 cm distal to the Ligament of Treitz) was isolated between two glass cannulae ligated at both ends. The intestinal segment was placed inside the abdomen and the cannulae were connected to a perfusion system, linked to a peristaltic pump (Gilson Minipulse 3) and to a heated bath to maintain the temperature of the perfusion medium at 37 °C. The intestinal loop was washed with 15 ml of Krebs–Ringer–Tris (KRT) saline solution at pH 7.4 or 6.0 and 37 °C. The composition of KRT in mmol/l was as follows: NaCl 140; KCl 5.6; $CaCl_2$ 3; KH_2PO_4 1.4; $MgSO_4$ 1.4; Tris 6.1; HCl 4.9 (Lostao *et al*. 1998). Air pumping was used to drain the remaining fluid in the intestine. Absorption was determined using the single-pass perfusion system, as described below. At the end of the experiment, the intestinal segment was excised and its length measured. The anaesthetized rat was then killed by cervical dislocation. The study protocol was approved by the Animal Research Ethic Committee of the University of Navarra, with the number 064-06.

Single-pass perfusion system

The intestinal loop was continuously perfused at 123 ml/min with a solution containing 5 mM galactose (Gal), 5 mM Gln, 2 mM Pro, or 5 mM β-Ala together with traces of the respective radiolabeled substrate. The radiolabeled sugar and amino acids ([^{14}C]-Gal, [^{14}C]-Gln, [^{3}H]-Pro, or [^{3}H]-β-Ala) were purchased from Perkin–Elmer and American Radiolabeled Chemicals. Consecutive fractions of the effluent were separately collected every 5 min. In each animal, control solution (without leptin) was perfused during the first 40 min and afterwards, 25 nM leptin was added and the solution pumped throughout the isolated loop for an additional 40 min. This leptin concentration was chosen on the basis of results from our previous *in vivo* studies (Iñigo *et al*. 2007) and studies by other authors (Buyse *et al*. 2001). The first four initial control samples were discarded. A similar protocol was carried out to investigate whether the effect of leptin on the absorption of amino acids was reversible. In this case, the intestinal loop was first perfused for 40 min with the substrate in the presence of leptin then the hormone was removed from the perfusion medium and the intestine was perfused for another 40 min. The samples of the initial medium and the effluent fractions were taken for counting of radioactivity. Each effluent fraction collected was weighed to determine the final volume. The variation in the volume of the solution after perfusion was minimal, but it was taken into account to correct for water transport in the calculation of the absorption values. Intestinal amino acid absorption was calculated using the difference between the initial and final amino acid concentrations in the perfusion solution with reference to the perfusion rate and the length of the intestinal segment (nmol amino acid/cm per min) (Vidal-Silvilla *et al*. 1950).

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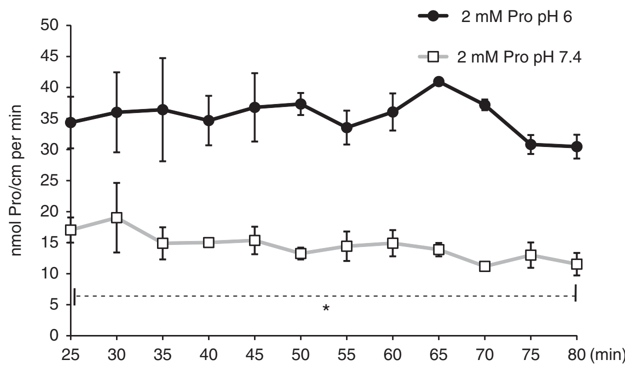

Figure 1
Proline absorption *in vivo*. Absorption of 2 mM Pro was measured at pH 6 and 7.4 throughout the experimental period (0–80 min); n=4–6, *P<0.05.

Statistical analyses

Statistical significances of differences were evaluated using the general linear model for repeated measures. Differences were considered to be statistically significant when P<0.05. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Inc.).

Results

One of the main transporters of Pro and β-Ala in the intestine is the H^+-dependent neutral amino acid transporter PAT1 (Bröer 2008). Optimal PAT1 activity occurs in Na^+ buffer at pH 6 (Iñigo *et al.* 2006, Fanjul *et al.* 2012). Before studying the effects of leptin on the absorption of Pro and β-Ala, we demonstrated the presence of PAT1 in rat intestine *in vivo*. Thus, based on our previous *in vitro* studies (Iñigo *et al.* 2007), we measured absorption of 2 mM Pro and 5 mM β-Ala at pH 6 and 7.4. As it is shown in Fig. 1, absorption of 2 mM Pro at pH 6.0 was 60% higher than at pH 7.4 (36.1±2.9 versus 14.9±2.1 nmol Pro/cm per min), indicating the presence of the PAT1 transporter. At both pHs, the absorption remained constant throughout the experiment (Fig. 1). Similar results were obtained with 5 mM β-Ala (data not shown). Gln absorption experiments were performed at 5 mM and pH 7.4 consistent with the functional characteristics of the intestinal transporters involved, ASCT2 and B^0AT1 (Bröer 2008, Ducroc *et al.* 2010).

In the control animals, amino acid absorption was assayed during the whole experimental period to confirm that absorption remained stable over time.

Effect of leptin on Pro, β-Ala, and Gln absorption

Absorption of 2 mM Pro, 5 mM β-Ala, and 5 mM Gln was measured for 40 min in the absence of leptin and then, for an during additional 40 min, in the presence of leptin. Figures 2A, 3A, and 4A show that leptin significantly (P<0.05) inhibited absorption of Pro, β-Ala, and Gln absorption by approximately 45% (29.5±2.4 versus 15.9±3.0 nmol Pro/cm per min; 79.5±0.6 versus 50.9±5.4 nmol β-Ala/cm per min; 136.0±2.7 versus 83.1±18.4 nmol Gln/cm per min). The inhibitory effect on absorption of Pro was observed after 15 min of perfusion of the amino acid with the hormone (Fig. 2A), whereas for β-Ala and Gln the inhibition was already statistically significant after 5 min (Figs 3A and 4A). For the three amino acids, the inhibition was maintained until the end of the experiment. Similarly, when the inhibition data were compared with the absorption levels of the corresponding control group of animals, the magnitude of the inhibition was of the same order (approximately 40–50%) for the three amino acids.

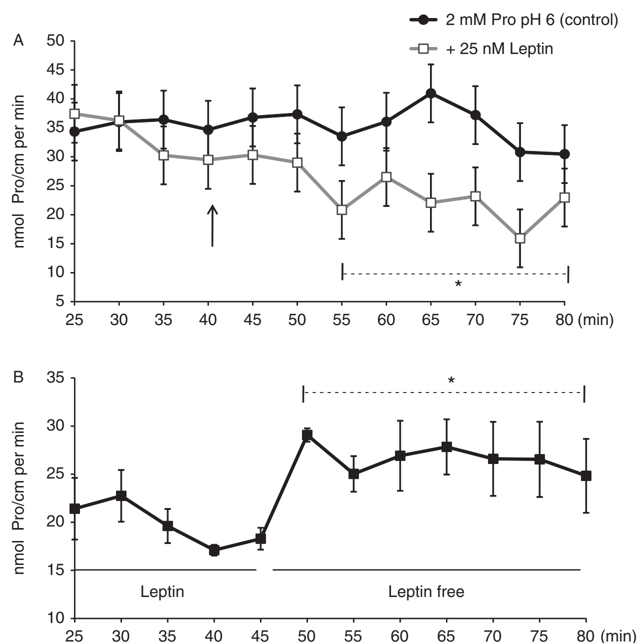

Figure 2
Effect of leptin on Pro absorption *in vivo*. (A) Absorption of 2 mM Pro at pH 6 (0–80 min) was measured in the absence of leptin (0–40 min) before the addition (↑) of the hormone (40–80 min). In the control animals, the amino acid absorption was measured throughout the experimental period (0–80 min) in the absence of leptin (B). The intestinal loop was perfused with 2 mM Pro in the presence of 25 nM leptin (0–40 min). Then, it was switched to perfusion with the amino acid in the absence of the hormone (40–80 min); n=4–6, *P<0.05 versus perfusion in the presence of leptin in the same group of animals.

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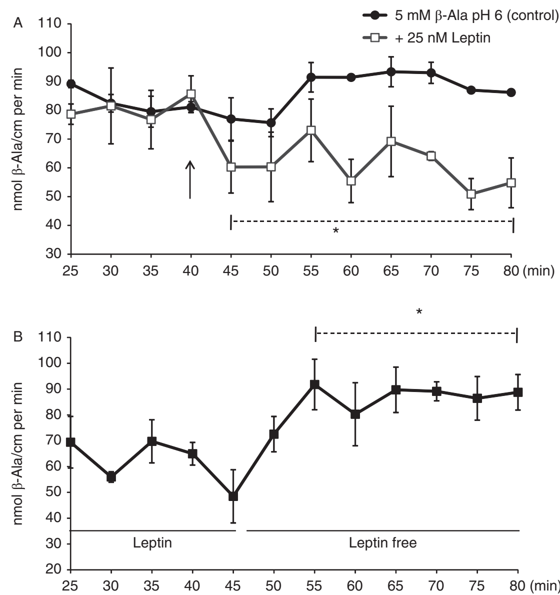

Figure 3
Effect of leptin on intestinal β-Ala absorption *in vivo*. (A) Absorption of 5 mM β-Ala at pH 6 (0–80 min) was measured in the absence of leptin (0–40 min) before the addition (↑) of the hormone (40–80 min). In the control animals, the amino acid absorption was measured throughout the experimental period (0–80 min) in the absence of leptin. (B) The intestinal loop was perfused with 5 mM β-Ala in the presence of 25 nM leptin (0–40 min). Then, it was switched to perfusion with amino acid in the absence of the hormone (40–80 min); n=4–6, *P<0.05 versus perfusion in the presence of leptin in the same group of animals.

Leptin inhibition of Pro, β-Ala, and Gln absorption is reversible

In order to check the reversibility of the inhibitory effect of leptin on intestinal amino acid absorption, the rat intestinal loop was first perfused with 2 mM Pro, 5 mM β-Ala, or 5 mM Gln in the presence of leptin for 40 min, followed by perfusion of the amino acid in the absence of the hormone for another 40 min (Figs 2B, 3B, and 4B). After removal of leptin from the perfusion solution, amino acid absorption levels significantly (P<0.05) increased within 5–10 min, reaching levels similar to those for controls (17.1±0.6 versus 29.1±0.7 nmol Pro/cm per min; 48.5±12.2 versus 91.8±3.7 nmol β-Ala/cm per min; 109.3±16.6 versus 142.0±15.8 Gln/cm per min) similar to those obtained in Figs 2A, 3A, and 4A.

These results indicated that the inhibitory effect of leptin could be completely and rapidly reversed by eliminating the hormone from the perfusion medium.

Leptin inhibits consecutive intestinal absorption of Gal and Pro

Previous results obtained by our group have indicated that sugar absorption is also inhibited by leptin *in vivo* (Iñigo *et al*. 2007). We wanted to verify whether leptin could inhibit the absorption of different nutrients in the same animal, which would further demonstrate the specific action of the hormone on individual transporters. Thus, a solution with 5 mM Gal at pH 7.4 was perfused for 20 min in the absence of leptin and afterwards, for an additional 25 min, in the presence of 25 nM leptin. Figure 4 shows that, as expected, leptin significantly (P<0.05) inhibited Gal absorption by approximately 40% (99.2±6.4 versus 61.9±9.2 nmol Gal/cm per min). After perfusion with Gal, the intestines were perfused with saline solution (KRT) for 15 min in order to eliminate the sugar that might still remain in the intestine. Following this wash out period, a solution containing 2 mM Pro at pH 6 was perfused for 20 min in the absence of leptin and then, for an additional 25 min, in the presence of leptin. As it happened for Gal, leptin inhibited Pro absorption also by approximately 40% (34.1±1.7 versus 20.6±3.41 nmol Pro/cm per min) (Fig. 4).

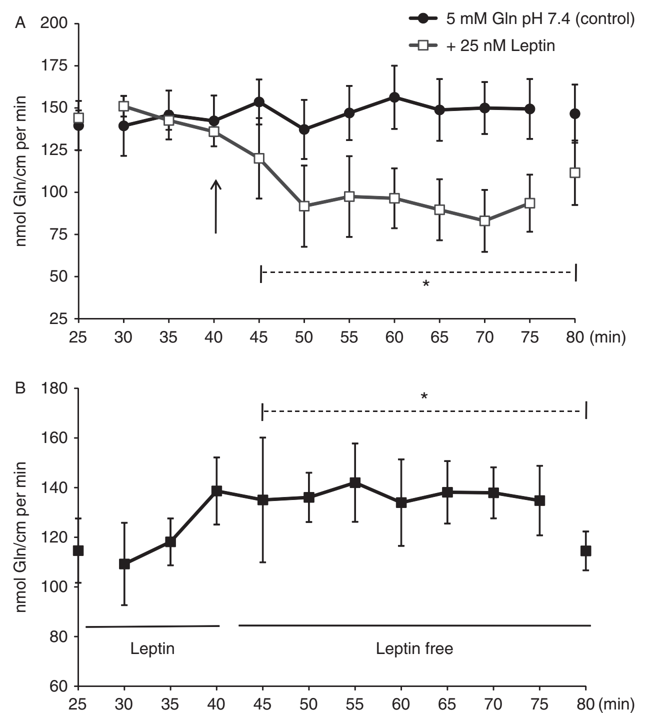

Figure 4
Effect of leptin on intestinal Gln absorption *in vivo*. (A) Absorption of 5 mM Gln pH 7.4 (0–80 min) was measured in the absence of leptin (0–40 min) before the addition (↑) of the hormone (40–80 min). In the control animals, the amino acid absorption was measured throughout the experimental period (0–80 min) in the absence of leptin. (B) The intestinal loop was perfused with 5 mM Gln in the presence of 25 nM leptin (0–40 min). Then, it was switched to perfusion with amino acid in the absence of the hormone (40–80 min); n=4–6, *P<0.05 versus perfusion in the presence of leptin in the same group of animals.

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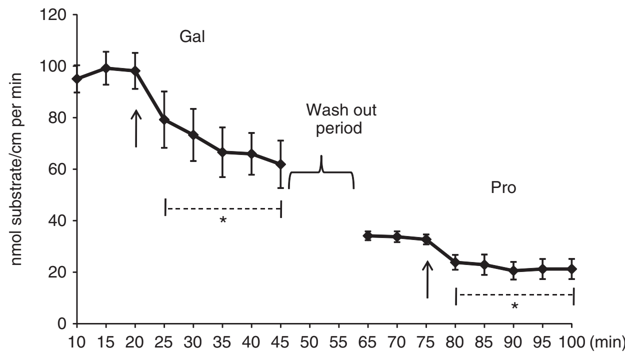

Figure 5
Effect of leptin on the consecutive intestinal absorption of galactose and Pro *in vivo*. Absorption of 5 mM glactose (Gal) at pH 7.4 was measured in the absence of leptin (0–20 min) before the addition (↑) of the hormone (20–45 min). Then, after a washout period of 15 min (45–60 min), absorption of 2 mM Pro at pH 6 was measured in the absence of leptin (65–75 min) before the addition (↑) of the hormone (75–100 min) $n=5$, *$P<0.05$ versus perfusion in the absence of leptin in the same group of animals.

These results indicated that leptin is able to regulate the absorption of two different substrates in the same animal, indicating that the hormone can separately modulate the activity of each of the transporters implicated in their absorption process (Fig. 5).

Discussion

The activity of the enterocytes results from the integration of multiple regulatory influences including hormones, mediators, neurotransmitters, and immune effector cells, among others (Barrett 1997). During the last 15 years, our group and the group of A Bado have been investigating the physiological role of leptin in absorption of nutrients using different physiological and biochemical approaches (Lostao *et al.* 1998, Barrenetxe *et al.* 2001, Ducroc *et al.* 2005, 2010, Iñigo *et al.* 2007, Sakar *et al.* 2009, Fanjul *et al.* 2012). All these results lead to the conclusion that leptin can be considered to be a new gastrointestinal hormone, which is secreted by the stomach (Cammisotto & Bendayan 2012), and rapidly regulates intestinal absorption of nutrients acting from the apical membrane of the enterocytes (Lostao *et al.* 1998, Ducroc *et al.* 2005, 2010, Iñigo *et al.* 2007, Fanjul *et al.* 2012).

In a continuation of our previous studies, the results from this work were indicative of *in vivo* short-term inhibition absorption of neutral amino acids by luminal leptin in rat intestine. The absorption of Pro, β-Ala, and Gln was decreased approximately 45% after 5–15 min of leptin perfusion; the effect remained constant until the end of the experiment and was rapidly and completely reversed when leptin was removed from the perfusion medium.

The *in vivo* intestinal absorption method results in experimental conditions close to the physiological state. These results described here confirm the findings from our previous *in vitro* studies in rat and in Caco-2 cells, where leptin rapidly inhibited Gln uptake by decreasing the expression in the brush border membrane of the enterocytes of the main Gln transporters present in the intestine, ASCT2 and B^0AT1 (Ducroc *et al.* 2010, Fanjul *et al.* 2012). We could, therefore, expect that the traffic of these two transporters from the plasma membrane to the intracellular compartments would also be directly regulated by leptin *in vivo*.

We also demonstrated the presence of a Na^+-independent, pH-dependent Pro and β-Ala transporter in rat intestine, whose activity was increased by Na^+, confirming the presence of the PAT1 transporter in the apical membranes of rat enterocytes (Iñigo *et al.* 2006). The results from *in vivo* experiments performed during this work indicated that absorption of Pro and β-Ala was also enhanced at acidic pH levels, demonstrating the proton-coupled amino acid transporter activity of PAT1. The absorption of these amino acids was also inhibited by leptin, which is consistent with our previous results obtained using Caco-2 cells where transport of Pro and β-Ala was reduced by leptin, as a result of decreased activity of PAT1, via leptin activation of an H-89-sensitive pathway (Fanjul *et al.* 2012). This effect occurred without modification of the expression of PAT1 in the brush-border membrane of the cells, and most probably was due to a decrease in the activity of the Na^+/H^+ exchanger, NHE3 (Fanjul *et al.* 2012). Whether the same mechanism occurs *in vivo* remains to be investigated, but all the results mentioned so far indicate that the *in vivo* data are consistent with the *in vitro* results.

Interestingly, luminal leptin increases peptide absorption by increasing the density of the proton-dependent peptide transporter PepT1 in the apical membrane of the absorptive cells both in rats *in vivo* and in Caco-2 cells (Buyse *et al.* 2001). Similarly, leptin can enhance the amount of GLUT2 and GLUT5 transporters in the apical membranes of enterocytes explaining the increase in absorption of Gal and fructose observed (Sakar *et al.* 2009).

At present, hormone regulation of digestive secretions and gastrointestinal tract motility is well established, nevertheless, little is known about endocrine regulation of intestinal absorption of nutrients, and information on this subject is not included in the text books yet. In this context, most of the information regarding the effects of leptin on absorption of nutrients, which is the major function of the

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intestine, has been demonstrated by our group. The present work and our previous results clearly indicate that leptin can regulate nutrient absorption, probably in relation to the specific metabolic needs of the enterocyte and its capacity for processing that specific nutrient.

The *in vivo* approach also revealed two relevant results. First, the effect of leptin is rapidly reversed once the hormone disappears from the lumen. Similar results have been obtained previously by our group for glucose absorption *in vivo*, where leptin regulates SGLT1 activity without modifying intestinal permeability (Iñigo *et al*. 2007). Second, leptin is able to separately regulate the absorption of Gal and Gln in the same animal, indicating an action on the specific transporters implicated in the absorption of each substrate.

In summary, the results of this study extend our previous findings and demonstrate that luminal leptin decreases absorption of amino acids *in vivo* in a short-term and reversible manner, as one of the physiological actions of leptin on the epithelial cells of the small intestine (Cammisotto & Bendayan 2012). Therefore, there is now enough information in the literature to consider leptin to be a new gastrointestinal hormone which provides the intestine with a control mechanism to handle absorption of nutrients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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