Luminal leptin inhibits L-glutamine transport in rat small intestine: involvement of ASCT2 and B^0AT1

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Leptin modulates intestinal glutamine absorption

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ABSTRACT

L-glutamine is the primary metabolic fuel for enterocytes. Glutamine from the diet is transported into the absorptive cells by two sodium-dependent neutral amino acid transporters present at the apical membrane: ASCT2/SLC1A5 and B^0AT1/SLC6A19. We have demonstrated that leptin is secreted into the stomach lumen after a meal and modulates the transport of sugars after binding to its receptors located at the brush border of the enterocytes. The present study was designed to address the effect of luminal leptin on Na^+-dependent Gln transport in rat intestine and identify the transporters involved. We found that 0.2 nM leptin inhibited uptake of Gln and phenylalanine (substrate of B^0AT1) using everted intestinal rings. In Ussing chambers, 10 mM Gln absorption followed as Na^+-induced short-circuit current was inhibited by leptin in a dose-dependent manner (maximum inhibition at 10 nM; I_{C50}~0.1 nM). Phe absorption was also decreased by leptin. Western blot analysis after 3 min incubation of the intestinal loops with 10 mM Gln, showed marked increase of ASCT2 and B^0AT1 protein in the brush border membrane that was reduced by rapid pre-incubation of the intestinal lumen with 1 nM leptin. Similarly, the increase in ASCT2 and B^0AT1 gene expression induced by 60 min incubation of the intestine with 10 mM Gln was strongly reduced after a short pre-incubation period with leptin. Altogether these data demonstrate that in rat, leptin controls the active Gln entry through reduction of both B^0AT1 and ASCT2 proteins traffic to the apical plasma membrane and modulation of their gene expression.

Key words

Ussing chamber, intestinal rings, leptin receptor, phenylalanine, qPCR

INTRODUCTION

Glutamine (Gln) is the most abundant amino acid in the plasma. It is absorbed by the intestine, making this organ a key player in the whole body Gln homeostasis. As Gln is precursor of the nucleosides and glucose synthesis and is involved in the acid-base balance in the kidney, it is crucial for the inter-organ nitrogen flux. Gln is also the most important energy source for the enterocytes, lymphocytes and fibroblasts, and necessary for the growth and viability of cells maintained in culture (5). Gln from the diet is transported into the enterocyte by two sodium-dependent neutral amino acid transporters present in the apical membrane: ASCT2 (System ASC) and B^0AT1 (System B) (6). ASCT2/SLC1A5 shows high affinity for Ala, Ser, Cys, Thr and Gln ($K_{0.5}$ ~20 μM), while B^0AT1/ SLC6A19 is a low affinity transporter ($K_{0.5}$ ranging from 1.4 to 4 mM) with preference for large neutral amino acids, including those with bulky lateral chain as Phe, a specific substrate for this transporter (6). In the cell, most of the Gln is metabolized to cover its energetic requirements and the rest is transported to the blood by the basolateral sodium-independent exchanger LAT-2/4F2hc, SLC7A8/SLC3A2 (system L) (6).

Leptin was initially described as an adipostatic signal controlling food intake and energy expenditure (32). Today, it is well known that leptin is a multifunctional hormone that is also involved in immune and neuroendocrine functions and nutrients absorption (22). This action is consistent with the production of leptin by many other tissues (1, 15, 23), as well as the expression of its receptors in peripheral tissues (14, 22). Indeed, we have demonstrated that leptin receptor is expressed in both the apical and basolateral membrane of intestinal absorptive cells (3). Other authors also showed that leptin is secreted into the gastric lumen after a meal by pepsinogen-containing secretory granules of chief cells (11), which also contain the leptin soluble receptor, indicating that this leptin receptor isoform is also released

into the gastric lumen (12). Bound to this receptor, leptin remains stable in the gastric juice, despite the severe conditions of pH and proteolytic activity in the gastric lumen, and is able to reach the intestinal lumen (17). Accordingly, we have demonstrated that leptin present in the intestinal lumen inhibits sugar absorption *in vivo* by regulating the Na^+/glucose cotransporter SGLT1 (19). Similar effect was previously reported *in vitro* (2, 16, 18, 21) with implication of both PKC and PKA activation (4, 16). Interestingly, luminal leptin enhances the intestinal transport of dipeptides by the H+/peptide transporter PEPT1 and CD147/MCT-1 mediated uptake of butyrate in mice and Caco-2 cells (9, 10), as well as fructose transport by the facilitative transporter GLUT5 in rat intestine *in vivo* (25). Given that leptin seems to regulate different nutrients transporters, one could anticipate that it may also modulate amino acid transporters in the intestine. However, there are not data on this respect yet. Considering the importance of Gln for the whole organism and in the intestine itself, the aim of the present study was to investigate the effect of luminal leptin on Gln transport and the target transporters of the hormone. We found that both ASCT2 and B^0AT1 are involved in Na^+-dependent uptake of glutamine in rat intestine and regulated by apical leptin. The present results give new insights into the role of leptin as a major gastrointestinal hormone regulating intake of rich energy molecules.

MATERIAL AND METHODS

Animals. Male Wistar rats weighing 220–260 g were obtained from Charles River Laboratories, L'Arbresle, France and the Applied Pharmacology Research Center (CIFA) of the University of Navarra, Pamplona, Spain. They were caged under standard laboratory conditions with tap water and regular food provided *ad libitum*, in a 12-h/12-h light/dark cycle at a temperature of 21–23°C and fasted for 16-18 h, with free access to water, before the

experiments. The animals were treated in accordance with the European Community Guidelines concerning the care and use of laboratory animals. The animal studies were performed under license from the veterinary department of Paris, France (to A. Bado and R. Ducroc, authorization no. 75-955 of September 22, 2004 and no. 75-174 of October 9, 2003, respectively; agreement no. B75-18-02; decision no. 05/12 established on July 12, 2005 by Prefecture de Police de Paris, France). The experimental protocol was approved by the Animal Research Ethic Committee of the University of Navarra, with the nº 064-06.

Everted intestinal rings uptake assays. Rats were anesthetized by i.p. injection of a mixture (4:1) of ketamine chlorhydrate (Ketolar, Merial S.A., Barcelona, Spain) and medetomidine chlorhydrate (Domtor, Pfizer Orion Corporation, Espoo, Finland), at a dose of 0.25 ml per 100 g body weight. Uptake of Gln or Phe by everted jejunal rings was determined as previously described (21). Briefly, rats were anesthetized and a segment (20-25 cm) of jejunum was quickly excised, rinsed with ice cold saline solution (NaCl 0.9%), everted and cut into ~30 mg pieces. Groups of 6 rings were incubated for 15 min at 37°C under continuous shacking and gassed with O_2, in Krebs-Ringer-Tris containing 0.5 mM Gln or 50 µM Gln (Sigma-Aldrich Co. USA) and 0.064 µCi/ml L-[^{14}C(U)]-glutamine (218 mCi/mmol; American Radiolabeled Chemicals, St Louis, MO, USA) or 0.5 mM Phe (Merck, Darmstadt) and 0.064 µCi/ml L-[^{14}C (U)]-phenylalanine (370 mCi/mmol; American Radiolabeled Chemicals, St Louis, MO, USA), in the absence (control) and in the presence of 0.2 nM recombinant rat leptin (Peprotech EC Ldt., London, UK). After the incubation period, rings were washed in ice-cold saline solution and radioactivity incorporated into the tissue was determined by liquid scintillation counting. Results are expressed as pmol amino acid/g wet weight/min (pmol/g/min).

Tissue preparation and short-circuit measurement. Animals were killed by i.p. pentobarbital overdose and the proximal intestine was dissected out and rinsed in cold saline solution. The mesenteric border was carefully stripped off using forceps and the small intestine opened along this border and rinsed in Krebs-Ringer bicarbonate solution (KRB). Four adjacent proximal samples were mounted in modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA). Exposed area was 0.50 cm^2. The tissue samples were bathed with 4 mL of carbogen-gassed KRB solution on each side. Serosal KRB solution had the following composition (in mM): NaCl 115.4, KCl 5, $MgCl_2$ 1.2, NaH_2PO_4 0.6, $NaHCO_3$ 25, $CaCl_2$ 1.2 and glucose 10. In mucosal KRB solution, glucose was replaced by mannitol. Each reservoir was gassed with 95% O_2-5% CO_2 and kept at constant temperature of 37°C (pH at 7.35).

Electrogenic ion transport was monitored continuously as short-circuit current (Isc) by using an automated voltage clamp apparatus (DVC 1000, WPI, Aston, England) linked through a MacLab 8 to a MacIntosh computer. Results were expressed as the intensity of the Isc ($\mu A/cm^2$) or as the difference (ΔIsc) between the peak Isc after amino acid challenge and the basal Isc measured just before the addition of the amino acid. Leptin was added in either the mucosal or the serosal bath 2-10 min before Gln challenge in the mucosal bath.

We found that there were no difference neither in the quantitative sodium-dependent Gln transport nor in the effect of leptin between middle jejunum and middle ileum, so the rest of studies were performed using middle small intestine (jejunum-ileum).

Western blot analysis. Fasted rats were anesthetized by i.p. administration of pentobarbital and laparotomized. Three small intestinal loops (7 cm length) per rat were prepared as previously described (16). They were filled with 3 mL of KRB-mannitol without (2 loops) or with 1 nM leptin (1 loop) using a syringe equipped with a 30-4/10 hypodermic needle

(Acierinox, Vincent, Paris, France). After 3 min *in vivo* incubation, the loop containing leptin and one loop without leptin were filled with ~1 ml of a 10 mM Gln solution and again incubated for 3 min. This protocol was performed in four different rats where the loops were treated in a randomized way. In some rats, one loop was injected with leptin but without Gln. After sacrifice, loops were removed, opened along the mesenteric border and the mucosa was scrapped off on ice with a glass blade. Brush border membrane vesicles (BBMV) were prepared from mucosa scrapings as previously described (16). Protein concentration was quantified using the BCA protein assay Kit (Pierce, Rockford II, USA). Solubilized proteins were resolved by electrophoresis on 12.5% SDS-PAGE gels. The resolved proteins were transferred onto nitrocellulose membranes and subjected to immunoblot analysis with a rabbit anti-ASCT2 polyclonal antibody (AB 1352, Chemicon Millipore, Temecula, CA) diluted 1:7,500, rabbit anti-B^0AT1 at 1:500 (generous gift from François Verrey) or with mouse monoclonal β-Actin antibody (sc-81178, Santa Cruz Biotechnology Inc., Santa Cruz CA. The intensity of the immunoreactive bands detected by enhanced chemiluminescence (Pierce, Rockford, IL) was quantified using NIH Image (Scion Corp. Frederick Maryland, USA). β-Actin was used as loading controls. The results were expressed in relation to control and the value of control was arbitrarily set to 1.

Real-time PCR analysis (qRT-PCR). Real-time PCR was used to examine the effect of leptin on Gln-induced ASCT2 and B^0AT1 gene expression. Rats were anesthetized and laparotomized. Three small intestinal loops of ~8 cm starting 15 cm from the cecum were prepared and filled with 1 nM leptin or saline. After 3 min *in vivo* incubation, loops were filled with 10 mM Gln for 60 min. After this time, rats were killed, loops were dissected and rinsed in saline and mucosa was scrapped off on ice. Total RNA was extracted from the mucosa samples with the Trizol reagent (Qiagen). The first-strand cDNA was synthesized by reverse transcription from 5 µg of total RNA with SuperScript II reverse transcriptase (Invitrogen,

Cergy-Pontoise, France). Quantification of cDNA was performed with a Light Cycler System (Roche Diagnostics, Meylan France) according to the manufacturer‘s instructions. Primers were as follows: rASCT2 f5’- CCACATGCGAAAAGGAATCT-3’, and r5’-CTCAAGAGCCCAATTTCCAA-3’; rB^{0}AT1 f5’-TTACCAAGTCAGGGGGTGAG-3’ and r5’-GATGAGGGCTTCATGACGAT-3’; r18S f5’–CCCTGGCCTTTGTACACACC-3’ r5’-GATCCGAGGGCGCTCACTA-3’. They were designed with oligo 4 software and synthesized by Eurogentec (UK). The comparative ΔΔCT-method was used for relative mRNA quantification of target genes, normalized to protein 18S and a relevant control equal to 2-ΔΔCT.

Chemicals. Recombinant murine leptin was purchased from PreProtech EC (PreProtech EC Ltd, London, United Kingdom). All other chemical reagents were purchased from Sigma (St. Louis, MO, USA). Leptin antagonist L39A/D40A (kind gift of Prof. Arieh Gertler) was diluted in saline.

Statistical analysis. All results were expressed as means ± SEM. Student’s t test or one-way ANOVA with Tukey-Kramer multiple comparison post test when appropriate were performed using GraphPad Prism version 4.0 for Windows (Graphpad software Inc., San Diego, CA) or SPSS. v.15. The level of significance was set at $p<0.05$.

RESULTS

Leptin inhibits glutamine uptake in everted rings The effect of leptin on Gln uptake was first studied on preparations of everted intestinal rings after 15 min incubation. As shown in Fig 1A, uptake of 0.5 mM Gln was significantly reduced (~35%) by 0.2 leptin. The hormone also significantly reduced, by ~25 %, uptake 50 µM Gln, a concentration close to the $K_{0.5}$ for ASCT2 (6) (Fig 1B). To evaluate the contribution of B^{0}AT1 on Gln absorption at both

concentrations, we examined the effect of 25 mM Phe (specific substrate of B^0AT1) on Gln uptake. As depicted in Fig. 1A, Phe reduced 0.5 mM Gln uptake by ~65%, indicating a higher contribution of B^0AT1 on Gln uptake in comparison to ASCT2. However, uptake of 50 μM was not inhibited by Phe (Fig 1B) demonstrating that at this concentration only ASCT2 was contributing to Gln absorption. The uptake of 0.5 mM Phe, assayed in the same tissue preparation as Fig. 1A, was found less than 10% of 0.5 mM Gln uptake but was also inhibited (~45%) by leptin (Fig 1C).

Luminal leptin reduces glutamine-induced Isc. Leptin effect was further investigated in Ussing chambers, a polarized system that permits the access to either side of the tissue preparation. After the intestinal mucosa was isolated in the chamber and allowed to reach a steady state (usually 40 min), tissues were challenged with 10 mM Gln, which induced a rapid (less that 2 min) and significant rise in Isc (~23 $\mu A/cm^2$). The increase in Isc was the result of the Na^+ mucosal-to-serosal movement that sustained the amino acid entry through sodium-dependent amino acid transporter/s. In accordance to the results obtained with intestinal rings (Fig 1.A and C), 10 mM Phe induced a small Isc, which was ~12 % of that for Gln (Fig. 2A). Luminal addition of 10 mM Ala also significantly raised Isc (ΔIsc ~15 $\mu A/cm^2$) whereas no change in Isc was observed after 10 mM methyl aminoisobutyric acid (data not shown), indicating the absence of system A participation in the electrogenic uptake of Gln.

Again, in line with the data obtained with intestinal rings (Fig. 1A), 20 mM Phe reduced the Gln-induced Isc by ~70 % when it was added to the mucosal bath 3 min before 10 mM Gln challenge (Fig. 2B) and leptin (10 nM) inhibited by ~60 % Gln and Ala-induced Isc (Fig. 2C)

Inhibition of 10 mM Gln transport across intestinal mucosa, following rapid incubation with leptin in the mucosal reservoir, was found concentration-dependent (Fig 2D). Inhibition was significant with 0.01 nM (~30 %), maximal for 10 nM (~80%), and decreased with 100 nM

leptin up to ~50 % The concentration producing a half-maximal inhibition of Gln transport (IC_{50}) was 0.1 nM (Fig. 2D). Leptin (10 nM) reduced the Isc induced by 30 mM Gln (26% inhibition, $p<0.05$) and also the small Isc induced by 1 mM Gln, although in this case the inhibition did not reach statistical significance (data not shown).

Mucosal vs. serosal leptin. The possible effect of leptin on Gln-induced Isc acting from the serosal side was also studied in Ussing chambers. As shown in figure 2E, 10 nM leptin after 2 min in the serosal bath also decreased the 10 mM Gln-induced Isc by ~ 40%. This decrease, however, was smaller than that produced by the hormone acting from the mucosal side, which was around 80 %. After 10 min, leptin from the serosal side had no more inhibitory effect while it was still active from the mucosal side (50 % inhibition).

Leptin inhibition of glutamine transport is blocked by leptin receptor antagonist. To examine whether the inhibitory effect of leptin was dependent upon leptin receptor, we studied the effect of L39A/D40A, a mutated leptin-based peptide acting as a leptin antagonist (26). As shown in Fig 2F, addition of 50 nM L39A/D40A to the mucosal bath immediately prior to 10 nM leptin, reversed leptin inhibition of Gln-induced Isc, whereas 5 nM L39A/D40A had no effect.

Leptin reduces glutamine-induced ASCT2 and B^0AT1 protein expression. Western blot assays were performed to investigate if leptin could modify the expression in the plasma membrane of ASCT2 and B^0AT1 transporters. As shown in Fig. 3, incubation of the intestinal loops with 10 mM Gln during 3 min increased the presence of ASCT2 and, in a lesser extent, of B^0AT1 in the brush border membrane vesicles (Fig. 3A and B). This increase was inhibited

when the loops had been previously incubated with 1 nM leptin for 3 min, showing that leptin reduced Gln-induced ASCT2 and B^0AT1 expression in the plasma membrane.

Leptin reduces glutamine-induced ASCT2 and B^0AT1 mRNA expression. Finally, the effect of luminal leptin on Gln-induced ASCT2 and B^0AT1 gene expression was studied. Luminal infusion of 10 mM Gln for 60 min markedly increased the level of ASCT2 and B^0AT1 mRNA (6.7 fold and 2.8 fold respectively when compared to their relative controls ; Fig. 4A and B). When the intestinal loops were first incubated with 1 nM leptin for 3 min before the Gln infusion, the increase in ASCT2 and B^0AT1 mRNA was significantly reduced (~55%). These results indicate that leptin can also regulate Gln-induce ASCT2 and B^0AT1 mRNA expression.

DISCUSSION

The present results demonstrate that, in rat intestine, leptin inhibits the absorption of Gln and the protein and mRNA expression of ASCT2 and B^0AT1, the two major Gln transporters in the apical membrane of the enterocytes (6), and that this effect is a clear result of the luminal action of the hormone.

Glutamine, the most abundant amino acid in the body, plays a central role in inter-organ nitrogen transfer (13). Circulating Gln concentration is maintained at relatively constant level which is critical to avoid Gln depletion in blood and organ dysfunction. The small intestine is the most important organ for the supply of exogenous Gln to the body. Gln is also the major fuel for intestine absorptive cells. Though the transport systems for the entry of L-glutamine by intestinal epithelia cells have been studied in different animal models (6), its regulation by hormones and peptides is not yet completely understood. Growth hormone (GH) and

epidermal-growth factor (EGF) can enhance intestinal Gln uptake (27), and a possible hormonal stimulation of ASCT2 *in vivo* has been suggested (24).

Leptin is now well documented as an important regulator of nutrients transporters from the apical membrane of the enterocytes. Indeed, leptin was shown to control butyrate MCT-1-mediated absorption (10) and di/tri-peptides transporter PepT-1 (9) and modulate the activity of glucose and fructose transporters through its action on SGLT1 (16, 19, 21) GLUT2 and GLUT5 (25). A significant role for leptin in controlling active transport of Gln was previously established in human placental villous fragments where leptin stimulates the activity of system A, thus controlling the availability of this important fuel to placenta cells (20).

Here, the functional studies were first performed *in vitro* using everted intestinal rings. The same technique was used to demonstrate for the first time that leptin inhibits sugar intestinal transport (21). Even though with this technique the mucosa is exposed to the medium and the access of the hormone to the enterocyte is mostly through the apical membrane, a possible basolateral action of leptin could not be discarded. Therefore, we further measured Gln transport in Ussing chambers, where contribution of mucosal *vs.* serosal leptin could be distinctly examined and mucosal-to-serosal Gln entry could be followed as sodium-induced short-circuit current. As observed with intestinal rings, leptin pre-treatment from mucosal side induced a rapid and marked reduction of the Gln-induced Isc. This effect was concentration-dependent with characteristics of inhibition found in the nanomolar range (16, 21). The effect of leptin from the serosal side was found significantly less pronounced and disappeared at the longest incubation time (10 min). These results may reflect the fact that leptin exposed to the serosal side needs to diffuse a few layers of muscle and connective tissues before reaching its receptors on the basolateral membrane of the enterocytes, with possible reduction of the effective concentration. In addition, the membrane surface area for leptin action is smaller when it acts from the serosal side compared to the apical side and the distance between the

receptor and the Gln transporters higher. After 10 min, the lack of leptin action from the serosal side can be also related to the decrease of its effect from the apical side.

The Ussing chamber technique mimics the relevant action of leptin which is produced and secreted by gastric cells (1, 11) together with its soluble receptor (12) and can flow along the digestive lumen to reach the small intestine and act as a physiological modulator. In fact, using leptin mutein, a leptin receptor antagonist (26) we demonstrate that this modulation requires an effective interaction of leptin with its specific receptor located at the brush border membrane of the small intestine (3, 11). Interestingly, a recent report shows that in leptin-receptor deficient obese Zucker rats, Gln transport is highly increased (29). This is in line with the inhibitory action of leptin as a regulator of the Gln uptake here reported. It is tempting to speculate, therefore, that leptin may act as a brake on the entry of Gln possibly in balance with other stimulating hormone (i.e. GH or EGF). Interestingly, this leptin-induced action is apparently different from the one recently proposed for angiotensin II which inhibits intestinal glucose uptake but not amino acid transport under normal and diabetic conditions (30, 31).

In the intestine Gln transport is assumed to be predominantly achieved by the sodium-dependent transporter B^0AT1 (28). In rat intestinal rings, uptake of Phe, a specific substrate of B^0AT1, was also inhibited by leptin, indicating that B^0AT1 was a target for the hormone. Interestingly, Phe uptake was very low compared to Gln uptake at the same concentration, which can be explained by the fact that B^0AT1 shows lower affinity for its substrates ($K_{0.5}$ ranging from 1.4 to 4 mM) compared with ASCT2 ($K_{0.5}$ ~20 µM) (6). Nevertheless, 25 mM Phe was able to inhibit by ~65 % Gln uptake, suggesting a higher contribution of B^0AT1 on Gln absorption in rat intestine under this experimental condition. By employing Ussing chamber, we also found that Phe-induced Isc was lower compared to the Gln-induced Isc. Aside to B^0AT1, ASCT2 is the other sodium-dependent Gln transporter present in the apical membrane of small intestine (6). From the functional studies using 50 µM Gln (concentration

at which only ASCT2 in responsible for Gln absorption), we also demonstrate that ASCT2 is regulated by leptin.

To assess directly the presence of the two Gln transporters in rat BBM and responsiveness to luminal substrate and/or leptin, we further used biochemical and molecular approaches. Our results clearly indicate that in basal condition, both B^0AT1 and ASCT2 are present in rat BBM. Their expression in the membrane can be rapidly increased by luminal Gln and this increase can be reduced by leptin at nanomolar range. Interestingly, the ratio of ASCT2/B^0AT1 protein expression in basal state was low (Fig. 3) and confirmed the reported predominance of B^0AT1 in intestinal BBM (28). However, after luminal challenge with Gln, more ASCT2 than B^0AT1 protein was observed to swing into the BBM. These biochemical data would support a major importance of ASCT2 on Gln uptake in rat intestine. However, due to the differences in the experimental conditions, it is not possible to compare the functional and biochemical results to draw conclusions about which transporter is the principal in Gln entrance in the enterocytes. Nevertheless, both functional and biochemical data hallmark the contribution of ASCT2 and B^0AT1 transporters in Gln uptake.

We further demonstrated that leptin, in addition to its effect on post-translational regulation, can rapidly trigger transcriptional control of the two Gln transporters. Thus, leptin inhibits the Gln-induced increase of ASCT2 and B^0AT1 mRNA levels. Regulation of *ASCT2* gene expression by Gln was previously reported in hepatic epithelial (7). The stimulation of expression in response to Gln was shown to involve in part binding of FXR/RXR to the ASCT2 promoter (8) .

In summary, we demonstrate that luminal leptin can modulate the intestinal activity and expression of the Gln transporters ASCT2 and B^0AT1 in the apical membrane of the enterocytes. Because Gln is known to have both acute and chronic effects on cell metabolism and function (13), the present findings of leptin involvement in the modulation of Gln uptake

in both short and long-term mode of action is believed to be of major importance for the biology of intestinal cells.

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FIGURE LEGENDS

Figure 1. Effect of leptin on glutamine and phenylalanine uptake by everted intestinal rings. Uptake of 0.5 mM Gln (A), 50 µM Gln (B) or 0.5 mM Phe (C) was measured in the absence or presence of 0.2 nM leptin after 15 min incubation. Uptake of Gln was also measured in the presence of 25 mM Phe (A and B) . Experiments in A and C were performed in the same experimental group. The results are expressed as mean ± SEM; ***, $p<0.001$ (n=18 from 3 animals).

Figure 2. Leptin inhibition of glutamine induced Isc in Ussing chamber. (A) Isc induced by 10 mM Gln, Ala and Phe (n=8-29) (B). Effect of 20 mM Phe on 10 mM glutamine-induced Isc (n=4) (C) Effect of 10 nM mucosal leptin on Isc induced by 10 mM Gln or Ala. (D). Dose-response for mucosal leptin inhibition of Gln-induced Isc. Leptin was added in the mucosal bath 2 min before tissues were challenged with 10 mM Gln. Values for Isc were standardized to control value. Each point represents the mean ± SEM of 4-6 non cumulative values from 4 separate experiments. (E). Effect of serosal leptin (sx) *vs*. mucosal leptin (mq) at 10 nM concentration on 10 mM Gln-induced Isc (control) (n=6) .*, $p<0.05$ *vs.* control; .#, $p<0.05$ *v.s.* 2 min mq. (F) Action of leptin is receptor specific: leptin antagonist L39A/D40A was added to the mucosal bath 5 min before the addition of leptin and 3 min later, tissues were challenged in the mucosal side with 10 mM Gln (n=6).

Figure 3. Effect of luminal leptin on Gln-induced expression of ASCT2 and B^0AT1 proteins in BBM. Brush border membrane vesicles were obtained from mucosa of NaCl (control), 1 nM leptin and 10 mM Gln treated (3 min) intestinal loops, and ASCT2 (A) and B^0AT1 (B) protein expression analyzed by Western blot. Representative Western blot images are shown. Intensity of the immunoreactive bands was quantified and expressed in relation to control.

Results are expressed as mean ± SEM for 3-4 rats per experimental group. **, p<0.01 and *** , p<0.001 *vs*. control. # P<0.05 *vs*. Gln.

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Figure 4. Effect of leptin on Gln-induced ASCT2 and B^0AT1 gene expression. Total RNA was extracted from mucosa of NaCl (control), 1 nM leptin and 10 mM Gln treated (60 min) intestinal loops, and ASCT2 (A) and B^0AT1 (B) mRNA expression analyzed by RT-PCR. Results are expressed as ratio of transporter/r18S mRNA expression in relation to control and presented as mean ± SEM of 3-4 rats per experimental group. * P<0.05; ** P<0.01 and *** P<0.001 vs. control; # P<0.05 *vs*. Gln