Universidad de Navarra

Faculty of Pharmacy

LEPTIN REGULATION OF INTESTINAL AMINO ACIDS ABSORPTION: *IN VITRO* AND *IN VIVO* STUDIES

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Facultad de Farmacia

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Memoria presentada por Dª Carmen Fanjul González para aspirar al grado de Doctor por la Universidad de Navarra

Carmen Fanjul González

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología (Nutrition, food Science, Physiology and Toxicology) y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, ... de ..... de 2012.

Dra. Mª Pilar Lostao Crespo

Dra. Jaione Barrenetxe Huici

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Abbreviations

AA^0	Neutral amino acids
AA^+	Cationic amino acids
ASCT2	Alanine, serine, cysteine transporter 2
ATB^0	Amino acid transporter B^0
$b^{0,+}$	$b^{0,+}$ type amino acid transporter
B^0AT1	Broad neutral amino acid transporter 1
BBB	Blood–brain barrier
BBMV	Brush border membrane vesicles
CCK	Cholecystokinin
CNS	Central nervous system
DMEM	Dulbecco‘s Modified Eagles medium
DNA	Deoxyribonucleic acid
DNAc	Complementary deoxyribonucleic acid
EGF	Epidermal grown factor
ERK	Extracellular regulated kinase fa/fa mutation
FBS	Foetal bovine serum
GH	Growth hormone
Gln	Glutamine
Glu	Glutamate
GLUT	Glucose transporter
GRB2	Growth factor receptor binding protein 2
HFS	High sucrose fat diet
Isc	Short circuit current
JAK	Janus tyrosine kinase
K_m	Affinity constant
LAT	Large amino acid transporter
LYAAT1	Lysosomal amino acid transporter 1
MAPK	Mitogen-activated protein kinase
MCT-1	Monocarboxylate transporter 1
mTORC1	Mammalian target of rapamycin 1
NHE3	Na^+/H^+ exchanger 3
Ob-Ra	Short isoform of leptin receptor
Ob-Rb	Long isoform of leptin receptor

PAT1	Proton amino acid transporter 1
Q-PCR	Quantitative polymerase chain reaction
PepT-1	Peptide transporter -1
Phe	Phenylalanine
pH_i	Intracellular pH
P_i	Inorganic phosphate
PI3K	Phosphatidy linositol 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPAR γ	Peroxisome proliferator-activated receptor γ
Pro	Proline
RNA	Ribonucleic acid
RNAm	Messenger ribonucleic acid
SCOCS-3	Suppresor of cytokine signalling-3
SGLT1	Sodium glucose cotransporter 1
SHP-2	SH domain containig protein tyrosine phosphatase 2
SIT	Sodium imino acid transporter
SLC	Solute carrier family
STAT	Signal transducer and activator of transcription
TER	Transepithelial resistance
4F2hc	4F2 heavy chain

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Introduction

1. STUCTURE OF THE ENTEROCYTES

The small intestine, among other layers, presents a specialized epithelial layer for its main function that is nutrient absorption. It consists of finger-like projections (villi) surrounded by openings of glandular structures called crypts of Lieberkühn (Figure 1). Both villi and crypts are covered by columnar epithelial cells. The cells lining the villi are considered to be responsible for both nutrient and electrolyte absorption, whereas the crypt cells participate in secretion.

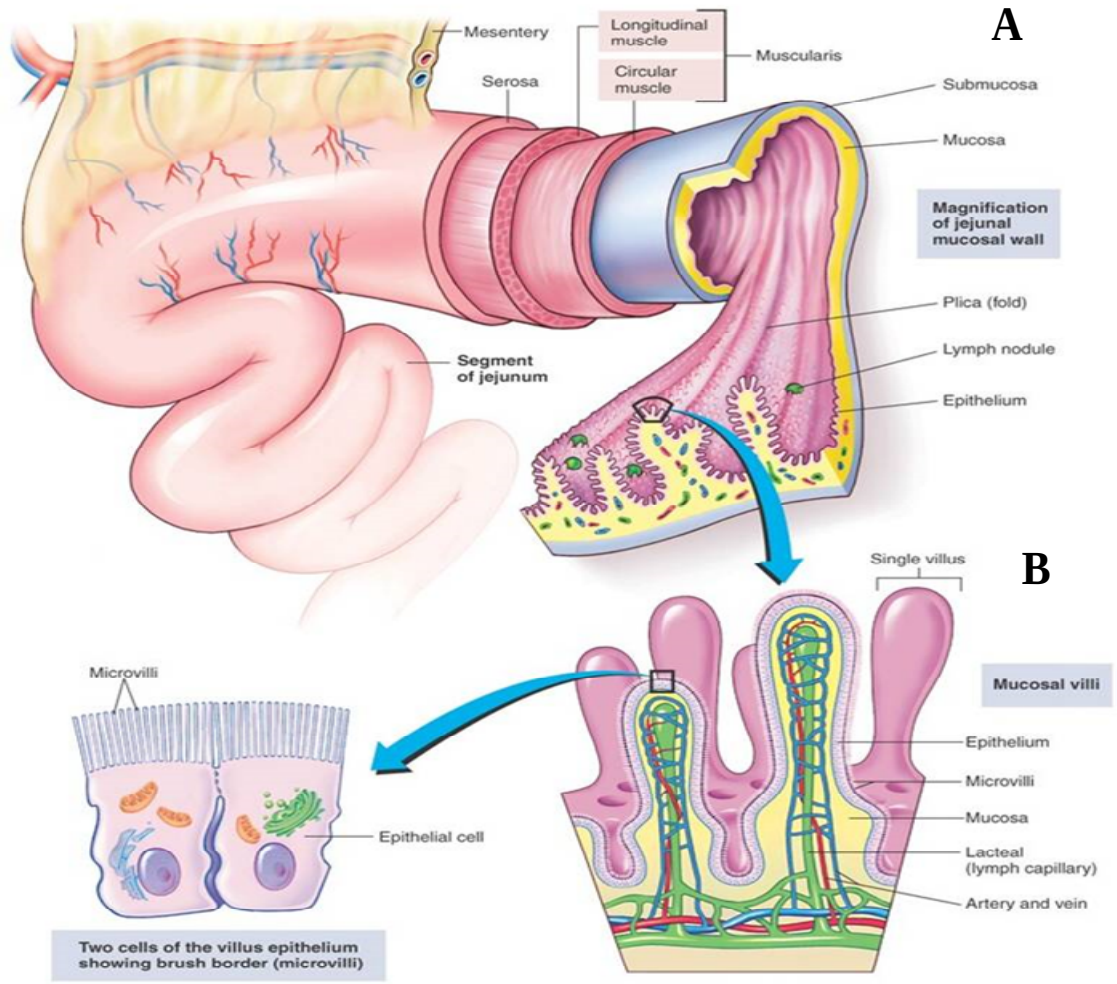

Figure 1. A. Structure of the small intestine wall. B Detail of the mucosal villi and the enterocytes.

The intestinal epithelium is composed by rapidly proliferating and perpetually differentiating cells. Stem cells located in the crypts of Lieberkühn differentiate into the 4 major epithelial cell types. These include columnar absorptive cells or enterocytes, mucous-secreting goblet cells, enteroendocrine cells, and Paneth cells. Enterocytic,

goblet, and enteroendocrine cell differentiation takes place during migration upward from the crypt to the tips of the adjacent villi or surface epithelium, whereas Paneth cells complete their differentiation at the crypt base (Sancho *et al*., 2003; Shaker *et al*., 2010).

Enterocytes are high cuboidal to low columnar epithelial cells that rest on a basement membrane. The cells are tightly joined to neighboring cells by cellular structures named tight junctions. The nucleus resides in the basal portion of the cell and the apical cell surface exhibits numerous microscopic projections (microvilli) that give the appearance of a "brush border" when seen with a light microscope and that greatly increase the absorptive surface area of the enterocyte.

The enterocytes are polarized cells that have two specialized and differentiated poles. On one hand, the apical membrane that is in touch with the intestinal lumen and, on the other hand, in touch with the blood, the basolateral membrane. Both membranes are separated by tight junctions that impede the passage of different molecules between the enterocytes, facilitating the selective uptake of nutrients and other substances (Figure 2).

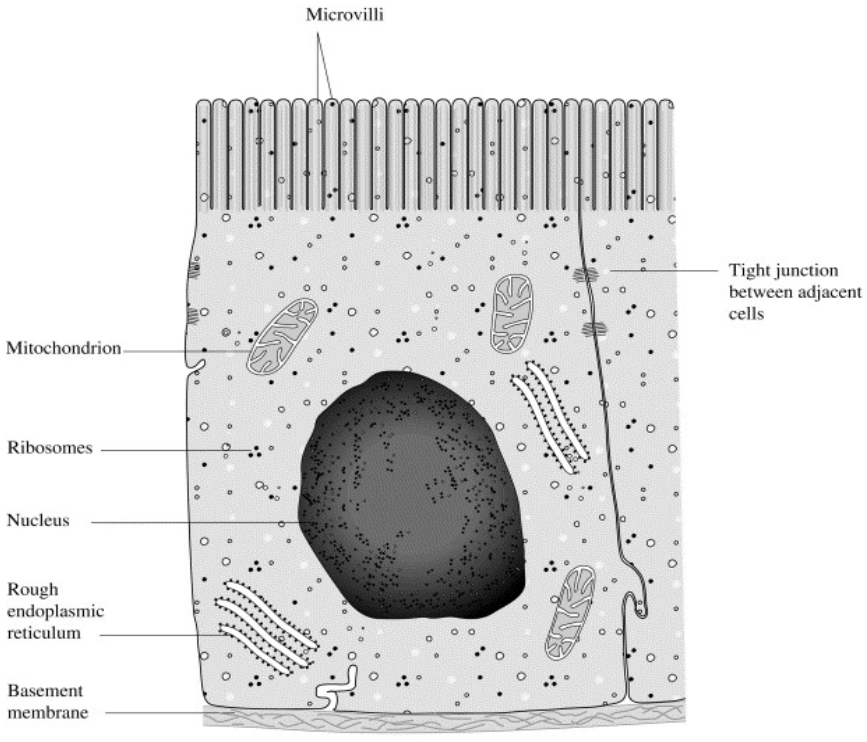

Figure 2. Diagram of a typical intestinal epithelial cell (enterocyte).

2. PASSAGE OF NUTRIENTS ACROSS THE PLASMA MEMBRANE

Once the digestive process is completed, the nutrients have to be transferred across the apical membrane of the enterocytes into the intracellular space, and then across the basolateral membrane into the circulation for its distribution throughout the body. This process is named intestinal absorption.

Passage of the nutrients across the membrane can be carried out by diffusion and active transport (Figure 3). Some elements such as water are absorbed exclusively by diffusion using osmotic forces. Ions can enter enterocytes by diffusion, but may need to be transported actively to the intracellular space. Molecules can move across membranes following concentration gradients, thus moving from more concentrated to less concentrated pools. They also can follow electric gradients whereby they move to areas of opposite charge.

2.1. Diffusion

Diffusion is the net movement of substances from a high concentration to a low concentration region so its overall net movement is along the concentration gradient. In simple diffusion substances diffuse across the membrane between the phospholipids. The facilitated diffusion, also called passive transport, is similar to simple diffusion but the movement of molecules across the membrane is performed through protein channels and carrier proteins.

2.2. Active transport

Active transport is the movement of a substance against its concentration gradient (from low to high concentration). If the process uses chemical energy from the hydrolysis of ATP, it is termed primary active transport. In secondary active transport, or coupled transport, the energy needed for the “uphill” movement of a molecule is obtained from the “downhill” transport of an ion, usually Na^+ in mammalian cells, along its electrochemical gradient. If the other molecule is moved in the same direction as Na^+, the coupled transport is called cotransport or symport. If the other molecule is moved in the opposite direction, the process is called countertranspor or antiport.

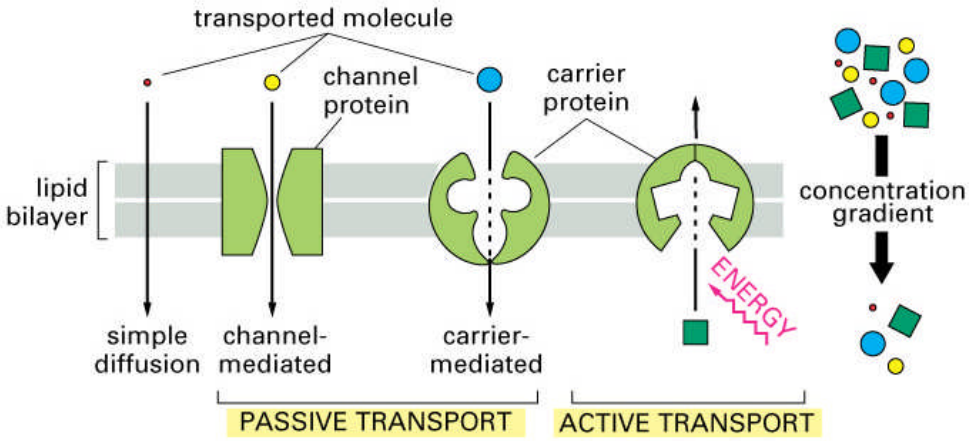

Figure 3. Passage of molecules across the plasma membrane

3. AMINO ACID ABSORPTION

The proteins partially digested in the stomach and by pancreatic enzymes in the duodenum, are hydrolyzed to peptides and free amino acids trough the action of intestinal aminopeptidases, carboxypeptidases and peptidases. The intestinal absorption of di-tri-peptides is mediated exclusively by the proton-dependent PepT-1 transporter, whereas multiple transports systems are implicated in the intestinal absorption of amino acids. A single amino acid can be transported into the enterocyte by more than one transporter, usually active transporter. Likewise, the exit of the amino acid to the blood is mediated by several transporters. Amino acid transport activities are frequently referred to as “systems”. This term indicates a functionally identified transport activity that appears to be similar in a variety of type cells. Table 1 provides an overview of systems related with amino acid absorption and their molecular identity.

Table 1. Epithelial amino acid transport system, their molecular identity, functional characteristics and location (tacked from Bröer *et al*., 2008)

System	cDNA	SLC	Amino Acid Substrates	Analogs	Affinity	Mechanism	Ions	Expression*
A	SNAT2	SLC38A2	G,P,A,S,C,Q,N,H,M	MeAIB	Medium	S	Na^+	Ub
	SNAT4	SLC38A4	G,A,S,C,Q,N,M,AA^+	MeAIB	Medium	S	Na^+	K
ASC	ASCT1	SLC1A4	A,S,C	Cysteic acid	High	A	Na^+	K
	ASCT2	SLC1A5	A,S,C,T,Q		High	A	Na^+	K,I (AM)
asc	4F2 hc/asc1	SLC3A2/SLC7A10	G,A,S,C,T	D-AA^0	High	A		K
B^0	B^0AT1	SLC6A19	AA^0	BCH	Low	S	Na^+	K,I (AM)
	B^0AT2	SLC6A15	P,L,V,I,M	BCH	High	S	Na^+	K
$B^{0,+}$	$ATB^{0,+}$	SLC6A14	AA^0, AA^+, β-Ala	BCH	High	S	Na^+, Cl^-	I (AM)
$b^{0,+}$	rBAT/$b^{0,+}$AT	SLC3A1/SLC7A9	R,K,O,cystine		High	A		K,I (AM)
β	TauT	SLC6A6	Tau, β-Ala		High	S	Na^+, Cl^-	K (AM,BM)
Gly	XT2	SLC6A18	G		NR	NR	NR	K (AM)
IMINO	IMINO	SLC6A20	P, HO-P	MeAIB	Medium	S	Na^+, Cl^-	K,I (AM)
L	4F2hc/LAT1	SLC3A2/SLC7A5	H,M,L,I,V,F,Y,W	BCH	High	A		
	4F2hc/LAT2	SLC3A2/SLC7A8	AA^0 except P	BCH	Medium	A		K,I (BM)
	LAT3	SLC43A1	L,I,M,F	BCH	Low	U		K
	LAT4	SLC43A2	L,I,M,F	BCH	Low	U		
N	SNAT3	SLC38A3	Q,N,H		Low	S	Na^+(S), H^+(A)	K (BM)
	SNAT5	SLC38A5	Q,N,H,S,G		Low	S	Na^+(S), H^+(A)	K
PAT (Imino acid)	PAT1	SLC36A1	P,G,A GABA, β-Ala	MeAIB	Low	S	H^+	K,I (AM)
	PAT2	SLC36A2	P,G,A	MeAIB	Medium	S	H^+	K
T	TAT1	SLC16A10	F,Y,W		Low	U		K,I (BM)
X^-_{AG}	EAAT2	SLC1A2	E,D	D-Asp	High	S	Na^+,H^+(S), K^+(A)	K (BM)
	EAAT3	SLC1A1	E,D	D-Asp	High	S	Na^+,H^+(S), K^+(A)	K,I (AM)
x^-_c	4F2 hc/xCT	SLC3A2/SLC7A11	E, $cystine^-$		High	A		Ub
y^+	CAT-1	SLC7A1	R,K,O,H		Medium	U		Ub
y^+L	4F2hc/y^+LAT1	SLC3A2/SLC7A7	K,R,Q,H,M,L		High	A	Na^+(S-AA^0)	K,I (BM)
	4F2hc/y^+LAT2	SLC3A2/SLC7A6	K,R,Q,H,M,L,A,C		High	A	Na^+(S-AA^0)	K,I (BM)

NR, not reported; A, antiport; AA^0, neutral amino acids; AA^+, cationic amino acids; U, uniport; S, symport; S-AA^0, symport together with neutral amino acids; K, kidney; I, intestine; AM, apical membrane; BM, basolateral membrane; Ub, ubiquitous. Amino acids are given in one-letter codes. O, ornithine; HO-P, hydroxyproline. Affinity: high, <100 μM; medium, 100 μM to 1 mM; low, >1 mM. * Expression in epithelial cells of kidney and intestine.

3.1. Glutamine absorption

There is biochemical and physiological evidence that glutamine supports the function of the intestinal mucosal system (Windmueller & Spaeht, 1978). Glutamine in the mucosal cells activates a number of genes associated with cell cycle progression and serves as an essential metabolic precursor in nucleotide, glucose and amino acids biosynthesis, glutathione homeostasis and protein synthesis (Neu *et al*. 1996; Reeds & Burrin, 2001). It is also necessary for the maintenance of the gut barrier function and intestinal cell proliferation and differentiation (Chun *et al*., 1997). Glutamine is also crucial in nitrogen metabolism. It is considered as a semi-essential amino acid because in certain physiopathological conditions as physical trauma, intense exercise, immune deficiencies or cancer, there is an increase in the requirements of glutamine by the organism. Supplementation of glutamine in the diet is estimated necessary in these conditions (Lenaerts *et al*., 2005).

Na^+-dependent glutamine transporters include Systems ASC, B^0, $B^{0,+}$, y^+L, A and N, and Na^+-independent transporters include Systems L, $b^{0,+}$ and n (Bode, 2001). Among these transporters, those present in the intestine are (see also table 1):

- **System B**: This system is a Na^+ dependent co-transporter that carries a large variety of neutral amino acids, including branched and bulky ones (Munck *et al*., 1994), which has been identified as the nineteen member of the solute carrier family 6 (SLC6) transporter family (Bröer *et al*., 2004). This transporter was initially characterized in intestinal brush border membrane vesicles (Fass *et al*., 1977; Maenz & Patience, 1992; Munck *et al*., 1994; Stevens *et al*., 1982), in bovine intestinal epithelial cells (Doyle & McGivan, 1992) and in Caco-2 cells (Hidalgo & Borchardt, 1990; Souba *et al*., 1992; Costa *et al*., 2000). Given the reported heterogeneity of B^0-like activities, particularly in the kidney, this transporter was denominated B^0AT1 (Broad neutral amino acid transporter 1). It is localized exclusively in the brush-border membrane of kidney and intestine (Romeo *et al*., 2005). Recent studies have been demonstrated that B^0AT1 needs specific partner proteins for its expression in the intestine at the plasma membrane, in particular the angiotensin-converting enzyme 2 (Kowalczuk *et al*., 2008; Camargo *et al*., 2009).

- **System ASC**: This system is a Na^+-dependent neutral amino acid exchanger, originally named for the three preferred substrates alanine, serine, cysteine (Christensen *et al*., 1967). The first mammalian glutamine transporter gene was isolated in 1996 from a mouse testis cDNA library. It encoded for a protein with functional properties of System ASC and was named ASCT2 (Utsunomiya-Tate *et al*., 1996). At the same time, Kekuda *et al.* (1997) isolated a cDNA from human choriocarcinoma and colon carcinoma cell line with functional properties nearly identical to ASCT2.
B^0AT1 can transport large neutral amino acids such as phenylalanine and tryptophan (Fass *et al*., 1977; Doyle & McGivan, 1992; Maenz & Patience, 1992; Bröer *et al*., 2004), which are not substrates of ASCT2. Therefore, phenylalanine can be used to inhibit B^0AT1 and discriminate its contribution on glutamine uptake.

- **System $B^{0,+}$:** This system was described by Van Winkle *et al*. in 1985 as Na^+ dependent zwitterionic and cationic amino acids. $ATB^{0,+}$ is the protein which correlates with this system. It localizes in the apical membrane and it has been demonstrated that is highly dependent on both Na^+ and Cl^-.

- **System y^+L**: This transport activity was first described in the plasma membrane of human erythrocytes (Deves *et al*., 1992). System y^+L is the only transporter that mediates the uptake of cationic amino acids with high affinity in a Na^+-independent manner, but requires Na^+ to efficiently transport zwitterionic amino acids such as glutamine. This transporter is thought to mediate the basolateral extrusion of dibasic amino acids in kidney and intestinal epithelia (Pfeiffer *et al*., 1999). The molecular correlate of system y^+L is the heterodimeric transporter $4F2hc/y^+LAT1$, which has been demonstrated to be an exchanger of neutral and cationic amino acids (Bröer, 2008). It is located in the basolateral membrane of the epithelial cells.

- **System L:** The broadly specific system L has been identified as the hterodimeric transporter 4F2hc/LAT2. It mediates neutral amino acids exchange and it is localized at the basolateral membrane of the enterocytes (Segawa *et al*., 1999; Fraga *et al*., 2005).

In summary, transport of glutamine across rat and human apical membrane is mediated by B^0AT1 and ASCT2, whereas the extrusion out of the cell could be mediated by 4F2hc/LAT2. It has been proposed that a Na^+-dependent mechanism with properties of system A is also implicated in basolateral Gln absorption (Taylor *et al*., 1989; Al-Mahroos *et al*., 1990; Wilde & Kilberg, 1991).

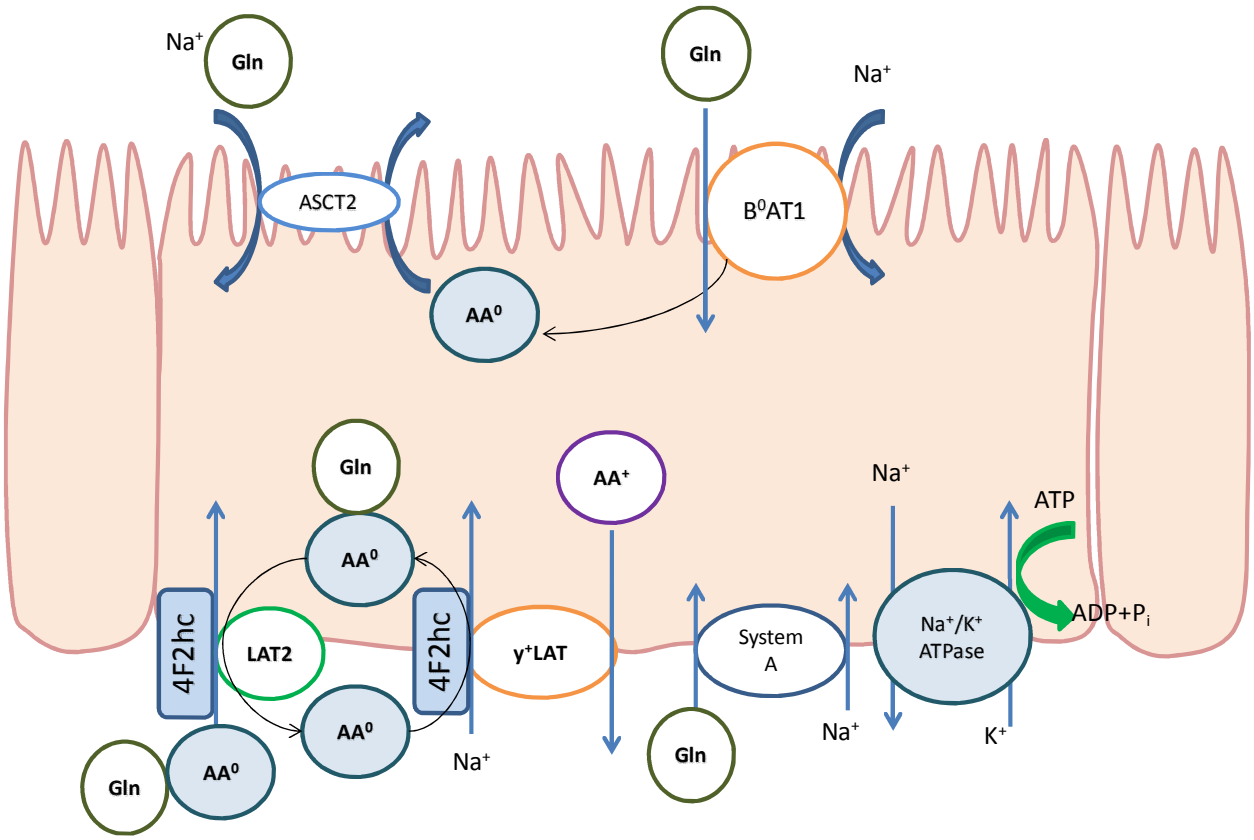

Figure 4. Main transporters involved in the absorption of glutamine. AA^0 (neutral amino acids); AA^+ (cationic amino acids)

3.2. Proline absorption

Proline is the only protein-forming amino acid with a secondary amino group. It is necessary to maintain collagen of the skin, joints, tendons, connective tissue and cartilage. Classical studies showed that proline absorption in the intestine and kidney was mediated by a specific transport system for imino acids, with differences between species. In rat intestine, proline transporters include system B (described above) and system Imino (Stevens & Wright, 1985; Munck *et al*., 1994; Munck & Munck, 1994) later identified as PAT1 (Anderson *et al*., 2004).

The Imino acid carrier was described in rat intestine as a Na^+-dependent Cl^- -independent transporter in contrast to system IMINO (alias SIT human XT3, mouse XT3s1, rat rb21a, XTRP3) described in intestinal brush border membrane of rabbit as a Na^+ and Cl^- dependent system (Kowalczuk *et al*., 2005).

PAT1 (SLC36A1) is a H^+-coupled, pH-dependent, low-affinity transporter of small, unbranched, zwitterionicα-, β- and γ-amino and imino acids, such as β-alanine. It also transports N-methylated amino acids and heterocyclic amino acids containing four- to six-membered rings (Thwaites & Anderson, 2007a, b). PAT1-like transport has been detected at the brush-border membrane of rat jejunum (Anderson *et al.* 2004; Iñigo *et al.* 2006). It can also function in two different modes, either as an electrogenic H^+-amino acid co-transporter or as an electroneutral H^+-anion co-transporter for short-chain fatty acids (Foltz *et al.* 2004). PAT1 is the first member of the solute carrier family 36, which includes three other related sequences in mammalian genomes (Chen *et al.* 2003; Bermingham & Pennington, 2004).

In human Caco-2 cell monolayers, PAT1-mediated amino acid-coupled H^+-influx across the apical membrane leads to an intracellular acidification. This acidification activates the apical Na^+/H^+exchanger NHE3, which recycles protons back across the apical membrane to maintain the pH_i and the H^+-electrochemical gradient (Thwaites *et al.*, 2002). This functional coupling with NHE3 means that in intact epithelia, such as human Caco-2 cells or rat small intestine, the Na^+-independent PAT1 becomes partially dependent upon extracellular Na^+ (Munck *et al.*, 1994; Anderson *et al.*, 2004) as NHE3 cannot function in the absence of Na^+.

As it happens with glutamine, proline extrusion from the cell is probably mediated trough the heterodimeric transporter 4F2hc/LAT2 (Segawa *et al.*, 1999; Fraga *et al.*, 2005). It has been proposed that a Na^+ -dependent mechanism with properties of system A is implicated in the basolateral uptake of proline (Taylor *et al.*, 1989; Al-Mahroos *et al.*, 1990; Wilde & Kilberg, 1991).

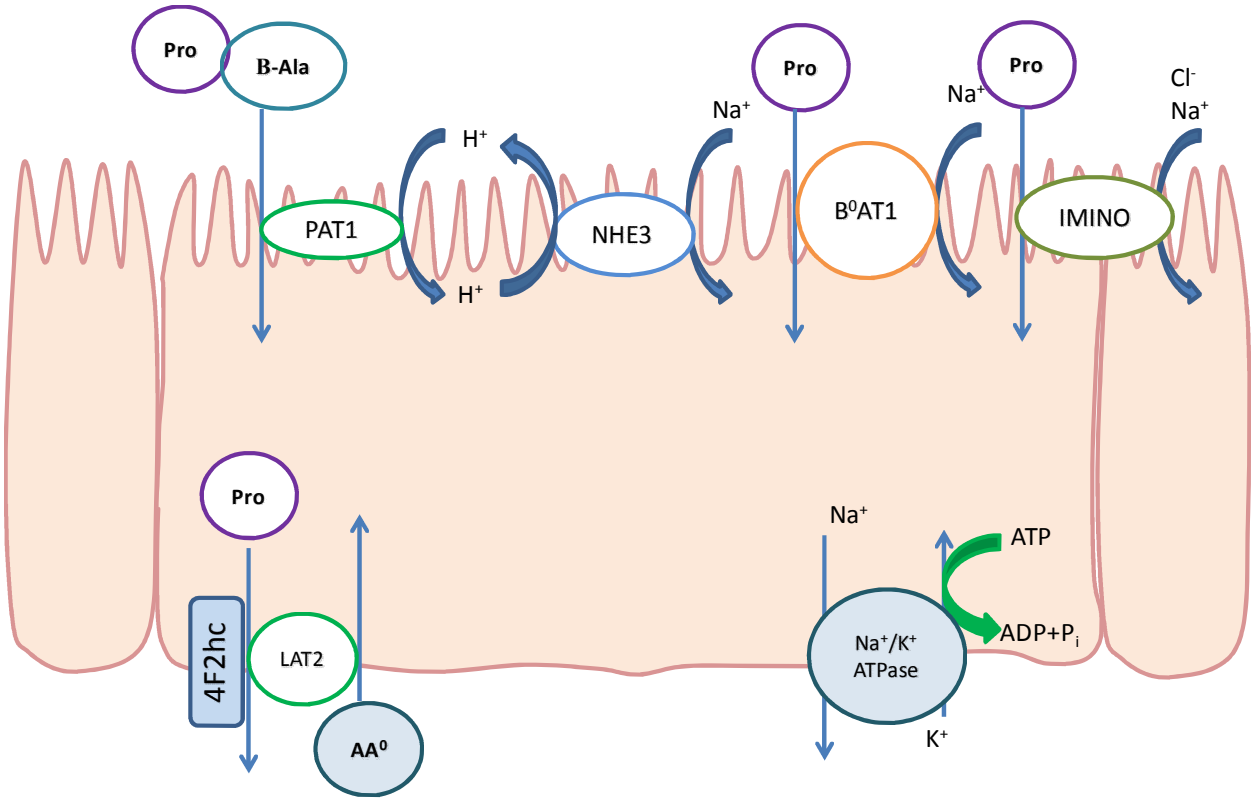

Figure 5. Main transporters involved in the absorption of proline. AA^0 (neutral amino acids)

3.3. Glutamate absorption

Glutamate is one of the most abundant amino acid in the food either in free form or in the form of peptides and proteins (Beyrehuther *et al*., 2007). In spite of its abundance in food, glutamate concentration in blood is almost the lowest (50 µmol/L) among all amino acis (Adibi *et al*., 1973), mainly because Glu is extensively oxidized by the small intestine in order to supply the high energy demand of the epithelium that is in rapid renewal (Blachier *et al*., 2009). Glutamate is the most important intracellular amino acid reaching concentrations up to 20 mmol/L and is central to numerous transamination and deamination reactions and synthesis of proteins. Intracellular glutamate increases the antioxidant defense by the generation of glutathione, and participates in the gluconeogenesis in the kidney and in the urea synthesis in the liver (Blachier *et al*., 2009).

In general, Glu uptake occurs by three different transport components: a high affinity Na^+ independent component, also carrying cystine, similar to system $x^-_{C;}$ a high affinity Na^+ dependent component corresponding to the ubiquitous system $X^-_{A,G;}$ and a low affinity Na^+ dependent component. The last component was proposed to be system B^0

(Schultz *et al.,* 1970), but other authors suggested that the low affinity transport system is mediated by system ASC (Munk *et al*., 1999), a system known to interact with acidic amino acids at low pH (Christensen *et al.,* 1984). Moreover, the order of inhibition of glutamate transport by neutral amino acids did not coincide with the relative affinity for system B^0 (Munk *et al*., 1999).

The molecular correlate of system x^-_c is a heteromeric transporter (4f2hc/xCT) involved in the defense against oxidative stress (Sato *et al.*, 1999). This transporter is a Na^+ -independent, anionic amino acid transporter, highly specific for cystine and glutamate. This transporter exchanges the anionic form of cystine for glutamate with a molar ratio of 1:1, and is expressed almost ubiquitously in cultured mammalian cell lines (Bannai *et al.,* 1986). A recent report suggests the presence of this transporter in the apical membrane of the enterocytes (Burdo *et al*., 2006).

The EAAT1-5 are the molecular correlates of system $X^-_{A,G.}$ EAATs cotransport three Na^+ together with each glutamate molecule, and the return of the carrier to its extracellular facing conformational state is facilitated by the intracellular binding of K^+ (Kanai *et al*., 1994). Later studies also demonstrated the contransport of one H^+ together with 3 Na^+ and 1 Glu (Zerangue & Kavanaugh, 1996). Auger *et al.* proposed that glutamate entrance in neurons does not require binding of extracellular H^+ (Auger *et al*., 2000), suggesting that the H^+ gradient is not essential for glutamate transport. Among all members, EAAT1 and EAAT3 are present in the intestine.

It has been reported that ASCT2 is also able to transport Glu under acidic conditions by being protonated at the carboxyl group (Utsunomiya-Tete *et al*., 1996). Therefore, ASCT2 would be also implicated in Glu transport across the apical membrane of the enterocytes.

It has been proposed that basolateral uptake of Glu in enterocytes could be across EAAT2, that is expressed in basolateral membrane of kidney (Welbourne *et al*., 1999; Bröer, 2008)

In summary, Glu uptake is mediated by both Na^+-dependent and Na^+-independent transporters without exclusion of H^+ coupling.

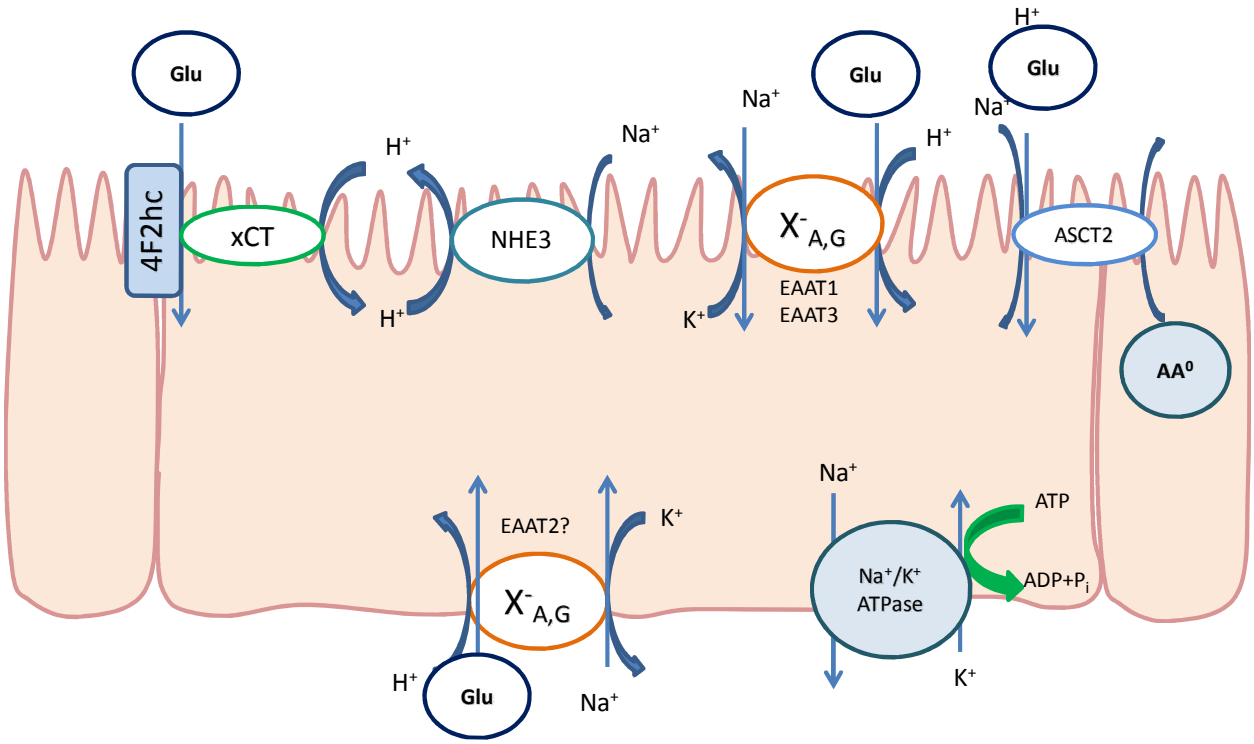

Figure 6. Main transporters involved in the absorption of glutamate.

4. LEPTIN

4.1. General comments

4.1.1. Discovery

The Jackson Laboratory identified in 1950 and 1965, two strains of mice that had identical phenotypes (morbid obesity, insulin resistance, infertility and lethargy): the *ob/ob* mice (obese) and the *db/db* mice (diabetic) (Kennedy, 1953; Hervey, 1959). Coleman and colleagues characterized the differences between the obese *ob/ob* and *db/db* lines by joining the circulatory systems of these mice with normal mice or each other and analyzing the resulting phenotype. These studies showed that the *ob/ob* strain was deficient in a blood-borne factor, while the *db/db* strain was deficient in the receptor for this factor (Coleman, 1973, 1978, 2010). Both animal strains thus displayed major food intake problems with related complications leading to morbid obesity.

In 1994 the *ob* gene was identified and cloned in rodent adipose tissue. Its product was named leptin after the Greek “leptos” meaning “thin” (Zhang *et al*., 1994). Initially, leptin receptors were localized in areas of the hypothalamus involved in the regulation of food intake and energy expenditure (Tartaglia *et al*., 1995), and leptin was considered

as an adipostatic signal acting from adipose tissue to the brain, as a part of a negative feedback loop, to regulate the size of the energy stores and energy balance. This theory was stabilized as the leptin-deficient *ob/ob* mice treated with leptin returned to control weight values as well as displayed a normal food intake and increased its energy expenditure (Camplfield *et al*., 1995; Pelleymounter *et al*., 1995). Besides, the complications related to the immune system and fertility disappeared, indicating a broad physiological role of leptin (Himms-Hagen, 1999; Considine, 2005).

4.1.2. Leptin receptors

The leptin receptors (ObRs) are members of the class I cytokine receptor family, and to date there are at least six alternatively spliced isoforms (ObRa–f; Fruhbeck, 2006). All receptors come from the same gene (*ob*) and have the same extracellular domain and the same affinity for the leptin, but differ in the amino acid sequences and length of their trans-membrane and intracellular domains (Chen *et al.,* 1996; Lee *et al.,* 1996). ObRa, c, d, and f possess relatively short cytoplasmic domain whereas ObRb, the so-called ‘‘long’’ isoform, has an extended C-terminal region and is the only variant capable of complete intracellular signal transduction (Baumann *et al.*, 1996). However, a degree of intracellular signaling has been also demonstrated for the short isoforms (Bjorbaek *et al.*, 1997; Yamashita *et al*., 1998; Han *et al.,* 2001). ObRe is a truncated form of the receptor which is secreted into the circulation and thought to play a role as a binding protein, regulating leptin bioavailability. This soluble isoform can regulate serum leptin concentration and serves as a carrier protein delivering the hormone to its membrane receptors. It is under the form of this leptin-leptin receptor complex how the hormone circulates in the blood. Complex formation allows for increasing the leptin half-life and modulates leptin action on target cells (Yang *et al.,* 2004). In addition, some studies have implicated ObRe in the regulation of leptin transport at the blood–brain barrier (BBB) by antagonizing the uptake of leptin and inhibiting its transport into the CNS (Tu *et al.*, 2007).

4.1.3. Leptin signaling

Binding of leptin to its receptors results in autophosphorylation of JAK2 and subsequent activation of the janus kinase/signal transducer and activator of transcription

(JAK/STAT-3) pathway (Banks *et al*., 2000). Parallel signaling pathways are also activated, including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways. Suppressor of cytokine signaling-3 (SOCS3) is robustly induced in response to leptin (Bjorbaek *et al*., 1998), and acts as a negative regulator of this signaling pathway.

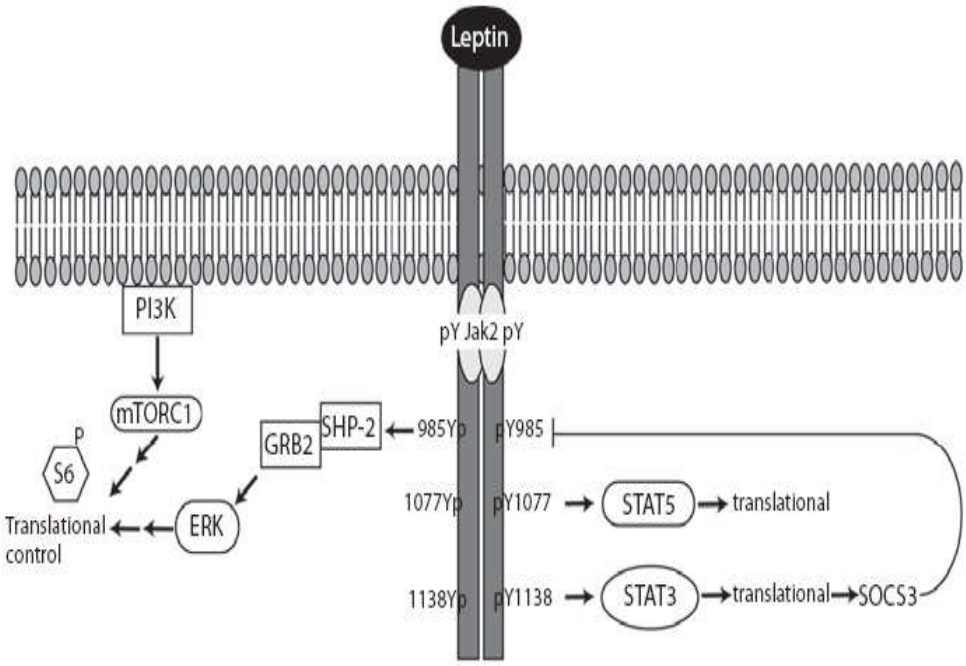

Figure 7. Intracellular signaling pathways engaged by the leptin receptor. The LepRb forms a homodimer in the membrane. Leptin binds to the extracellular domain leading to a conformational change in the receptor; activating Janus kinase 2 (Jak2) which then phosphorylates (designated by 'p') three evolutionarily conserved tyrosine residues within the cytoplasmic domain of LepRb. The Y 985 is required for activation of the SH2-containing tyrosine phosphatase-2 (SHP2)/extracellular signal regulated kinase (ERK) cascade (activation of ERK via growth factor receptor binding protein 2 – GRB2) which leads to the phosphorylation of ribosomal protein S6 and increased translation. Y 1077 is required for signaling by signal transducer and activator of transcription 5 (STAT5), while Y 1138 mediates STAT3 signaling. STAT3 trans activates the suppressor of cytokine signaling 3 (SOCS3) gene, and SOCS3 mediates negative feedback on LepRb signaling via Y 985. The LepRb also activates the phosphatidylinositol 3 kinase (PI3K) and mammalian target of rapamycin 1 (mTORC1) pathways, but the mechanism is not understood (Taken from Denver *et al*., 2011).

4.2. Leptin and the gastrointestinal tract

4.2.1. Leptin and the stomach

Although adipose tissue is the major source of leptin, the hormone can be produced by different peripheral tissues such as stomach, salivary glands, placenta and kidney, where

leptin receptors are also expressed (De Matteis *et al.,* 1998; Barrenetxe *et al.*, 2002; Margetic *et al.*, 2002; Gertler *et al* 2009). It has been reported that the stomach produces and secretes leptin into the gastric lumen by pepsinogen-containing secretory granules of chief cells (Bado *et al.*, 1998). These granules also contain the leptin soluble receptor that is released into the gastric lumen together with leptin (Cammisotto *et al.*, 2006). Leptin remains stable in the gastric juice because the binding to its soluble receptor protects it from the acidic pH and the proteolytic activity present in this environment (Guilmeau *et al.*, 2003, 2004).

Secretion of leptin by the gastric mucosa is regulated by the nutritional status of the body together with nutrients and hormones. Food intake is a strong stimulus for gastric leptin secretion together with acetylcholine released by the vagus nerve (Sobhani *et al*., 2002), secretin, cholecystokinin (CCK), insulin, glucocorticoids and trans-retinoic acids (Table 2).

Table 2. Agents controling leptin expression and release from the gastric mucosa, Modified from Cammisotto *et al.*, 2010b.

Leptin expression		
• **48 h fasting**	**-**	**Zhao *et al*., 2008**
• **Substained feeding**	**+**	**Bado *et al*., 1998**
• **High sucrose fat diet (HFS)**	**+**	**Lindqvist *et al*., 2005**
• **Carbohydrate-rich diet**	**+**	**Sanchez *et al*., 2004**
• **fa/fa mutation**	**+**	**Picó *et al*., 2002**
Leptin release		
• Secretin	+	Sobhani *et al*., 2000
• Cholecystokinin	+	Sobhani *et al*., 2000
• Insulin	+	Goïot et al., 2005
• Glucocorticoids	+	Goïot et al., 2005
• All trans-retionic acids	+	Goïot et al., 2005
• Pentagastrin	+	Sobhani *et al*., 2000
• Vagal stimutaltion	+	Sobhani *et al*., 2002

4.2.2. Leptin and intestine

Leptin receptors are expressed in both apical and basolateral membrane of the intestinal absorptive cells (Barrenetxe *et al.,* 2002) indicating a possible action of the hormone from both the blood and the intestinal lumen. Thus, once secreted into the gastric lumen, leptin reaches the intestine where it can bind to its brush border receptor to regulate various functions of the intestinal absorptive cells which include nutrients absorption, secretion and cell proliferation (Cammisotto *et al*., 2010b). Besides, circulating leptin could also act as an endocrine hormone on the enterocytes by binding to the receptors expressed in the basolateral membrane.

4.2.2.1. Transcytosis of gastric exocrine leptin trough the intestinal wall

Plasma leptin rises significantly and rapidly within 15 minutes after the onset of food intake or upon cholecystokinin stimulation (Bado *et al*., 1998). This rapid increase in blood levels cannot be due to adipose leptin since it takes over 60 minutes for adipose tissue to respond to any secretory stimuli (Cammisotto *et al*., 2010b). Therefore, it has been suggested that the sudden increase in plasma leptin levels may be due to the postprandial leptin secretion, which next crosses intestinal mucose by transcytosis and reaches blood circulation without being altered (Cammisotto *et al*., 2007, 2010a). At the ultrastructural level, leptin has been found to be associated with enterocytes microvilli, in endocytotic vesicles and Golgi apparatus, and at the level of the basolateral membrane (Cammisotto *et al*., 2010a).

4.2.3. Effects of leptin in the gastrointestinal tract

4.2.3.1. Nutrients absorption

Several aspects of the intestinal physiology, in particular nutrient absorption, are influenced by leptin. In rodents using *in vitro* and *in vivo* techniques, it has been shown that leptin, acting from the luminal side, inhibits sugar absorption by reducing the density of the active transporter SGLT1 in the brush border membrane (Lostao *et al*., 1998, Barrenetxe *et al.,* 2001, Ducroc *et al.*, 2005, Iñigo *et al*., 2004, Iñigo *et al*., 2007,). This effect is mediated by PKA and PKC (Barrenetxe *et al.,* 2004, Ducroc *et al.*, 2005)

and is reversible (Iñigo *et al.,* 2007). In addition, it has been demonstrated that luminal leptin also increases GLUT2 and GLUT5 insertion in the apical membrane of the rat enterocytes enhancing galactose and fructose absorption (Sakar *et al.,* 2009).

In contrast, luminal leptin increases peptide absorption in rat intestine *in vivo* and in Caco-2 cells (Buyse *et al.,* 2001a). This effect is due to an increase in the density of the proton-coupled peptide transporter (PepT1) in the apical plasma membrane, without any changes at the mRNA level. Similarly, leptin increases butyrate uptake in the same cell line by enhancing intracellular pools and translocation of the monocarboxylate transporter 1 (MCT-1) (Buyse *et al.,* 2002).

Altogether, these observations support the view that gastric leptin can exert a rapid regulatory function on intestinal nutrients absorption in the postprandial state.

Contrary to the increase on peptide transport produced by leptin acting from the apical membrane in Caco-2 cells, leptin acting from the basal membrane did not have any effect (Buyse *et al.,* 2001a). Nevertheless, it has been reported in Caco-2 cells that leptin acting from the basolateral membrane, affects lipid handling by decreasing export of triglycerides to the basolateral medium without affecting the monoglyceride, diglyceride and cholesterolester classes (Stan *et al.,* 2001). Leptin also decreases the release of de *novo* synthesis of apolipoproteins B-100 and B-48, as well as newly formed chylomicrons and low density lipoproteins. Interestingly, *in vivo* injection of leptin attenuates apolipoprotein A-IV increase elicited by intraduodenal infusion of lipids (Morton *et al*., 1998; Doi *et al*., 2001). Finally, serosal leptin can inhibit glucose absorption but indirectly, by stimulation of endogenous CCK secretion (Ducroc *et al*., 2005).

Therefore, these data also suggest a role of systemic leptin in the intestinal physiology.

4.2.3.2. Motility

Afferent and efferent vagus nerve endings contain leptin receptors (Buyse *et al*., 2001b). In the small intestine, leptin can cause excitatory and inhibitory effects on mechanoreceptors and thus has a complex effect on intestinal motility. It has been shown that leptin deficiency increases the rate of gastric emptying (Asakawa *et al*., 1999), increases transit activity in the jejunum, and shortens total transit time in the

small intestine (Kiely *et al*., 2005) The leptin-induced inhibition of food intake and the stimulation of pancreatic exocrine secretions can be blocked by a cholecystokinin-1 (CCK-1) receptor antagonist (Barrachina *et al*., 1997). CCK itself increases the release of leptin from gastric glands, suggesting that leptin and CCK comprise a positive feedback loop. Locally injected intra-arterial leptin stimulated motility in the small intestine in cats pretreated with CCK, whereas leptin alone did not induce such activity (Guilmeau *et al*., 2003; Gaigé *et al*., 2003). This suggests that modulation of vagal fibers in the presence of CCK plays a key role in the observed effects of leptin on intestinal motility.

4.2.3.3. Growth

Although there is some evidence in the literature suggesting that leptin does not stimulate growth in the GI tract mucosa, several studies have shown that leptin acts as a trophic factor in the GI tract and can stimulate gut epithelial cell proliferation when given exogenously (Hardwick *et al*., 2001; FitzGerald *et al*., 2005). Moreover, leptin might have a role in the regulation of the antitumor immune response in colonic cells (Abolhassani *et al*., 2008; Aloulou *et al*., 2008).

4.2.3.4. Immune function and gut mucosal inflammation and injury

Leptin has important effects on the immune system by modulating the inflammatory and anti-inflammatory responses in the GI tract (Gabay *et al*., 2001). Leptin might also prevent gastric ulcer formation by increasing the activities of the cyclooxygenase and nitric oxide pathways and by enhancing mucus secretion (Adeyemi *et al*., 2005). Others have reported that serum concentrations of leptin decrease in patients with Crohn's disease (Franchimont *et al*., 2005; Karmiris *et al*., 2006). The role of leptin in ischemic injury and healing of the GI tract has also been studied; thus, leptin administration accelerated the healing of colonic anastomosis (Tasdelen *et al*., 2004).

Leptin effects on the GI tract are summarized in figure 8.

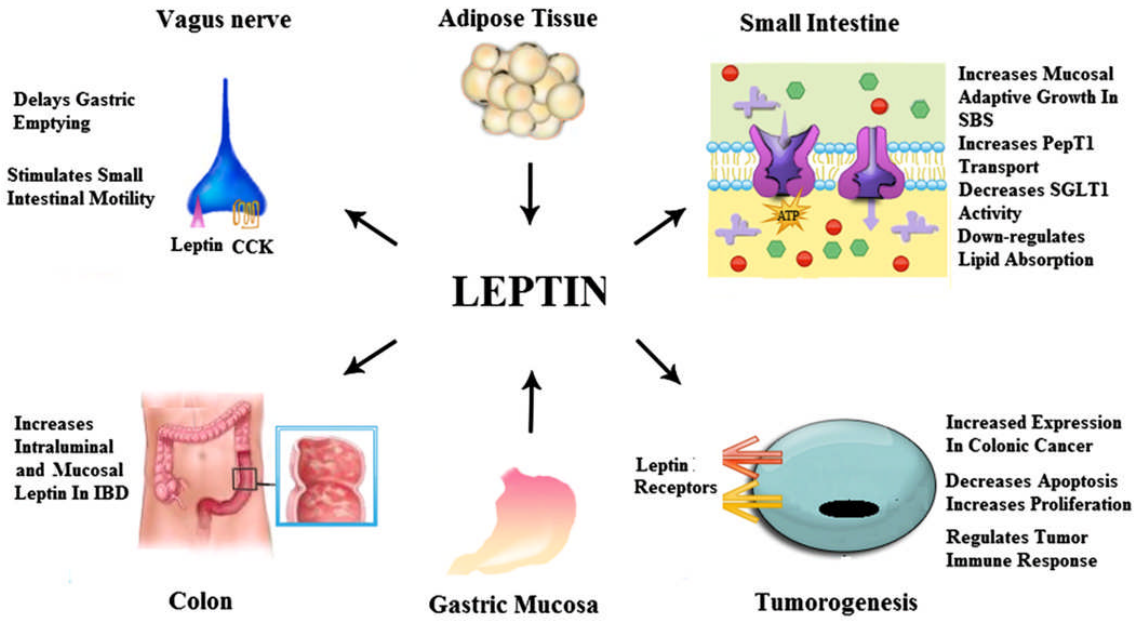

Figure 8. Effects of leptin on the gastrointestinal tract. Leptin, produced by adipose tissue and gastric mucosa, modulates numerous aspects of gastrointestinal function. Leptin, mainly produced by the gastric mucosa, regulates motility of the stomach and small intestine through its interaction with CCK and the vagus nerve. Leptin can act as a proinflammatory cytokine in colonic IBD. Leptin influences macronutrient transport in the small intestine in part by its action to regulate PepT1 and SGLT-1 transports of di-/tripeptides and glucose, respectively. Leptin exerts proliferative and antiapoptotic effects, suggesting that leptin is a potential tumorigenic factor. Several immunoregulatory effects relevant to colon cancer have been attributed to leptin. CCK, cholecystokinin; IBD, inflammatory bowel disease; SBS, short bowel syndrome; SGLT-1, sodium–glucose transporter-1. Taked from Yarandi *et al*., 2011.

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Aims

Shortly after leptin identification (Zhang *et al*., 1994), our group demonstrated for the first time that leptin inhibits sugar absorption in rat intestine *in vitro* by short-term regulation of the Na^+/glucose cotransporter SGLT1 (Lostao *et al*., 1998). We later described that leptin receptors are expressed in both apical and basolateral membrane of the human and murine enterocytes (Barrenetxe *et al.*, 2002). In the same year, another group reported that the stomach chief cells secrete leptin into the gastric lumen after a meal (Bado *et al.*, 1998). This study was followed by the demonstration that the chief cells also secrete leptin soluble receptor (Cammisotto *et al.*, 2006), and that leptin remains stable in the gastric juice because the binding to this receptor protects it from the acidic pH and the proteolytic activity of the stomach, and favours its arrival to the small intestine (Guilmeau *et al*., 2004). All these data supported our initial discovery about the role of luminal leptin as a regulator of intestinal sugar absorption and were followed by further studies on leptin modulation of sugars transport *in vitro* and *in vivo* (Barrenetxe *et al.*, 2001, 2004, Iñigo *et al.*, 2004, 2007, Ducroc *et al.*, 2005; Sakar *et al*., 2009).

Thus, in continuation with our previous studies, the general goal of the present work has been to investigate the possible effect of leptin on the intestinal absorption of a series of amino acids and identify the transporters and the possible intracellular mechanisms implicated. The specific objectives have been the following:

1. To determine apical leptin effect on the uptake of the neutral amino acids glutamine and phenylalanine in rat everted intestinal rings and to identify the transporters involved.

2. To study the effect of luminal leptin on the amino acids glutamine, proline and β-alanine absorption in rat intestine *in vivo*.

3. To study the effect of apical leptin on glutamine, phenylalanine, proline and β-alanine transport in the human intestinal cell line Caco-2 and to identify the transporters and the intracellular mechanisms involved.

4. To determine the effect of basal leptin on glutamine and β-alanine transport in Caco-2 cells.

5. To investigate the transport systems involved in glutamate uptake in rat intestine and Caco-2 cells and the possible effect of leptin.

Materials and Methods

1. EXPERIMENTS IN THE ANIMAL MODEL

1.1. Animal model

All the experiments were performed in Wistar rats (180-260 g), purchased from the Applied Pharmacobiology Research Centre (CIFA) of the University of Navarra, (Pamplona, Spain) or from Janvier, Le Genest St Isle, France. The animals were housed at room temperature (20-22 ºC), with a 12 h light/12 h dark cycle, and had free access to water and a standard chow diet (Harlam Ibérica, Barcelona, Spain). Food was removed 16-18 h prior to the experiments. The ethics committee of the University of Navarra approved the study protocols.

1.2. Surgical procedure

Rats were anaesthetized by intraperitoneal 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 per 100 g of body weight. The animal was placed in a dissection table and was submitted to a laparatomy. The initial portion of the jejunum was identified by the localization of the Treitz's ligament. This process was common for all animal studies but each technique required different tissue preparation. At the end of the experiments the rats were killed by cervical dislocation.

1.3. Everted jejunal rings

The accumulation of the substrate in intestinal everted rings was determined following the procedure described by Crane & Mandelstam (1960).

1.3.1. Mediums and Reagents

The incubation medium used was the Krebs-Ringer-Tris (KRT) saline solution composed by (mM): NaCl 140; KCl 5.6; $CaCl_2$ 3; KH_2PO_4 1.4; $MgSO_4$ 1.4; Tris 6.1; HCl 4.9. The pH was adjusted at 6 or 7.4, depending on the experimental requirements.

In the Na^+ free incubation medium NaCl was replaced by an isosmolar concentration of choline chloride. The pH of the medium was adjusted to the desired value with 10 mM Hepes/Tris (pH 7.5) or 10 mM Mes/ Tris (pH 6).

The following substrates were then dissolved in the incubation medium:

- 0.5 mM Gln or 50 μM Gln (Sigma-Aldrich, St. Louis, MO) in the presence of 0.064 μCi/ml L-[^{14}C(U)]-glutamine (American Radiolabeled Chemicals, St. Louis, MO) presenting a specific activity of 218 mCi/mmol.
- 0.5 mM Phe (Merck, Darmstadt, Germany) in the presence of 0.064 μCi/ml L-[^{14}C(U)]-phenylalanine (American Radiolabeled Chemicals) presenting a specific activity of 370 mCi/mmol.
- 6 mM L-glutamate (Sigma) in the presence of 0.1 μCi/ml L-[^{3}H(G)]-glutamic acid (American Radiolabeled Chemicals, St. Louis, MO) presenting a specific activity of 30 Ci/mmol.
- 0.2 nM of recombinant rat leptin (Peprotech Inc.) (Depending on the experimental condition).

1.3.2. Experimental procedure

The tissue was rapidly soaked in physiological serum (NaCl 0.9 %) at 4ºC. Using a syringe, the intestine was washed with serum, in order to eliminate the possible food content that could be present inside the tissue and, carefully with tweezers, the remaining fat adhered to the serous cap was also removed. Then, the intestine was everted and cut in small fragments of approximately 0.2-0.5 cm and 10-30 mg weight. These rings were kept in physiological cold serum, until the beginning of the experiment and, those presenting Peyer's patches were rejected.

Intestinal rings (5-8) were distributed in tubes containing 10 mL of incubation medium (KRT) with the corresponding substrate at the desired concentration and traces of the corresponding radiolabeled substrate. According to the experimental condition, leptin at the concentration in study was also added.

The incubation was performed at 37ºC ± 0.1 ºC in a thermostatic bath with controllable agitation (Unitronic 320 OR, Select). In all the uptake experiments, constant oxygenation of the samples during the incubation period was present.

Before the beginning of the incubation period a 200 μL sample of the uptake medium was taken by duplicate (initial medium) to determine the radioactivity present before the incubation period. At the end of the incubation (5 or 15 min) the intestinal fragments were washed with cold KRT, dried on a filter paper and weighed individually in an analytical balance (Newclassic ML 104/01; Mettler Toledo). Finally, the rings were

incubated for 24 h in a solution containing 0.1 $HN0_3$ to denature the proteins and to allow the exit of the cellular radioactivity.

After 24 hours, duplicated 200 µL samples from each tube were collected and, after the addition of 2 mL of scintillation liquid (Ecoscint H; National Diagnostics), the radioactivity was counted in a liquid scintillation counter (Wallac 1409, Pharmacia). The uptake in each ring was calculated by the following formula:

$$\mu\text{mol substrate/g wet weight} = \frac{cpm_{ts}}{cpm_i} \times \frac{[\text{substrate}] \times V_t \times V_i}{V_{ts} \times Pf}$$

[Substrate] = substrate concentration (mM) in the incubation medium.

Pf = wet weight of the intestinal fragment (g).

cpm_{ts} = counts per minute in 0.1 HNO_3 solution.

cpm_i = counts per minute of the initial medium.

V_t = volume of nitric acid (0.5 mL).

V_i = volume of initial medium sample (0.2 mL).

V_{ts} = volume of denaturised medium sample (0.2 mL).

For the calculation of the substrate accumulation in the intestine, it was considered that the tissue has approximately 70 % of water content.

1.4. Ussing Chambers

1.4.1. Mediums and reagents

The physiological solution used is Krebs-Ringer bicarbonate solution (KRB) that has 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. For sodium-free experiment, the medium has the following composition (in mM): Cho Cl 110, KCl 5, $CaCl_2$ 3.6, $MgCl_2$ 1.2, $KHCO_3$ 26, and glucose (serosal) or mannitol (mucosal) 10. The pH solution was adjusted to 5, 6 or 7 with HCl. To this solution the following substances were added:

- Different concentrations of Glutamate (Glu) were tested (0-50 mM) (Sigma-Aldrich)

- Leptin in final concentrations ranging from 10^{-8} to 10^{-15} M of recombinant rat peptide (Preprotech)

1.4.2. Description of the Ussing chamber

The Ussing chamber is a technique for measuring transepithelial currents first described by H. Ussing and K. Zerahn in 1951 who studied ion exchanges across the frog skin. Ussing chamber consists of two half-chambers forming two compartments which are inserted between the epithelial tissues on a support (Ducroc & Samouelian, 2009).

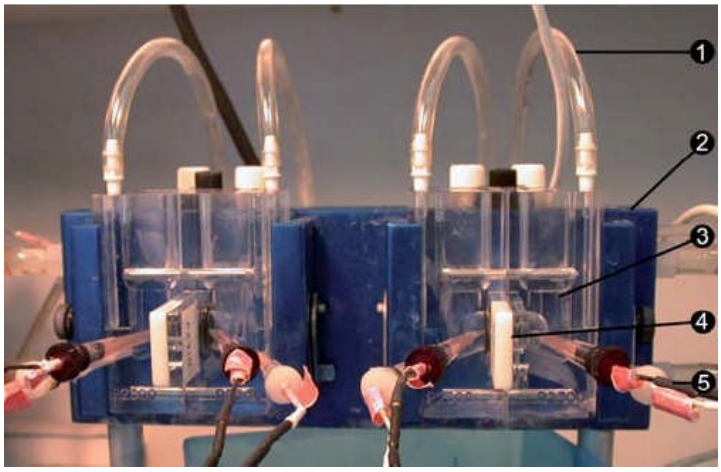


Figure 9. Modified Ussing chamber. (Physiologic Instrument, San diego, CA).
1. Pipes connected to an oxygen tank for oxygenation (95%O_2-5% CO_2).
2. Heated plate behind the two systems.
3. Survival fluid compartments distributed in serosal (left) and mucosal (right).
4. Vertical drawer with the epithelial tissue located between the two compartments (exposed area 0.5 cm^2).
5. Measuring electrodes, potential difference (PD) and short-circuit current (Isc).

1.4.2.1. Experimental procedure

The intestine is rinsed in cold KRB solution to remove luminal content. The mesenteric border is carefully stripped off using forceps. Intestine is then opened along the mesenteric border and adjacent pieces are placed between the two halves of the Ussing chamber (EasyMount Diffusion system, Physiologic Instruments, San Diego CA, USA). The exposed area of tissue is 0.50 cm^2 (Easy Mount P2312).

The tissues are bathed with 4 mL of Krebs Ringer Buffer (KRB) bicarbonate solution on each side gassed with 95% O_2 5% CO_2 and kept at constant temperature of 37°C. Stock solution of leptin is prepared in 0.9% NaCl containing stored frozen at –20 °C

and thawed immediately before use. Working solutions are prepared by serial dilution of the stock solution in KRB to achieve final concentration in the range of 10^{-6} – 10^{-9} M in the Ussing chamber compartments. All solutions are added as 40 µL samples to the mucosal bath.

Electrogenic ion transport is monitored continuously as the 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. Every 50 s, the tissue is automatically clamped at ± 1 mV for 5 s to calculate the electrical conductance according to the Ohm's law. Note that any increase in Isc is in the direction of a cation absorption and/or anion secretion.

Results are expressed as the intensity of the Isc (µA/cm^2) or as the difference (ΔIsc) between the Isc peak (measured within 10 min) and the basal Isc (measured just before addition of glutamate).

1.5. *In vivo* single-pass perfusion system

This technique is directed to reproduce the experimental conditions as close as possible to the physiological ones over the experiment, in order to obtain the most faithful information. It consists in the study of the substrate absorption through an isolated intestinal segment ligated at both ends with two glass cannulae. After that, the intestinal segment is placed inside the abdomen and the cannulae are connected to a perfusion system, with a peristaltic pump (Harvard Apparatus 1201 model or Gilson Minipulse 3). The perfusion medium is maintained over the experiment at 37^{o}C with a heated water bath (JB 1, Grant Instruments, Barrington). Absorption is determined using the single-pass perfusion system, as described below.

1.5.1. Mediums and reagents

The intestinal loop is perfused with KRT saline solution (composition already showed in the *in vitro* absorption protocol) at pH 6 or 7.4. In these *in vivo* experiments, different substrates were tested:

- 2 or 5 mM Pro (Merck) with traces of 0.064 µCi/mL of L - [2,3-^{3}H]-proline (Perkin Elmer Inc) presenting a specific activity of 40 Ci/mmol.

- 2 or 5 mM β-Ala (Sigma) with traces of 0.064 μCi/mL of β-[3-^{3}H]-alanine (American Radiolabeled Chemicals) presenting a specific activity of 50 Ci/mmol.
    - 5 mM Gln (Sigma-Aldrich, St. Louis, MO) in the presence of 0.064 μCi/ml L-[^{14}C(U)]-glutamine (American Radiolabeled Chemicals, St. Louis, MO) presenting a specific activity of 218 mCi/mmol.
- 5 mM D-galactose (Merck) in the presence of 0.0125 μCi/mL D-[14C]-galactose (Perkin Elmer Inc) presenting a specific activity of 56.5 mCi/mmol.

1.5.2. Description of the perfusion system

All the experiments were made in a temperature-controlled cabinet (30-35 ^{0}C) as it is shown in figure 9:

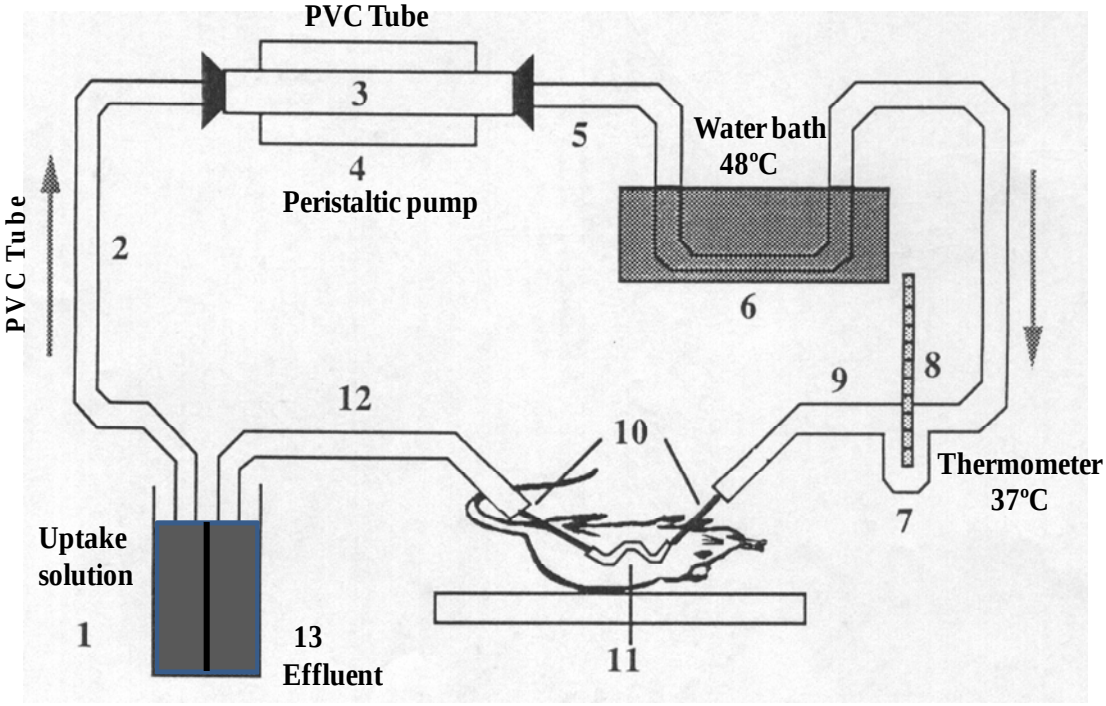


Figure 10. Scheme of the perfusion system used for the tests of absorption experiments *in vivo*. The circuit consists of the following components:

The perfusion solution (1) is taken by the peristaltic bomb (4) that drives it through the tubes system (2, 3 and 5) and is warmed in the water bath (6). In this way, solution when reaching the intestinal segment is at 37ºC. Finally, the liquid is collected every 5 min in previously weighed glass tubes (13).

1.5.3. Experimental procedure

Once isolated, the intestinal loop was washed with 15 mL saline solution (KRT) at pH 6 or 7.4 at 37 ºC (Lostao *et al.,* 1998) followed by air pumping to drain the remaining fluid in the intestine. After that, intestine is perfused with the KRT solution containing the substrate in study. The moment in which the liquid appears in the exit cannula was taken as time zero and consecutive fractions of the effluent were collected every 5 min.

Samples from the initial medium and from each effluent tube were taken for radioactivity counting. Each effluent fraction collected was weighed to determine final volume. Intestinal substrate absorption was calculated by the difference between initial and final radioactivity counts in the perfusion solution and referred to the perfusion rate and the length of the intestinal segment analysed (nmol amino acid cm^{-1}. min^{-1}) (Vidal-Silvilla *et al.,* 1950).

We first performed several uptake experiments with different Pro and β-Ala concentrations and pH (pH 6 or pH 7.4) to select the best experimental conditions for each substrate and confirm that the absorption of the analyzed nutrient remained stable over the time. Once the best conditions were selected, the intestinal loop was continuously perfused with a solution containing: 5 mM D-galactose (D-Gal), 5 mM glutamine (Gln), 2 mM proline (Pro) or 2mM β-alanine (β-Ala) and traces of [^{14}C]-galactose, [^{14}C]-glutamine, [^{3}H]-proline or [^{3}H]-β-alanine at a rate of 1,23 mL min^{-1}. In each animal, control solution (absence of leptin) was perfused during the first 20 or 40 min and afterwards, 25 nM leptin was added and the solution pumped throughout the isolated loop during additional 25 or 40 min. The four first initial control samples were discarded. A similar protocol was performed to investigate whether the effect of leptin on intestinal amino acid absorption was reversible. The intestinal loop was first perfused during 40 min with the same substrates and concentrations previously indicated in the presence of 25 nM leptin and then the perfusion medium was changed for a medium containing the substrate alone for the next 40 min.

At the end of the experimental absorption period, the intestinal segment was excised and its length measured.

To determine the substrate absorbed by the intestine we used the formula described below (Ponz *et al*., 1979).

$$ABS\ (\mu mol/min) = [substrate] \times Flow \times [1-(cpm_f/cpm_i) \times (V_f/V_i)]$$

[substrate] = substrate concentration (mM) in the perfusion medium.

V_f = final volume.

V_i= prefunded volume, flow x time (6.15).

cpm_i = counts per minute in the initial perfusion medium.

cpm_f = counts per minute in the perfusion medium after the absorption process.

Flow= perfusion volume per min (1.23).

Taking into account the perfusion flow used, the initial volume was calculated whereas the final volume was obtained by the effluent withdrawal. The radioactivity measured in the effluent liquid was corrected to avoid the possible variation of the perfused volume across the intestinal handle. The effluent volume was weighed, considering a density of 1 g/mL (Sutton *et al*. 2001).

2. EXPERIMENTS IN THE HUMAN MODEL: CACO-2 CELLS

2.1. Human model

We used a clone of the human epithelial colorectal adenocarcinoma cells (Caco-2 cells), named PD7, kindly provided by Dr. Edith Brot-Laroche (*Univesité Pierre et* Marie *Curie*, INSERM U505, Paris). This cellular line is widely used for intestinal physiology studies because of its ability to grow as a monolayer with the morphological and phenotypical characteristics of the enterocytes. This cell clone was selected due to its particular metabolic characteristics and the expression of its transporters, summarized as follow: (Mahraoui *et al*., 1994):

- Low glucose metabolism.
- Low glycogen content.
- High expression of metabolic enzymes.
- High expression of basolateral membrane transporter, GLUT2, and apical membrane transporters, GLUT5 and SGLT1.
- Low expression of some transporters like GLUT3 (apical membrane) and GLUT1 (basolateral membrane).

2.2. Cell culture

2.2.1. Mediums and reagents

For the maintenance of the cellular line Caco-2 in the laboratory, the Dubelcco's Modified Eagle medium (DMEM) was used. This growth medium present the following composition in mg/L: $CaCl_2.2H_2O$ 264; Fe (NO_3) 0.1. H_2O 9; KCl 400; $MgSO_4.7H_2O$ 200; NaCl 6400; $NaHCO_3$ 3700; $NaH_2PO_4.7H_2O$ 141; glucose 4500; phenol red 15; hypoxanthine 13.6; inositol 4; L-arginine 84; L-cysteine 48; L-glutamine 584; 30 glycine; L-histidine 42; L-isoleucine 105; L-leucine 105; L-lysine 146; L-methionine 30; L-phenylalanine 66; L-serine 42; L-threonine 95; L-valine 94; choline chloride 4; folic acid 4; nicotinamide 7.2; pyridoxal 4; riboflavin 0.4; thiamine 4 (Gibco BRL). This medium was supplemented with:

- 10% foetal bovine serum (Gibco BRL).
- 1% solution of non-essential amino acids (MEN eagle NEAA (100x); Lonza).
- 1 % solution of penicillin - streptomycin (1000 U/mL of penicillin, 1000µg/mL of streptomycin, Gibco BRL).
- 1% solution of fungizone/ amphotericin B (250 µg/mL, Gibco invitrogen).

All these substances were used in sterile conditions, aliquoted and frozen at -20ºC for its conservation.

2.2.2. Passage and cell culture

The cells were frozen dissolved in the culture medium supplemented with 10 % dimethyl sulfoxide (DMSO) (Sigma) in an ultra-low temperature freezer (-140 ºC model; Sanyo) at a concentration of 1-1.5 million cells per tube. They were thawed rapidly to avoid possible cellular damage caused by DMSO and were transferred into a 25 cm^2 surface plastic flask (Costar) with 5 mL of fresh culture medium. The cells were maintained in a CO_2 incubator (MCO-18 AIC UV; Sanyo) at 37ºC with 5 % CO_2, 95 % O_2 and 90 % of relative humidity. The medium was changed every 2-3 days until the cells reached a 80 % cellular confluence in the flask (3-5 days).

In this moment, the cells were dissociated with 0.5 mL of a trypsin-EDTA solution (Composition in g/L: trypsin 5, EDTA 2 and NaCl 8.5 (Gibco BRL) without Ca^{+2} and

Mg^{+2} previously warmed at 37ºC. After 10 min in the CO_2 incubator, the reaction was stopped by adding fresh culture medium, the cellular suspension was homogenized with a Pasteur pipette and the cells were counted in a Neubauer's chamber of 0.1 mm^3 depth.

The number of cells/mL was calculated applying the following formula:

$$cells/mL = n/v = n \times 10^4$$

v = counted cell volume that is equal to 0.1 mm^3 or 10^{-4} mL.

n = average of the cells number counted in at least four fields of the chamber.

Once this value was calculated, the cells were seeded in 25 cm^2 flasks at a density of 200,000 cells per cm^2, in order to maintain cell culture.

The substrate uptake experiments were performed in cells seeded on 24 well-plates or 12 well-filters, respectively (TranswelTM Costar). In both cases, the cells were seeded at a density of 60,000 cells per cm^2. The filters consisted in a support with a polycarbonate membrane of 12 mm of diameter and an area of 1 cm^2 and micropores of 0.4 µM (TranswelTM Costar), that allows to distinguish between and apical and a basal compartment over the cell monolayer. (Figure 10)

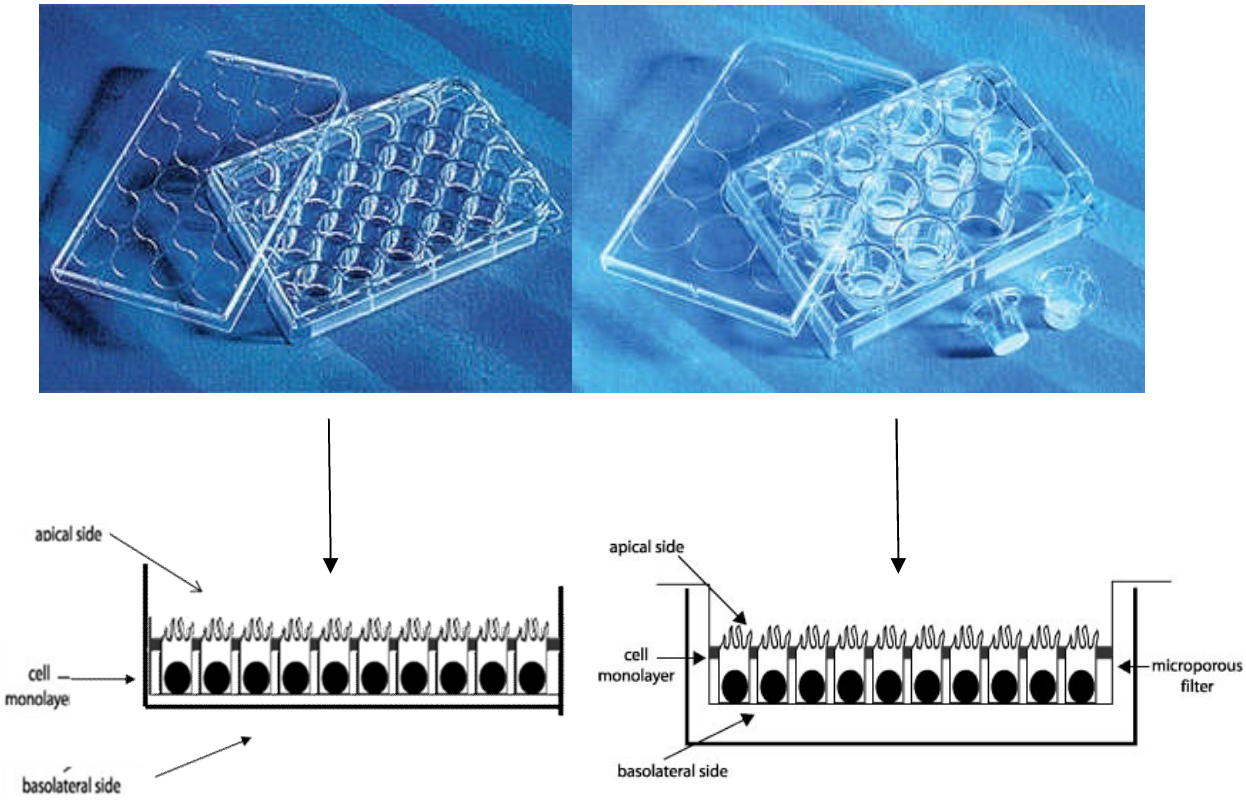


Figure 11. Distribution of the cells in plates and filters

Before seeding the cells on filters, 1 mL of growth medium was added to each well. After that, the filters were introduced in each well and the cells were seeded inside the filters. The cells were observed routinely with an optical microscope to control its development. After 15-21 days the cells are ready to use for the uptake experiments.

Transepithelial resistance (TER) was measured (ohms per cm^2) with a Millicel ERS electrode (Millipore) to verify the integrity of the cell monolayer in the filter. The transepithelial resistance increases as the cell monolayer grows. When it reaches a maximum TER value between 450-500 $ohm.cm^2$, we consider that the monolayer presents optimal conditions for the experiments.

2.3. Amino acids uptake experiments

2.3.1. Mediums and Reagents

The medium used is a Krebs modified saline solution (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10, HEPES or MES 10 (adjusted with TRIS base to pH 7.4 or 6, respectively). For the experiments performed in Na^+ free conditions, NaCl was replaced with the same concentration of choline chloride and the NaH_2PO_4 was omitted. The amino acid with its respective radiolabelled substrate was dissolved in the medium to the desired concentration, in the absence or in the presence of leptin.

Different substrates and reagents have been used in this set of experiments:

- 1 mM β-alanine (Sigma) in the presence of 2 µCi/mL of β-[3-^{3}H]-alanine (American Radiolabeled Chemicals) with a specific activity of 50 Ci/mmol.
- 0.1 mM L-glutamine (Sigma) in the presence of 2 µCi/mL L-[3,4-3H (N)]-glutamine (Perkin Elmer Inc) with a specific activity of 30 Ci/mmol.
- 0.1 mM L-phenylalanine (Sigma) in the presence of 2 µCi/mL L-[3,4-3H]-phenylalanine (Perkin Elmer Inc) with a specific activity of 30 Ci/mmol.
- 0.1 mM L-glutamate (Sigma) in the presence of 0.25 µCi/mL L-[^{3}H(G)]-glutamic acid (American Radiolabeled Chemicals, Inc.) with a specific activity of 30 Ci/mmol.

- 10 mM L-alanine (Sigma) and 25 mM L-phenylalanine (Sigma) for the competitive substrate experiments.
- 0.2 or 8 nM recombinat rat leptin (Peprotech Inc. UK).
- The PKA inhibitor H-89 at a concentration of 1 µM. The concentration used was the one recommended by the distributor to specifically inhibit PKA (<10µM).
- 1 % Triton X-100 in 0.1 N NaOH (Sigma).
- Dimethyl sulfoxide (DMSO) (Sigma).

2.3.2. Leptin effect on amino acid uptake

A similar experimental procedure was performed in plates and in filters. Uptake assays were usually made without leptin preincubation excepting a set of experiments in which cells grown on filters were preincubated with leptin for 6 h before the beginning of the uptake period.

First, cells were washed twice with the incubation medium in the absence of the substrate. Then, the medium was removed by aspiration and 500 µL of amino acid uptake solution were added in the absence or in the presence of leptin. Before the beginning of the experiment, 10 µL of the uptake solution were taken for triplicate to measure the initial medium radioactivity.

After 5, 15 or 30 minutes incubation at 37ºC, uptake was stopped by the addition of 500 µL ice-cold free-substrate buffer followed by aspiration. Then, cells were again washed twice with ice-cold buffer to eliminate non-specific radioactivity. Cells (grown in plates or filters membranes), were solubilised in 500 µL of 1 % Triton X-100 in NaOH 1M and kept at 37ºC for two hours to liberate the radioactive cell content.

After this time, 100 µL of samples were taken for duplicate and, after the addition of 2 mL of scintillation liquid (Ecoscint H; National Diagnostics), the radioactivity was counted in a liquid scintillation counter (Wallac 1409, Pharmacia). The protein content was measured by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories).

In the experiments performed in cells grown in filters the basolateral compartment was refilled with 1 mL of modified KRT with or without 0.2 or 8 nM leptin and 500 µL of amino acid uptake solution were added to the apical compartment. At the end of the

incubation period apical medium was decanted and the filter was introduced in cold free substrate medium. The membrane of every filter was cut and, as described previously, introduced in the Triton X-100 solution.

The final amino acid uptake was calculated by applying the following formula:

$$\text{Capt (nmol/mg prot)} = \frac{Cpm_m}{Cpm_i} \times \frac{[\text{substrate}] \times V_i \times V_d}{V_c \times \text{mg prot}} \times 1000$$

[substrate] = substrate concentration (mM).

cpm_s = counts per minute in the TritonX-100 medium.

cpm_i = counts per minute in the initial medium.

V_i = sample volume of initial medium (0.01 mL).

V_d Triton X-100 volume (0.5 mL).

V_c = Triton X-100 sample volume (0.1 mL).

2.3.3. Reversal of leptin effect on amino acids uptake

To determine the possible reversal of leptin effect in Gln and β-Ala uptake, cells were preincubated with KRT modified medium without (control) and with apical (plates) or basal (filters) leptin (8 nM) during 15 min in the absence of the substrate (leptin and reversion groups). After that substrate uptake (15 min) was measured in the presence or the absence of leptin.

Condition	**Pre-incubation (15 min)**	**Uptake (15 min)**
Control	KRT modified medium	Substrate
Leptin	Leptin	Substrate +leptin
Reversion	Leptin	Substrate

During the incubation period leptin concentration remained unaltered.

2.3.4. Leptin effect on amino acid uptake in the presence of PKA inhibitor (H-89)

After 30 min pre-incubation of the cells grown in plates with or without 1 µM H-89, the substrate (Gln or β-Ala) was added in the absence (control; H-89) or in the presence of 8 nM leptin (leptin; leptin + H-89) and the uptake measured for additional 30 min.

Condition	**Pre-incubation (30 min)**	**Uptake (30 min)**
Control	DMSO	Substrate+ DMSO
H-89	H-89	Substrate +H-89
Leptin	DMSO	Substrate +DMSO+Leptin
H-89+Leptin	H-89	Substrate +H-89+Leptin

During the uptake period H-89 concentration remained unaltered.

2.4. Western blot experiments

The brush border membrane expression of the sugar (sodium glucose transporter, SGLT1) and amino acids transporters (alanine, serine, cysteine transporter 2, ASCT2; B^0 amino acid transporter, B^0AT1 and proton amino acid transporter 1 PAT-1) was analysed by Western blot.

2.4.1. Cell treatment

Cells grown in plastic flasks of 75 cm^2 were incubated with 20 mL of uptake medium during 30 min in the presence of 0.1 mM MG and Gln or 1mM β-Ala without (control) or with 8 nM leptin. After the end of the incubation period the cells were processed for the isolation of brush border membrane vesicles (BBMV).

2.4.2. BBMV isolation

BBMV were isolated following the method of Kessler and Shirazi-Beechey with slight modifications (Kessler *et al*., 1978; Shirazi-Beechey *et al*., 1990).

2.4.2.1. Mediums and reagents

- Buffer A, composed by (in mM): mannitol 100; Phenylmethyl sulfonyl fluoride (PMSF) 0.2; Hepes/TRIS 2; sodium azide 0.00041; benzamidine 0.2. The buffer was adjusted at pH 7.4.
- Buffer B, composed by (in mM): mannitol 300; Hepes/TRIS 20; $MgS0_4.7\ H_2O$ 0.1; sodium azide 0.00041. The buffer was adjusted at pH 7.4.
- Protease inhibitor cocktail (Roche).
- $MgCl_2$ 1 M.

2.4.2.2. Experimental procedure

All the manipulations were made at 4 ºC to avoid protein degradation of the samples.

After incubation period with the substrate in the absence or the presence of 8 nM leptin, 5 mL of buffer A was added to the flask and the cells were homogenized. This cell suspension was mixed with an homogenizer and an aliquot of approximately 300µL was freezed to use it as a reference to determine vesicles enrichment in BBM.

1 mM of $MgCl_2$ was diluted in the cell homogenate until a final concentration of 20 mM $MgCl_2$. After 20 min at room temperature and constant agitation, the solution was centrifuged at 3,000xg during 15 min to remove precipitated cellular material and organelles. The supernatant containing mainly brush border membranes was centrifuged at 27,000xg during 30 min. The pellet was finally resuspended in buffer B (100 µL).

Finally, the pellet was homogenized with a 27G syringe to cycle the vesicles that were stored at -80 º C.

2.4.2.3. Determination of alkaline phosphatase activity

To determine the purity of the vesicles, the pellet was analyzed by measuring alkaline phosphatase activity in both, the initial homogenate and the final resuspended precipitate.

The alkaline phosphatase is an enzyme located in the apical membrane of the cells, so a high activity of the enzyme in the final sample indicates apical membrane enrichment.

Alkaline phosphatase enrichment of the vesicles was determined by Cobas Mira S auto analyser (Roche Diagnostics; French Forest, NSW, Australia) and the vesicles were considered highly pure when enzymatic activity was 2.5- to 3.0- fold higher than the cell homogenate.

2.4.2.4. Determination of protein concentration

In order to verify the performance of the isolation, protein concentration was also measured in the vesicles by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories).

2.4.3. Western blot

This technique involves the separation of the proteins present in a determined sample by polyacrylamide gel electrophoresis under denaturing conditions and subsequent transfer to a membrane in which, using specific antibodies, the protein of interest is immunodetected.

2.4.3.1. Sample preparation

The vesicles were slowly defrosted on ice and then diluted to load in the gel the required protein concentration (20 µg of solubilised proteins).

2.4.3.1.1. Mediums and Reagents

- Water of molecular use or DEPC-treated water
- Loading Buffer: Laemmli sample buffer; Bio-Rad. 50 µL of β-mercaptoehtanol was added to 950 µL of sample buffer.

Each sample was prepared by adding 20 µg of protein and mixed 1:1 with Laemmli buffer. Then the samples were heated at 90ºC during 5 min and after, put on ice during 1 min.

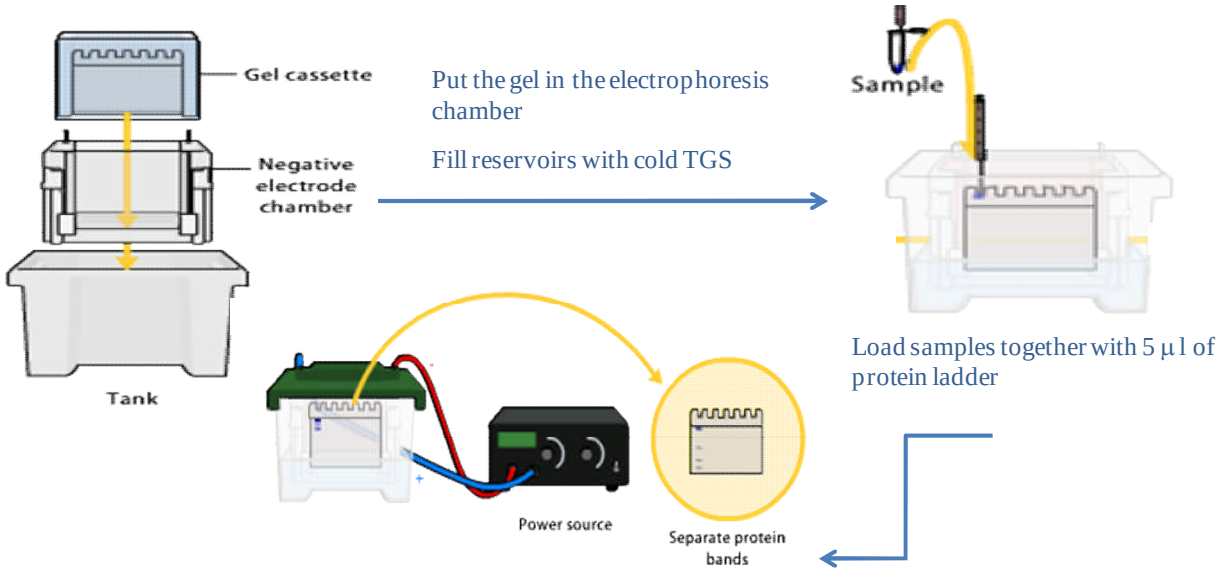
2.4.3.2. Electrophoresis

2.4.3.2.1. Mediums and reagents

- Mini-PROTEAN TGX gels (Bio-Rad)

- Running Buffer 10X: Tris/Gkycine/SDS buffer (TGS); (Bio-Rad) diluted the 1/10 for use.
- Ladder: Prestained SDS-PAGE standards, low range; Bio-Rad.

2.4.3.2.2. Experimental procedure



Run the gel first at 20 mA until the bromo phenol blue reached the limit of the stacking and separating gels, then run the gel at higher voltage (40 or 60 mV) until the blue front was at the bottom of the gel.

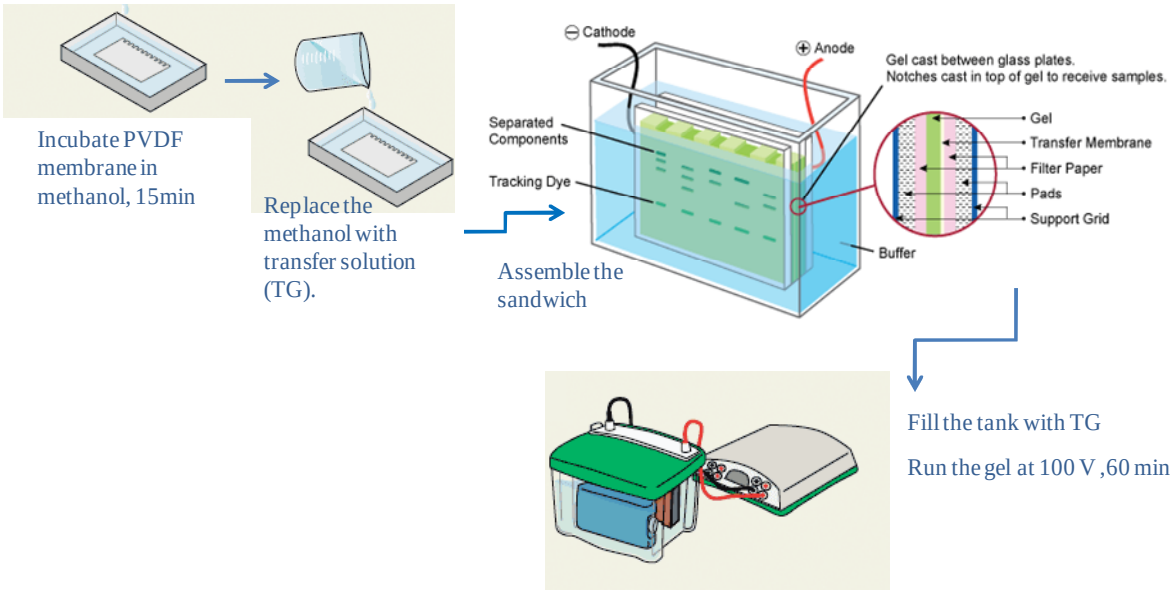
2.4.3.3. Protein transfer

Once the proteins were separated by electrophoresis, the blot was transferred onto a polyacrylamide membrane (PVDF)

2.4.3.3.1. Mediums and reagents

- Transfer solution: Tris/glycine buffer (TG) 10X (Bio-Rad) dilute 1/10 for use.
- PVDF transfer membranes (Amersham)

2.4.3.3.2. Experimental procedure



2.4.3.4. Protein immunodetection

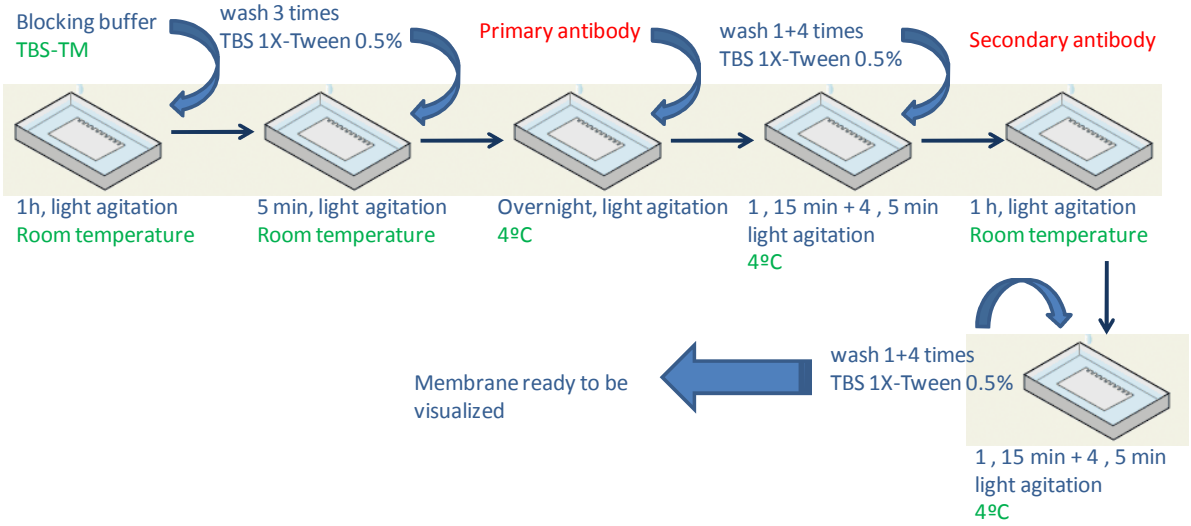
Immunodetection blotting provides a simple and effective method for identifying specific antigens in a complex mixture of proteins.

2.4.3.4.1. Mediums and reagents

- Tris Buffered Saline 10X (TBS 10X) composed by: 250mM Tris – HCl; 2M NaCl. pH 7.5 diluted 1/10 for use.
- TBS Tween 0.5%: 5ml Tween 20 (Sigma) +995ml TBS 1X.
- Tris Buffered Saline-Tween (TBS-T) composed by: 250mM Tris – HCl; 2M NaCl.; Tween 0.5%, pH 7.5.
- Membrane blocking reagent TBS-TM composed by: 10% BSA; TBS-Tween 1X.
- Anti-SGLT1 antibody made in rabbit against the residues 604-613 of SGLT-1 protein; generous gift from Ernest M. Wright (Lostao et al. 1995). The antibody was used at a 1:1000 dilution in TBS-T.
- Anti-B^0AT1 antibody generous gift from Françoise Verrey (Romeo et al. 2006)
- Anti-ASCT2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA). The antibody was used at a 1:1000 dilution in TBS-T.
- Polyclonal anti-PAT-1 antibody raised against residues 107-119 of human PAT-1 (Abytenk, Spain). The antibody was used at a 1:1000 dilution in TBS-T.

- Monoclonal β-Actine (Santa Cruz, Biotech)
- Secondary antibodies HRP-conjugated: Goat anti-rabbit (Pierce) and anti-mouse (St. Cruz Biotech)

2.4.3.4.2. Experimental procedure



2.4.3.5. Chemiluminescence

2.4.3.5.1. Mediums and reagents

- Revelation buffer: Termo scientific 34076 Supersignal west dura.
- High performance chuminescence film (Amershan)
- Hypercassette (Amershan)

2.4.3.5.2. Experimental procedure

- Mix equal parts of the reagents 1 and 2
- Incubate the membrane during 3 min

Once in the darkness, the membrane was exposed to the high performance chuminescence film. According to the result obtained in the first exhibition period, the time was changed until obtaining the desired resolution.

2.4.3.6. Stripping

Stripping is the term used to describe the removal of primary and secondary antibodies from a western blot membrane. Stripping is useful to investigate more than one protein on the same blot. When probing for multiple targets, stripping and re-probing a single membrane, instead of running and blotting multiple gels, has the advantage of saving samples, materials, and time.

2.4.3.6.1. Mediums and reagents

- Tris Buffered Saline-Tween (TBS-T) composed by: 250mM Tris – HCl; 2M NaCl; Tween 0.5%, pH 7.5.
- Mild Antibody Stripping Solution: Re-blot Plus (Millipore). Dilute Re-Blot Antibody Stripping Solution 10x with distilled water to obtain a 1x solution.

2.4.3.6.2. Experimental procedure

The membranes were washed 2 times during 10 min in TBS 1X-Tween 0.5% solution with light agitation. Then, each membrane was incubated at room temperature with 1 mL of the stripping solution 1X during 15 min with gentle agitation. After that, membranes were washed with distilled water during 5 min followed by 2 consecutive incubations whit TBS 1X-Tween 0.5% during 10 min. Then, the blot was ready for the incubation with blocking solution and then for the incubation with the new antibodies.

2.5. Gene expression of sugar and amino acids transporters: B^0AT1, ASCT2 and PAT-1

2.5.1. Preincubation of the caco-2 cells

Cells grown in 75 cm^2 of plastic flasks were incubated for 1 hour in the presence of 0.1 mM MG, Gln or 1 mM β-Ala with or without 8 nM leptin. 20 mL of incubation medium were added to each flask and maintained in the $C0_2$ incubator during 30 min.

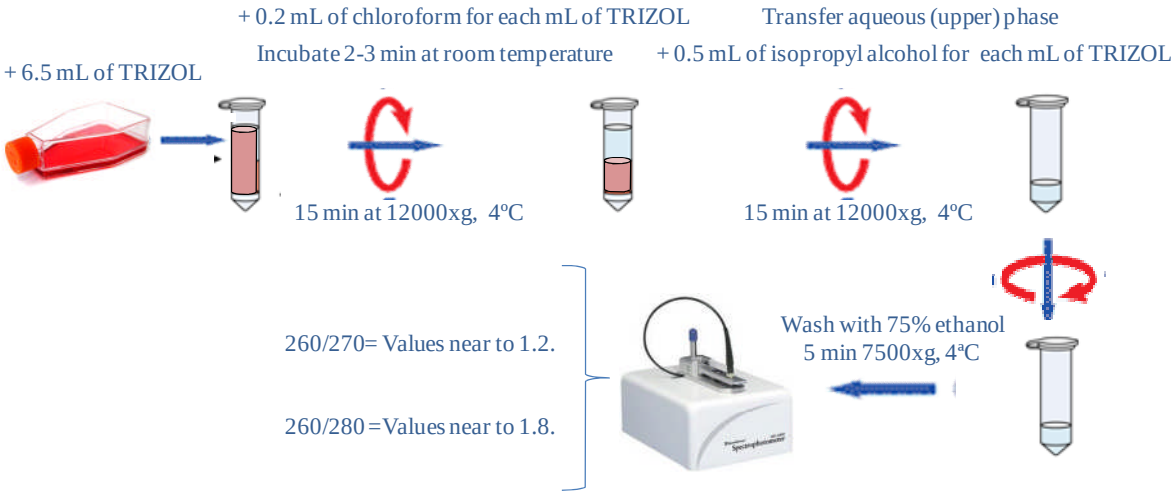
2.5.2. RNA extraction using TRIZOL method

This technology is based on the cells homogenization in a solution of phenol that protects the sample to the RNAses, avoiding its degradation.

2.5.2.1. Mediums and Reagents

- TRIZOL
- Chloroform
- Isopropyl alcohol
- Ethanol at 75 % (With DEPC-treated water 0.01 %)
- Water of molecular use or DEPC-treated water
- Spray anti-RNAses (RNAse Zap, Ambion)

2.5.2.2. Experimental procedure



2.5.3. Agarose gel

The agarose gel allows to visualize the RNA and to evaluate its quality identifying the ribosomal RNA portions with different molecular weight. This technology consists in submitting the mixture of RNA molecules, placed in an agarose gel to an electrical field. Exposure of the gel to UVs light allows the visualization of the different bands because the samples have been previously marked with ethidium bromide. Two bands must be observed in the gel: First corresponding to 28s and the second one to 18 s.

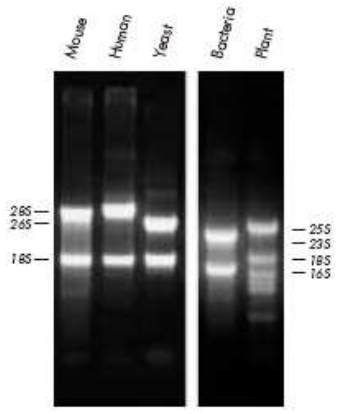


Figure 12. Tacked from Qiagen

2.5.3.1. Mediums and Reagents

- Agarose
- Ultrapure mili Q water
- Buffer TBE 1X
- DEPC treated water to 0.01 %
- Ethidium bromide

2.5.4. DNAse Treatment

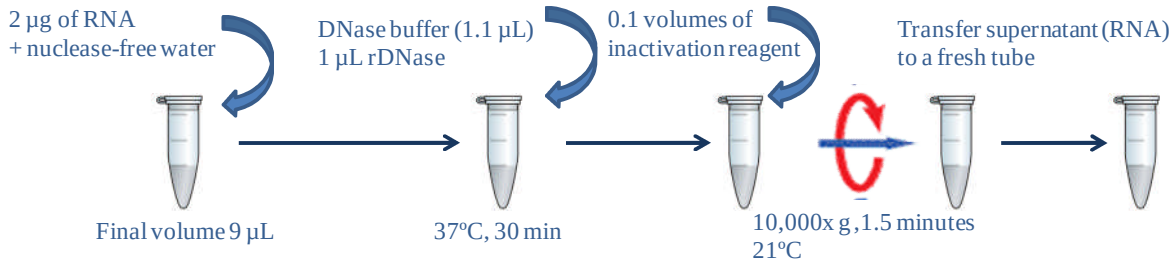
This technology is designed to remove contaminating DNA from RNA preparations, and to subsequently remove the DNase and divalent cations from the sample.

2.5.4.1. Mediums and Reagents

The kit DNAfree (Ambion, TX, the USA) contains the following reagents:

- rDNase I
- 10X DNase I Buffer
- DNase inactivation reagent
- Nuclease-free Water

2.5.4.2. Experimental procedure



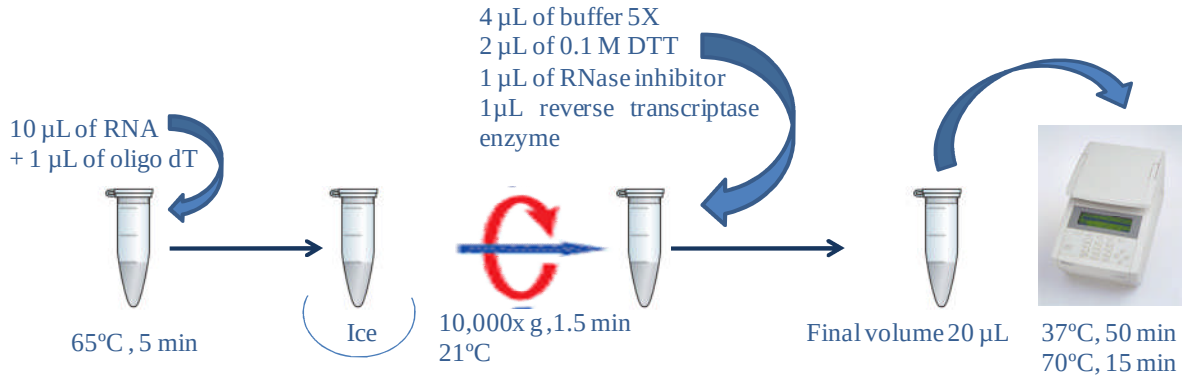
2.5.5. Reverse transcription

The reverse transcription is the synthesis of complementary DNA or cDNA using as reference a filament of RNA.

2.5.5.1. Mediums and Reagents

- d NTP (10 mm of dATP, dCTP, dGTP, and dTTP at neutral pH) (Bioline)
- Oligo dT (Boehringer Mannheim)
- Buffer 5X (Invitrogen)
- 0.1 M DTT (Invitrogen)
- RNasin® Plus RNase Inhibitor (Promega)
- DEPC treated water to 0.01 %
- M-MLV Reverse Transcriptase (Invitrogen)

2.5.5.2. Experimental procedure



2.5.6. Real-time PCR

Quantitative PCR is a method used to detect relative or absolute gene expression level. All qPCR involves the use of fluorescence to detect the threshold cycle (Ct) during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplified curve. This Ct value is responsible for the accurate quantization of qPCR. SYBR Green is a dye that penetrates within the double-stranded DNA. The qPCR machine (LightCycler 480 Real-time PCR system, Roche) detects the fluorescence and the software calculates Ct values related to fluorescence.

2.5.6.1. Mediums and Reagents

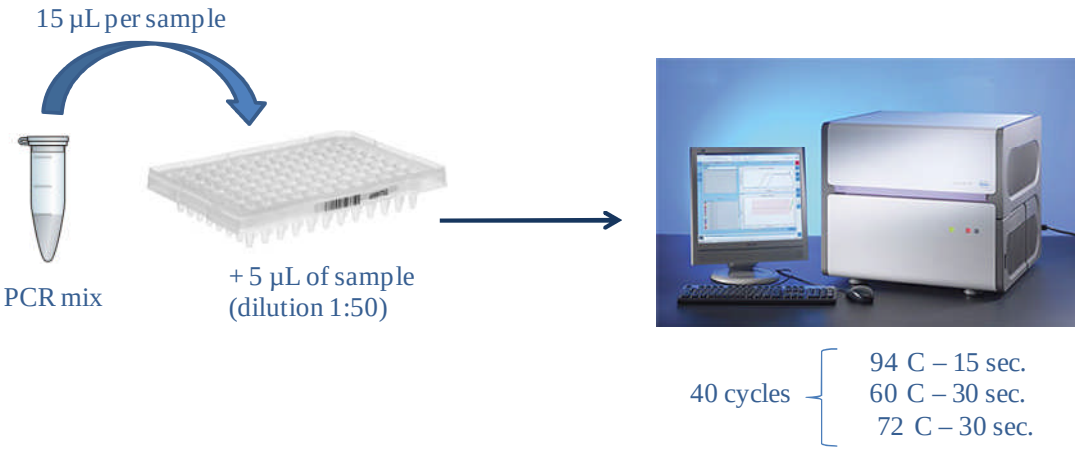
- SYBR® Green PCR Master Mix
- RNase/DNase-free H_2O
- Primers were as follows:
  - hB^0AT1 f5'-ACCCTGGCTACGAGGAATTT-3' and r5'-GTACTTCAGGTCCCCGTTCA-3´.
  - hASCT2 f5'-ACATCCTGGGCTTGGTAGTG-3' and r5'-GGGCAAAGAGTAAACCCACA-3´.
  - hPAT1 f5´- CTTGGCTGAGGACTCAAAGG -3´, and r5´ CTCTCACTCAGCCACCCTTC -3´.
  - hGAPDH f5´- CCATCACCATCTTCCAGGAG-3´, and r5´ CCTGCTTCACCACCTTCTTG -3´.

They were designed with oligo 4 software and synthesized by Eurogentec (Southampton, UK).

The cDNA samples were diluted 1:50 and the following mix was added to 5 µL cDNA of each sample in the PCR plate.

Mix	**µL per sample**
SYBR Green	10
Forward Primer (10µM stock)	0.5
Reverse Primer (10µM stock)	0.5
RNase/DNase-free H_2O	4
	Final Volume= 15 µL

2.5.6.2. Experimental procedure



The melting curve was generated and analyzed because SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. The melting curve ensures that the desired amplicon was detected. The comparative ΔΔCT-method was used for relative mRNA quantification of target genes, normalized to mRNA GAPDH and a relevant control equal to 2-ΔΔCT.

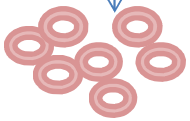
3. STATISTICAL ANALYSIS

Statistical differences were evaluated by Student t-test for parametric analysis and U Mann–Whitney test for non-parametric one. Differences were considered as statistically significant when $p < 0.05$. The calculations were performed using the SPSS/WINDOWS Version 15.0 statistical package (SPSS, Chicago, IL, USA).

4. SUMMARY

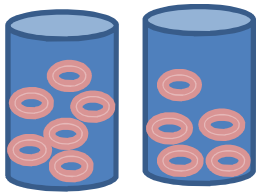
Chapter 1

Jejunum extraction



Everted jejunal rings

$^{14}C/^{3}H$-substrate



- 37ºC
- Oxygenation
- Agitation

Control + Leptin

$HN0_3$

Scintillation counter

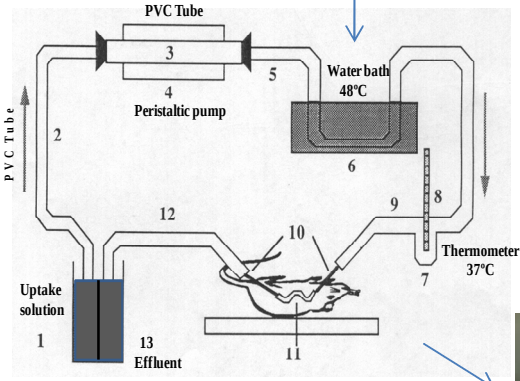
Chapter 2

Jejunum isolation

Perfusion solutions

- Control
- + Leptin

$^{14}C/^{3}H$-substrate

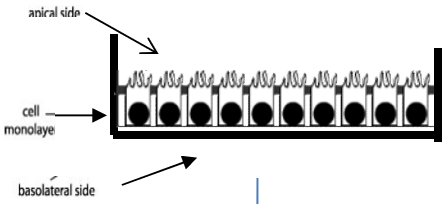


In vivo single pass perfusion system

Scintillation counter

Chapter 3

Caco-2 cells
(plates)



37ºC

$^{14}C/^{3}H$-substrate

- Control
- + Leptin
- + H-89 + Leptin

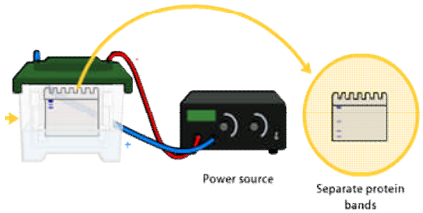
Triton X-100

Scintillation counter

Caco-2 cells
(flaks)

- Incubation
  Control
  + Leptin

BBMV preparation

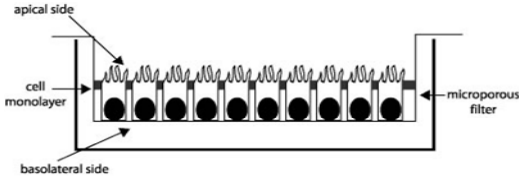


RNA extraction
Reverse transcription

Q-PCR

Chapter 4

Caco-2 cells
(filters)



37ºC

$^{14}C/^{3}H$-substrate

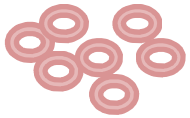
- Control
- + Leptin

Triton X-100

Scintillation counter

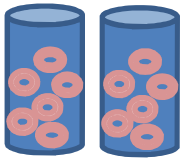
Chapter 5

Jejunum extraction



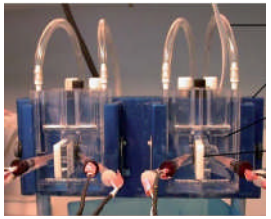
Everted jejunal rings

- 37ºC
- Oxygenation
- Agitation



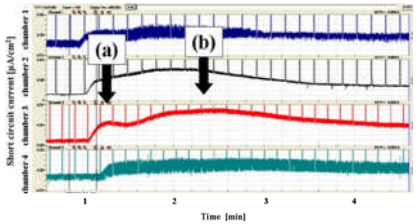
$^{14}C/^{3}H$-substrate

Scintillation counter



Ussing chambers

- Control
- + Leptin



Short circuit current data

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Results

Chapter 1:

Preliminary studies

Chapter 1

The preliminary studies of this thesis were published in the article: “Luminal leptin inhibits L-glutamine transport in rat small intestine: involvement of ASCT2 and B^0AT1” *Amer. J. Physiol. Gastrointest Liver Physiol* 299:G179-G185, 2010. These studies were performed in collaboration with the laboratory of Dr. André Bado of the “Institut National de la Santé et de la Recherche Médicale, U773, CRB3 Faculté de Médicine Xavier Bichat” in Paris, and were the beginning of a collaborative work that continues to date.

Chapter 2:

Regulation of amino acids absorption by leptin in rat intestine *in vivo*.

Regulation of amino acids absorption by leptin in rat intestine *in vivo*

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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 implicated in the regulation of food intake and energy expenditure by binding to its receptor located in the hypothalamus (Tartaglia *et al*. 1995, Pelleymounter *et al*. 1995). Nevertheless, the studies performed since its discovery almost 20 years ago (Zhang *et al*. 1994) have largely demonstrated that leptin is a multifunctional hormone that can be synthesized and can act in many different peripheral tissues (Margetic *et al.* 2002, Gertler 2009). In this regard, shortly after leptin identification, we demonstrated that leptin inhibits sugar absorption in rat intestine *in vitro* by short-term regulation of the Na^+/glucose cotransporter SGLT1 (Lostao *et al.* 1998). We later described that leptin receptors are expressed in both apical and basolateral membrane of human and murine enterocytes (Barrenetxe *et al.* 2002). In the same year, another group reported that the stomach chief cells secrete leptin into the gastric lumen after a meal (Bado *et al.* 1998). This study was followed by the demonstration that the chief cells also secrete the leptin soluble receptor (Cammisotto *et al.* 2006), and that leptin remains stable in the gastric juice because the binding to this receptor protects it from the acidic pH and the proteolytic activity of the stomach, and favours its arrival to the small intestine (Guilmeau *et al.* 2003, 2004). All these data 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 sugars transport (Barrenetxe *et al.* 2001, 2004, Iñigo *et al.* 2004, 2007, Ducroc *et al.* 2005, Sakar *et al.* 2009). Regarding amino acid absorption, we have recently shown in rat intestine *in vitro* and in Caco-2 cells that leptin also inhibits glutamine 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). Given that those studies were performed using *in vitro* models, the purpose of the present study was to investigate whether leptin modulation of amino acids absorption could also be observed using *in vivo* techniques, which are closer to the real physiological conditions.

The results show that leptin inhibits the absorption of glutamine, proline and β-alanine *in vivo*, after short-time exposure of the intestinal mucosa to the hormone, and that this effect is reversible.

MATERIALS AND METHODS

Animals and surgical procedure

The studies were performed in 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 ºC), with a 12 h light/12 h dark cycle, and had free access to water and standard chow diet (Harlam Ibérica, Barcelona, Spain). Food was removed 16-18 h prior to the experiments. Rats were anaesthetized by intraperitoneal injection of a mixture (4:1) of ketamine (Ketolar, Parke-Davis, Barcelona, Spain) and medetomidinechlorhydrate (Domtor, Pfizer Orion Corporation, Espoo, Finland), at a dose of 0.25 ml per 100 g body weight. The rat was then placed in a temperature-controlled cabinet (30-35 ^oC) for the whole of 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

(Harvard Apparatus 1201 model or Gilson Minipulse 3) and to a heated bath to maintain the temperature of the perfusion medium at 37^{o}C. The intestinal loop was washed with 15 ml of Krebs Ringer Tris (KRT) saline solution (Lostao *et al.* 1998) at pH 7.4 or 6 and 37 ºC followed by air pumping to drain the remaining fluid in the intestine. Absorption was determined using a 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 sacrificed by cervical dislocation. The ethics committee of the University of Navarra approved the study protocol.

Single-pass perfusion system

The intestinal loop was continuously perfused with a solution containing 5 mM galactose (Gal), 5 mM glutamine (Gln), 2 mM proline (Pro) or 5 mM β-alanine (β-Ala) and traces of [^{14}C]-galactose, [^{14}C]-glutamine, [^{3}H]-proline or [^{3}H]-β-alanine. The perfusion rate was 1.23 ml^{-1} min^{-1}. The radiolabelled amino acids were from Perkin Elmer or American Radiolabeled Chemicals. Consecutive fractions of the effluent were separately collected every 5 min. In each animal, control solution (absence of leptin) was perfused during the first 40 min and afterwards, 25 nM leptin was added and the solution pumped throughout the isolated loop during additional 40 min. This leptin concentration was chosen based on our previous *in vivo* studies (Iñigo *et al.* 2007). The four first initial control samples were discarded. A similar protocol was performed to investigate whether the effect of leptin on the amino acids absorption was reversible. In this case, the intestinal loop was first perfused during 40 min with the substrate in the presence of 25 nM leptin and then, the medium was changed to another which only contained the substrate that was perfused for the next 40 min. Samples of the initial medium and the effluent were taken for radioactivity counting. Each effluent fraction

collected was weighed to determine the final volume. The variation in 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 by the difference between initial and final amino acid concentration in the perfusion solution and referred to the perfusion rate and the length of the intestinal segment (nmol amino acid cm^{-1} min^{-1}) (Vidal-Silvilla *et al.* 1950).

Statistical analysis

Statistical differences were evaluated by the general linear model for repeated measures. Differences were considered as statistically significant when $p<0.05$. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS Inc, Chicago, USA).

RESULTS

One of the main transporters of Pro and β-Ala in the intestine is the H^+-depedent neutral amino acid transporter PAT1 (Bröer 2008). Before studying leptin effect on the absorption of those amino acids, we wanted to demonstrate the presence of PAT1 in *in vivo* conditions. Thus, based on our previous *in vitro* studies (Iñigo *et al.* 2007), we measured 2 mM Pro and 5 mM β-Ala absorption at pH 6 and 7.4. As it is shown in figure 1, absorption of 2 mM Pro at pH 6 was 60 % higher than at pH 7.4. (36.06 ± 2.9 *vs.* 14.91 ± 2.1 nmol Pro cm^{-1} min^{-1}), indicating the presence of the PAT1 transporter. At both pHs, the absorption remained constant along the whole 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 in agreement with the functional

characteristics of the intestinal transporters involved, ASCT2 and B^0AT1 (Bröer 2008, Ducroc *et al.* 2010).

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

Effect of leptin on amino acids absorption

To study the possible regulation by leptin on amino acids absorption *in vivo*, 2 mM Pro, 5 mM β-Ala and 5 mM Gln absorption was measured for 40 min in the absence of the hormone and then, during additional 40 min, in the presence of 25 nM leptin. Figures 2a, 3a and 4a show that leptin inhibited Pro, β-Ala and Gln absorption by ~ 45 % (29.47 ± 2.45 *vs.* 15.92 ± 2.99 nmol Pro cm^{-1} min^{-1}; 79.5 ± 0.58 *vs.* 50.88 ± 5.39 nmol β-Ala cm^{-1} min^{-1}; 135.98 ± 2.67 *vs.* 83.07 ± 18.36 nmol Gln cm^{-1} min^{-1}, $p< 0.05$). The inhibitory effect on Pro absorption was observed after 15 min of perfusion of the amino acid with the hormone, whereas for β-Ala and Gln the inhibition was statistically significant already after 5 min. This decrease on each amino acid absorption due to leptin was maintained until the end of the experiment.

The magnitude of inhibition was the same when the data were compared with the absorption levels in the control group of animals.

Leptin effect on intestinal amino acid absorption is reversible

In order to check the reversibility of leptin inhibition 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 25 nM leptin during 40 min, followed by perfusion of the amino acid in the absence of the hormone for another 40 min. After removal of leptin

from the perfusion solution, amino acid absorption levels significantly increased in 5-10 min, reaching control absorption levels (17.10 ± 0.56 *vs.* 29.07 ± 0.69 nmol Pro cm^{-1} min^{-1}; 48.48 ± 12.20 *vs.* 91.79 ± 3.68 nmol β-Ala cm^{-1} min^{-1}; 109.26 ± 16.59 *vs.* 142.02 ± 15.78 Gln cm^{-1} min^{-1}, $p< 0.05$).

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 D-gal and Pro

Previous work from our group had shown that sugar absorption was also inhibited by leptin *in vivo* (Iñigo *et al.* 2007). We wanted to verify that leptin inhibition on different nutrients could occur in the same animal. For that, a solution with 5 mM Gal at pH 7.4 was perfused for 20 min in the absence of leptin and afterwards during additional 25 min in the presence of 25 nM leptin. Figure 4 shows that, as expected, leptin inhibited Gal absorption by ~ 40 % (99.17 ± 6.39 *vs.* 61.87 ± 9.20 nmol Gal cm^{-1} min^{-1}, $p< 0.05$). After Gal perfusion, the intestine was perfused with saline solution (KRT) for 15 min in order to eliminate the sugar that could still remain in the intestine. Following this wash out period, a solution containing 2 mM Pro at pH 6 was perfused during 20 min in the absence of leptin and then, for additional 25 min, in the presence of 25 nM leptin. As it happened for Gal, leptin inhibited Pro absorption also by ~ 40 % (34.13 ± 1.72 *vs.* 20.57 ± 3.41 nmol Pro cm^{-1} min^{-1}, $p< 0.05$) (fig. 4).

These results indicated that leptin is able to regulate the absorption of two different substrates in the same animal by acting on each of the transporters implicated in their absorption process.

DISCUSSION

Normal 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 on nutrients absorption using different physiological and biochemical approaches (Lostao *et al.* 1998, Barrenetxe *et al.* 2001, Ducroc *et al.* 2005, Iñigo *et al.* 2007, Shakar *et al.* 2009, Ducroc *et al.* 2010, Fanjul *et al.* 2012). In this regard, more and more it is becoming clear that leptin can be considered as a new gastrointestinal hormone, secreted by the stomach (Cammisotto *et al*. 2006), which rapidly regulates intestinal nutrients absorption 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 continuation with our previous studies, the present work shows in rat *in vivo* short-term inhibition of neutral amino acids absorption by luminal leptin. The absorption of Pro, β-Ala and Gln was decreased about ~ 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.

In vivo intestinal absorption method is directed to reproduce experimental conditions close to the physiological state. The results here found confirm our previous studies in rat *in vitro* and in Caco-2 cells, where leptin rapidly inhibits Gln uptake by decreasing the expression in the brush border membrane of the enterocytes of the main Gln transporters 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 had demonstrated in rat intestine the presence of a Na^+-independent, pH-dependent Pro and β-Ala transporter, whose activity was enhanced by Na^+, which confirmed the existence of PAT1 transporter in the apical membrane of the rat enterocytes (Iñigo *et al.* 2006). In the *in vivo* experiments here presented, absorption of Pro and β-Ala was also enhanced at acidic pH, demonstrating the proton-coupled amino acid transporter PAT1 activity in this experimental condition. The absorption of these amino acids was also inhibited by leptin, which is in agreement with our previous results in Caco-2 cells where transport of Pro and β-Ala was reduced by leptin, as a result of a decreased activity of PAT1, via leptin activation of an H-89 sensitive pathway (Fanjul *et al.* 2012). This effect occurred without modification of PAT1 expression in the brush-border membrane of the cells, and most probably was due to a decrease in the activity of the Na^+/H^+ exchanger (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 in line with the *in vitro* results.

Interestingly, luminal leptin increases peptide absorption by enhancing the density of the proton-dependent peptide transporter PepT-1 in the apical membrane of the absorptive cells both in rat *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 membrane of the enterocytes which explains the increase on galactose and fructose absorption due to the hormone (Sakar *et al.* 2009).

All these results clearly indicate that leptin can either increase or decrease nutrients absorption, probably in relation with the metabolic needs of the enterocyte and its capacity for processing that specific nutrient.

The *in vivo* approach also shows two relevant results. First, leptin effect reverses quickly once the hormone disappears from the lumen, as we previously demonstrated for glucose *in vivo*, where leptin regulates SGLT1 but does not modify intestinal permeability (Iñigo *et al.* 2007). And second, leptin is able to regulate the absorption of galactose and proline in the same animal, indicating direct action on the specific transporters implicated in the absorption of each substrate.

In summary, the present results extend our previous findings and demonstrate that luminal leptin decreases amino acids absorption *in vivo* in a short-term manner, as one of the physiological effects of leptin on the epithelial cells of the small intestine (Cammisotto & Bendayan 2012). Therefore, leptin can be considered as a hormone which provides the intestine with a control mechanism to handle nutrients absorption.

Acknowledgments

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Pablo was a recipient of a fellowship from “Departamento de Industria” Navarra Government.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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LEGENDS TO FIGURES

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.

Figure 2 Effect of 25 nM 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 rat, the amino acid absorption was measured throughout the experimental period (0-80 min). (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 *vs.* perfusion in the presence of leptin in the same group of animals.

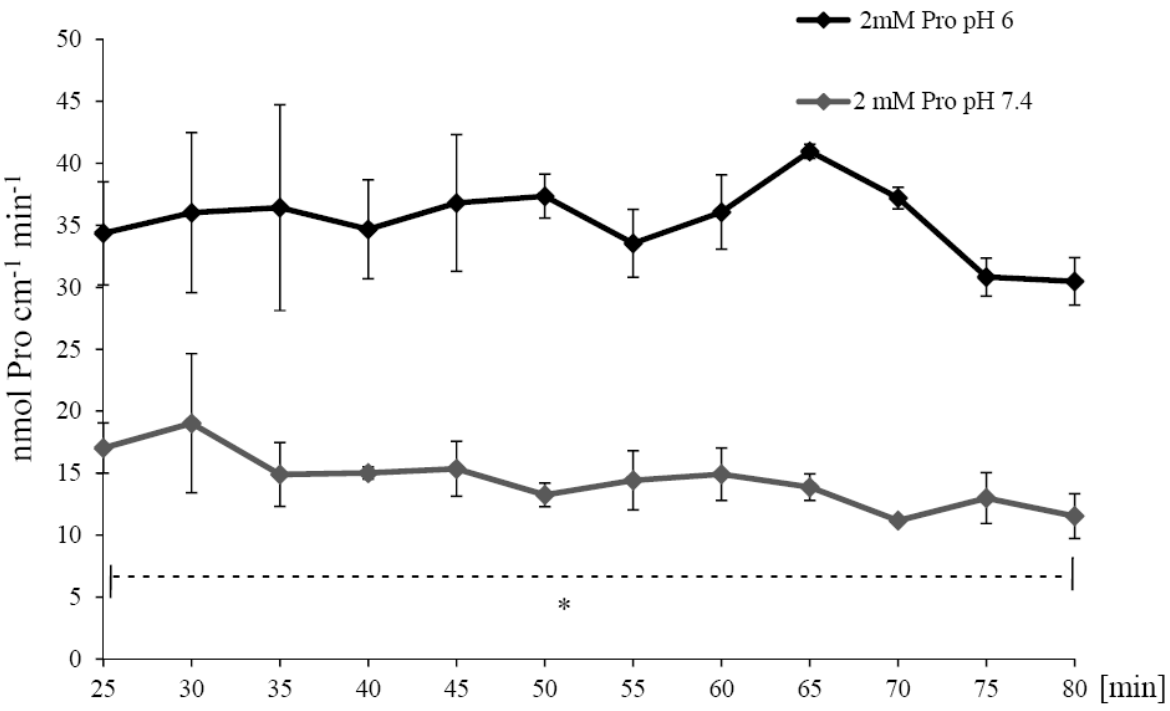
Figure 3 Effect of 25 nM leptin on intestinal β-Ala absorption *in vivo*. (a) Absorption of 5 mM β-Ala at pH 6 (0-80 min) was measured in the absence of leptin (0-40 min) before the addition (↑) of the hormone (40-80 min). In the control rat, the amino acid absorption was measured throughout the experimental period (0-80 min). (b) The intestinal loop was perfused with 5mM 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 *vs.* perfusion in the presence of leptin in the same group of animals.

Figure 4 Effect of 25 nM leptin on intestinal Gln absorption *in vivo*. (a) Absorption of 5mM 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 rat, the amino acid absorption was measured throughout the experimental period (0-80 min). (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 *vs.* perfusion in the presence of leptin in the same group of animals.

Figure 5 Effect of 25 nM 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). All the experiment was performed in the same animal. n=5, * p< 0.05 *vs.* perfusion in the absence of leptin in the same group of animals.

FIGURE 1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nmol Pro cm-1 min-1
50
45
40
35
30
25
20
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25
30
35
40
45
50
55
60
65
70
75
80
[min]

FIGURE 2

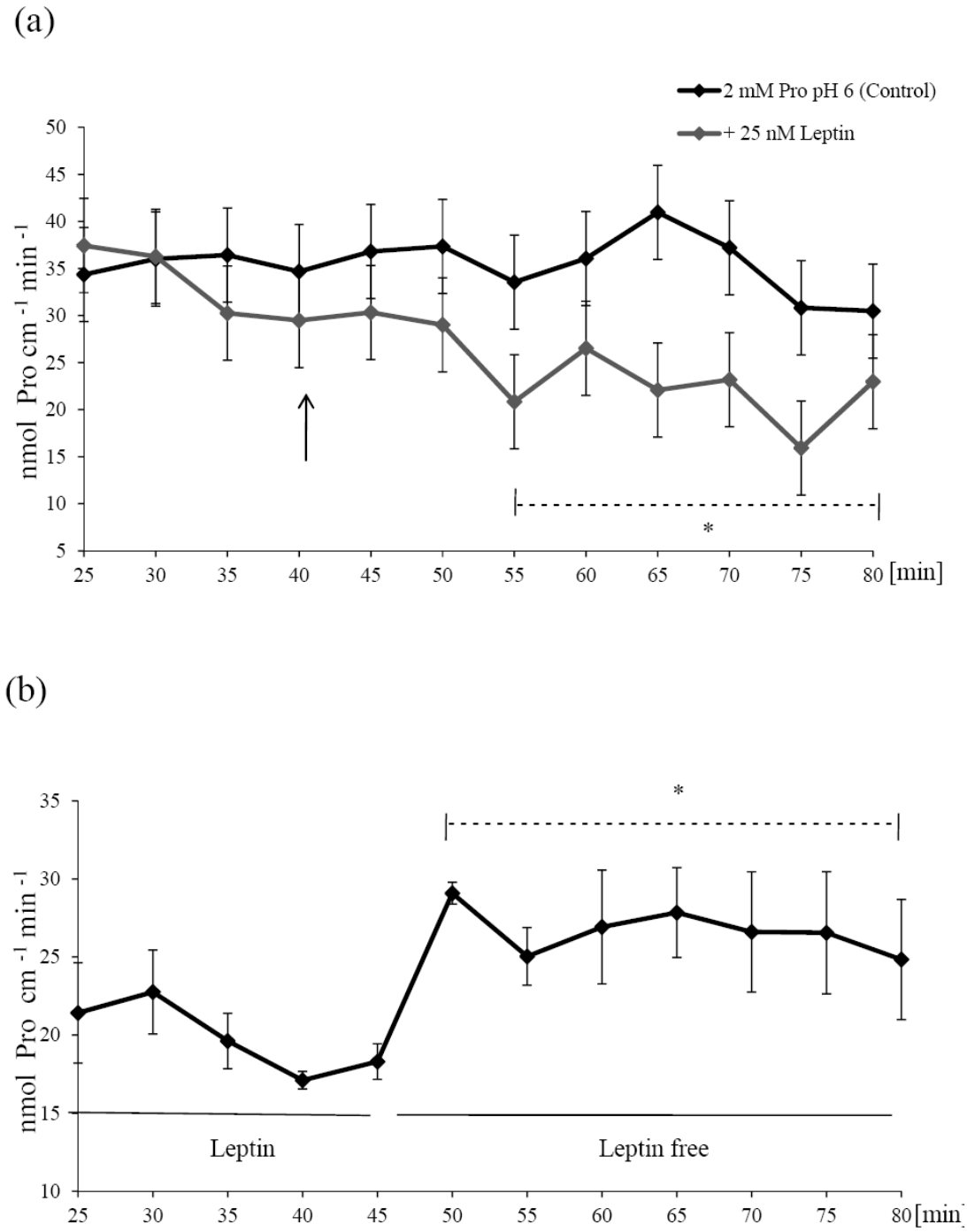
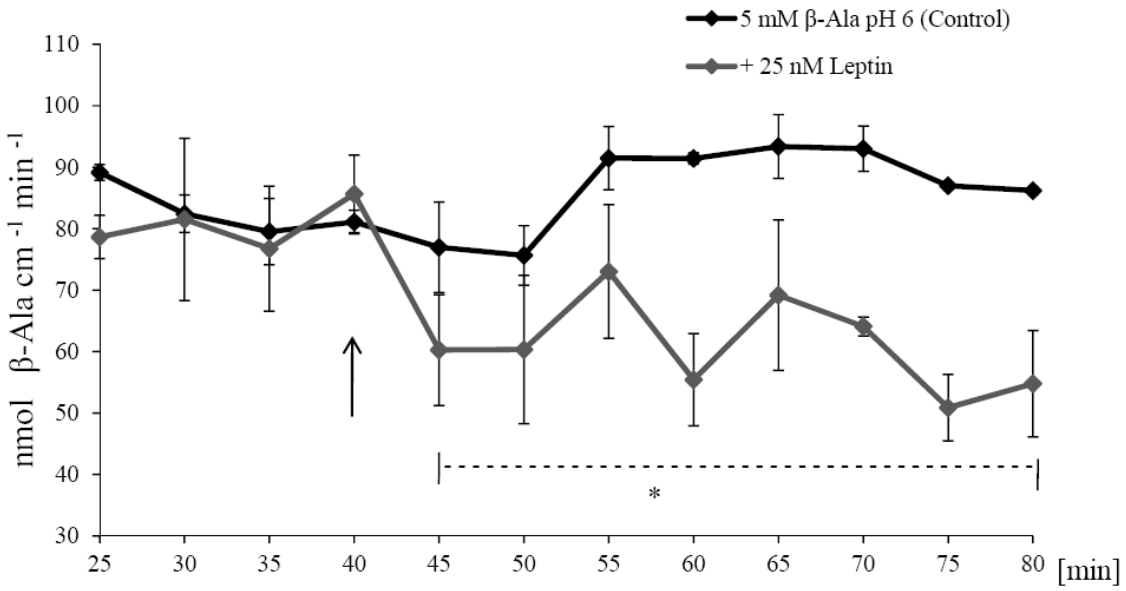

(a)
2 mM Pro pH 6 (Control)
+ 25 nM Leptin
nmol Pro cm -1 min -1
50
45
40
35
30
25
20
15
10
5
25 30 35 40 45 50 55 60 65 70 75 80 [min]
*
(b)
nmol Pro cm -1 min -1
35
30
25
20
15
10
*
Leptin
Leptin free
25 30 35 40 45 50 55 60 65 70 75 80 [min]

FIGURE 3

(a)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+ 25 nM Leptin
nmol β-Ala cm -1 min -1
110
100
90
80
70
60
50
40
30
*
25
30
35
40
45
50
55
60
65
70
75
80
[min]

(b)

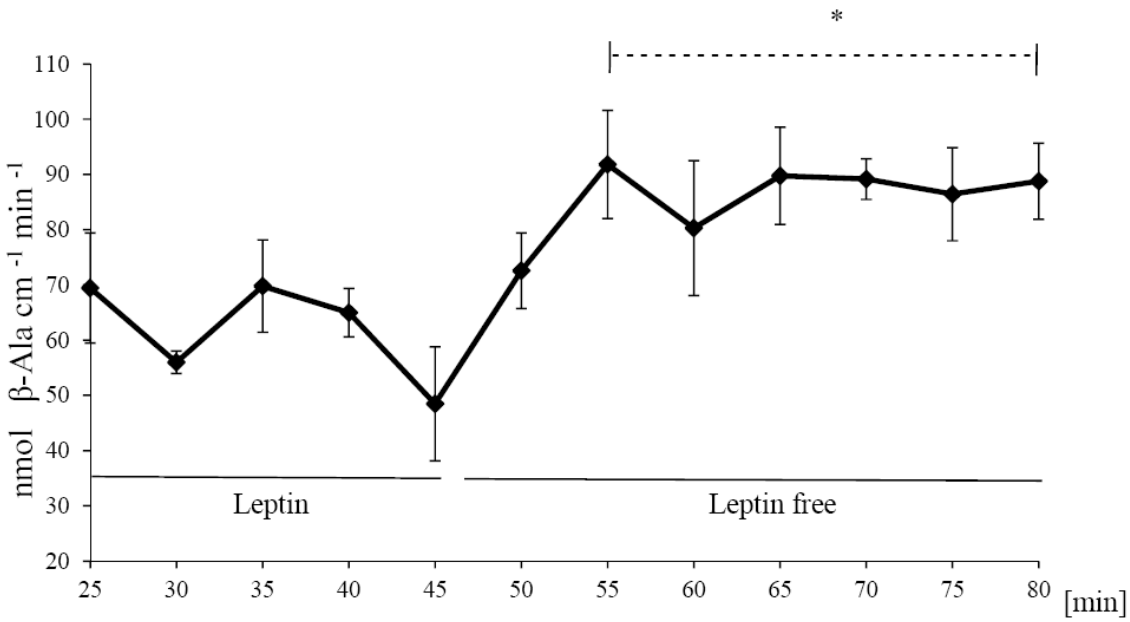
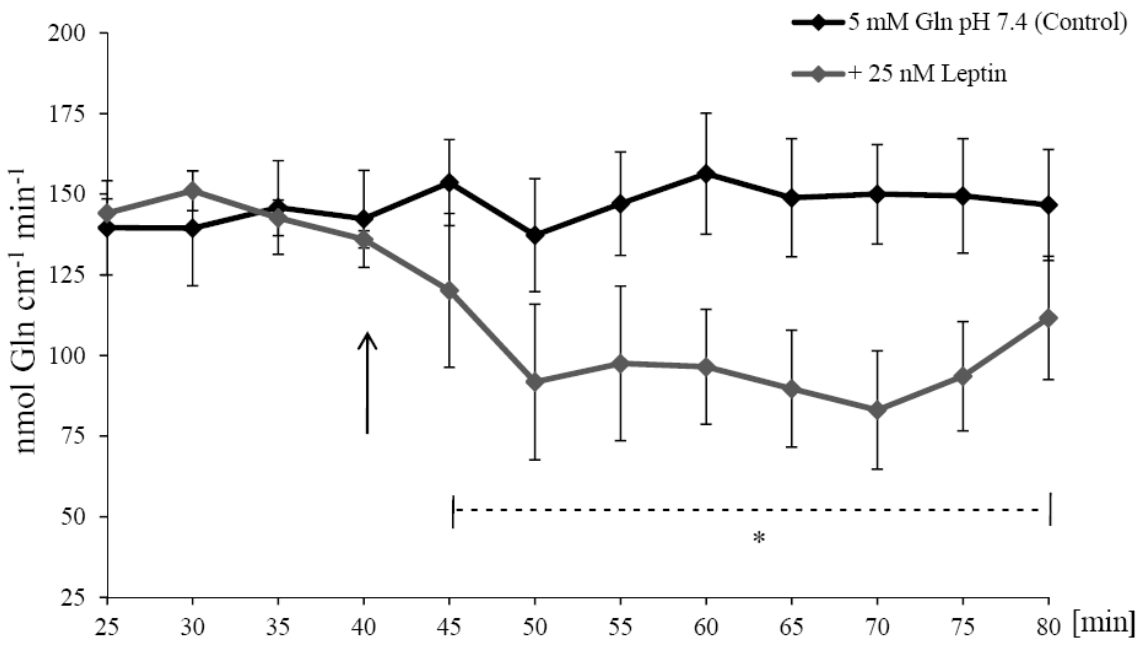

*
nmol β-Ala cm -1 min -1
110
100
90
80
70
60
50
40
30
20
Leptin
Leptin free
25
30
35
40
45
50
55
60
65
70
75
80
[min]

FIGURE 4

(a)


5 mM Gln pH 7.4 (Control)
+ 25 nM Leptin
nmol Gln cm-1 min-1
200
175
150
125
100
75
50
25
25 30 35 40 45 50 55 60 65 70 75 80 [min]
*

(b)

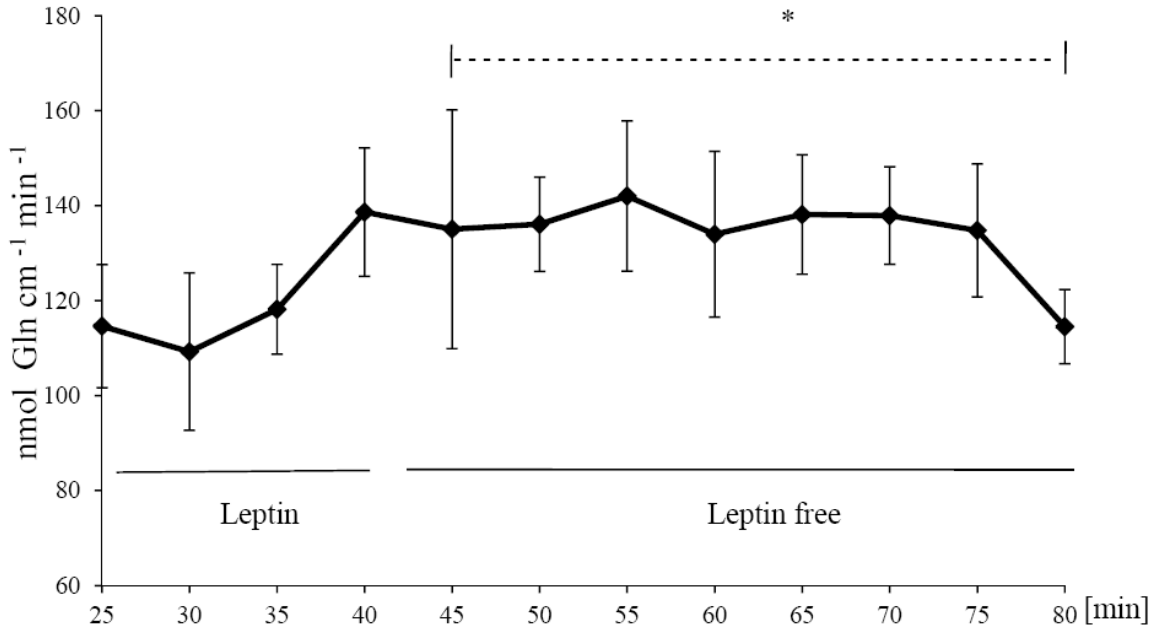
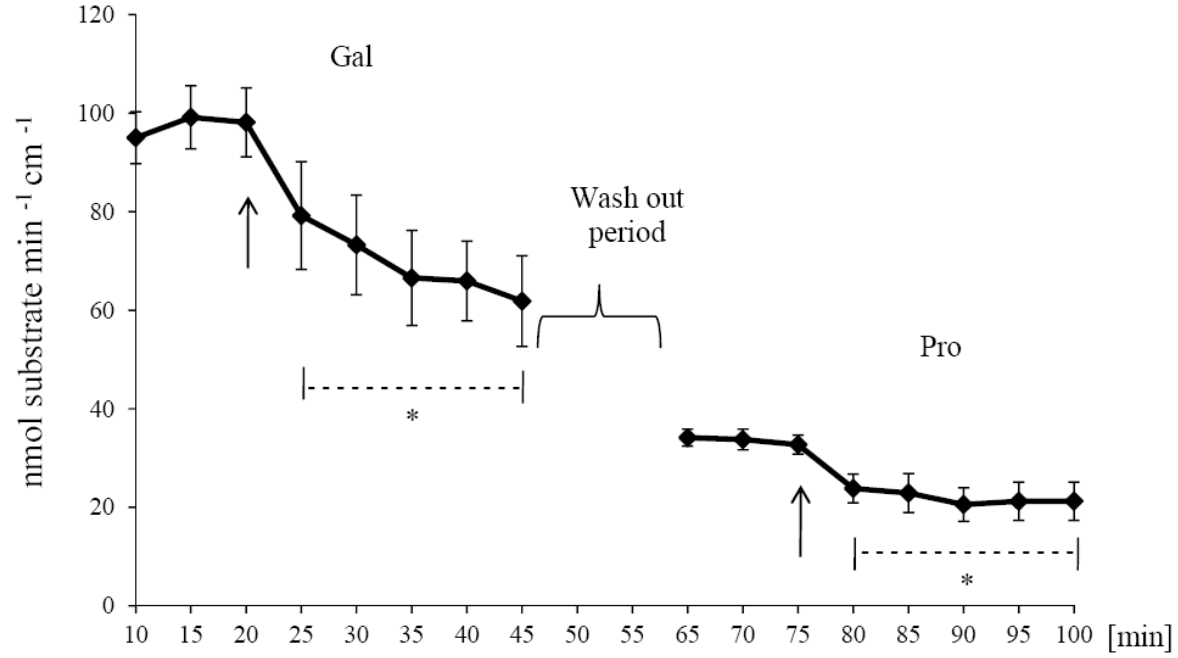

nmol Gln cm -1 min -1
180
160
140
120
100
80
60
25 30 35 40 45 50 55 60 65 70 75 80 [min]
*
Leptin
Leptin free

FIGURE 5


120
100
80
60
40
20
0
nmol substrate min -1 cm -1
Gal
Wash out
period
Pro
*
*
10
15
20
25
30
35
40
45
50
55
65
70
75
80
85
90
95
100
[min]

Chapter 3:

Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2

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Chapter 4:

Intestinal amino acid uptake regulation by basal leptin in Caco-2 cells

Intestinal amino acid uptake regulation by basal leptin in Caco-2 cells

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Aim Leptin is secreted by gastric mucosa and is able to reach the intestinal lumen where its receptors are located in the apical membrane of the enterocytes. We have previously demonstrated that apical leptin inhibits sugar and amino acids uptake *in vitro* and glucose absorption *in vivo*. Since leptin receptors are also expressed in the basolateral membrane of the enterocytes, the aim of the present work was to investigate leptin effect on amino acid uptake acting from the basolateral side.

Methods Gln and β-Ala uptake was measured in Caco-2 cells grown on filters in the presence of basal leptin at short incubation times (5 and 30) and after 6 h of preincubation with the hormone. For comparison, Gln and β-Ala uptake was also measured in the presence of leptin acting from the apical membrane in cells grown on filters. Amino acid uptake was measured by the radioactivity method.

Results Basal leptin (8 mM) inhibited by ~15-30 % the uptake of 0.1 mM Gln and 1 mM β-Ala quickly, after 5 min of exposure of the cells to the hormone, and the effect was maintained after long preincubation periods. Apical leptin had the same effect. Moreover, the inhibition was rapidly and completely reversed when leptin was removed from the apical or basolateral medium.

Conclusion These results extend our previous findings and contribute to the vision of leptin as an important hormonal signal for the regulation of intestinal absorption of nutrients

Key words Amino acids transport, basolateral compartment, leptin, Caco-2

Introduction

Leptin is an adipocyte-derived hormone [25] which regulates food intake and energy expenditure by providing afferent signals to the hypothalamus [22, 9]. Leptin levels in the plasma are highly correlated with body fat mass and adipocyte size, and regulated by diverse factors such as hormones, nutritional status and the sympathetic nervous system [15, 20, 7]. Leptin can be also produced by different peripheral tissues such as stomach, salivary glands and kidney [10, 3, 21, 14] where it can exert its action in a paracrine way. Thus, gastric mucosa releases leptin in a rapid and exocrine manner into the gastric juice [1] where it remains stable by binding to its soluble receptor, also secreted by the stomach, and is able to reach the duodenal lumen in an intact and active form [6]. Accordingly, long leptin receptor isoform (Ob-Rb) is expressed in the apical and basolateral membrane of human and murine enterocytes [3]. More recently, the presence of the short and long leptin receptor isoform and the different pattern of regulation have been described in Caco-2 cells [8]. All this information explains the well documented physiological role of gastric leptin as modulator of nutrients absorption. In this regard, we have demonstrated, in rodents and Caco-2 cells, that luminal leptin rapidly inhibits sugar absorption by reducing the amount of the Na^+/glucose cotransporter (SGLT-1) in the brush border membrane [2, 11, 13, 17-19]. Similarly, leptin decreases neutral amino acid transport by the regulation of ASCT2, B^0AT1 and PAT1 transporters [12, 13]. Interestingly, uptake of other nutrients such as dipeptides, butyrate or fructose is increased by the hormone also acting from the apical membrane [4, 5, 23].

Since leptin receptors are expressed in the basolateral membrane of the enterocytes [24, 3] systemic leptin may have a role on nutrients absorption as well, however, the studies

are scarce or no effect has been found [4]. Therefore, the aim of the present work was to extend our previous studies to investigate whether leptin can regulate amino acid uptake by the enterocytes acting from the basolateral plasma membrane.

Material and Methods

Cell culture

The Caco-2 cell line PD7 clone was kindly provided by Dr. Edith Brot-Laroche. Cells were maintained in a humidified atmosphere of 5% CO^2-95% at 37 °C. Cells (passages 50-70) were grown in Dulbecco‘s Modified Eagles medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin (1000 U. mL^{-1}), 1% streptomycin (1000 g. mL^{-1}) and 1% amphoterycin (250 U mL^{-1}). Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin-EDTA and subcultured on 25 or 75 cm^2 plastic flasks at a density of 200,000 cells cm^2. For transport studies, the cells were seeded at a 60,000 cells cm^2 density on 24 well-plates or 12 well-filters (Transwel TM Costar), which allows the access to both apical and basolateral membrane. Culture medium was replaced every 2 days. Cell confluence was confirmed by microscopic observance. Experiments were performed 17–21 days postseeding.

Uptake measurements

The amino acids (0.1 mM Gln and 1 mM β-Ala) with traces of their respective radiolabelled substrates (2 µCi mL^{-1} of L-[3, 4-3H]-glutamine, 30 Ci mL^{-1}, and □-[3-3H]-alanine ,50 Ci mL^{-1}, from American Radiolabeled Chemicals,St Louis, MO, USA) were diluted in Krebs modified buffer: 5.4 mM KCl, 2.8 mM $CaCl_2$, 1mM $MgSO_4$, 0.3 mM NaH_2PO_4, 137 mM NaCl, 0.3 mM KH_2PO_4, 10 mM glucose and 10 mM

HEPES/Tris (pH 7.5) or Mes/Tris (pH 6). Cells were gently washed with substrate-free buffer before the starting of the experiment. In some assays, cells were preincubated with leptin before the beginning of the uptake period. Uptake was initiated by adding 0.5 mL uptake buffer in the apical compartment and 1 mL of substrate-free buffer, without or with 8 nM leptin, in the basal or apical compartment. This leptin dose corresponds to plasma leptin basal levels in obese subjects [16] and was used in our previous studies [13, 19]. After an incubation period of 5 or 30 min at 37 °C, uptake was stopped with ice-cold free-substrate buffer. Cells were again washed twice with ice-cold buffer to eliminate non-specific radioactivity fixation and were finally solubilized in 500 µL 1% Triton X-100 in 0.1 N NaOH. Samples (100 µL) were taken to measure radioactivity by liquid scintillation counting. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad laboratories, Hercules, CA, USA).

Uptake of β-Ala was performed in Na^+-contained buffer (Krebs modified buffer) at pH 6, which are the conditions for PAT1 activity [13]. Glutamine presence in the culture medium did not affect the results [13].

Statistical analysis

Results of transport experiments are expressed as nmol mg^{-1} of protein. All data are presented as % compared to controls which are normalized to 100%. Statistical differences were evaluated by Student t-test for parametric analysis and U Mann–Whitney test for non-parametric one. Differences were considered as statistically significant when $p< 0.05$. The calculations were performed using the SPSS/WINDOWS Version 15.0 statistical package (SPSS, Chicago, IL, USA).

Results

We have previously demonstrated that apical leptin inhibits Gln and β-Ala uptake in Caco-2 cells grown on plates [13]. In order to study the effect of leptin acting from the basolateral membrane on the amino acids uptake, which would mimic systemic leptin, cells were grown on filters. Cells grown on plates are not in the same conditions that those grown on filters and, therefore, could differently express membrane proteins. In order to compare apical and basal leptin effect on amino acids uptake we study again the effect of leptin acting from the apical membrane but in cells grown on filters. As shown in figure 1a, 8 nM leptin present either in the apical or in the basal compartment inhibited 0.1 mM Gln uptake by ~20% after 5 min incubation of the cells with the hormone and the amino acid. When the incubation time was 30 min, the inhibitory effect increases up to ~40 % in those cells in which leptin was acting on the apical membrane, and remained at 20% when the hormone was in the basal compartment. In the case of 1mM β-Ala, the magnitude of inhibition was also ~20 % in all conditions except for that in which leptin acted from the basal membrane for 5 min, where leptin did not have any effect (figure 1b).

These results demonstrated that leptin inhibits in a short-time manner Gln and β-Ala uptake acting from the apical and basal membrane of Caco-2 cells grown on filters, although the effect from the basal membrane seemed to be less powerful that the effect from the apical membrane in some conditions. Another interesting information derived from these studies is that leptin effect acting from the apical membrane in cells grown on filters is the same than in cells grown on plates [13], indicating similar expression of the proteins implicated.

Since the basolateral membrane of the enterocytes is continuously exposed to circulating leptin while, in comparison, exposure of bush border membrane to gastric leptin is shorter (systemic *vs.* postprandial leptin), we wanted to investigate leptin effect on the amino acids uptake after 6 h preincubation of the cells with 8 nM leptin present in the basal compartment. Figure 2 shows that leptin again inhibited apical uptake (5 min) of 0.1 mM Gln and 1 mM β-Ala by ~40 and ~30 % respectively.

Our previous studies had demonstrated that leptin inhibits galactose absorption in rat intestine *in vivo* and that this effect is rapidly reverted in the absence of leptin in the intestinal lumen [18]. We wanted to check whether in Caco-2 cells the inhibitory effect of leptin, acting from that apical or basolateral membrane, could also be reverted.

The studies on the reversion of leptin inhibition from the apical membrane were performed on cells grown on plates. Cells were preincubated for 15 min in the absence (control) or in the presence of 8 nM leptin placed in the apical (figure 3a) or the basal (figure 3b) compartment. Then, uptake of 0.1 mM Gln or 1 mM β-Ala in the presence of leptin in its corresponding compartment was measured for additional 15 min. As shown in figure 3, leptin inhibited the uptake of both amino acids by ~15 % when it acted from the apical membrane (figure 3A) and by ~20-30 % when it was present in the basal compartment (figure 3B). For the reversion experiments, cells were preincubated for 30 min with leptin (present in the apical or basal compartment). Then, leptin was removed and uptake of 0.1 mM Gln or 1 mM β-Ala was measured for 15 min in the absence of the hormone. For both amino acids, inhibition of leptin acting from either side was reverted (figure 3A and B)

Discussion

It has been demonstrated that the long leptin receptor isoform, Ob-Rb, is expressed at the basolateral membrane of Caco-2 cells [24]. Incubation of those cells with 200 nM leptin for 20 h resulted in a decreased on the export of triglicerydes in the basolateral medium and reduced the output of de novo-synthesized apolipoproteins [24]. In the present work we have demonstrated that basal leptin at lower concentration (8 nM) and shorter incubation periods (5 and 30 min) than those used by Stan *et al.*, is able to inhibit apical Gln and β-Ala uptake. The magnitude of inhibition is similar to that obtained when leptin acted on apical membrane [13], and lower than 40 %, which is the maximum percentage of inhibition on nutrients transport due to leptin [2, 12, 13, 17-19].

Apical leptin diminishes Gln uptake by decreasing the amount of the Gln transporters, ASCT2 and B^0AT1, in the apical membrane of the absorptive cells [12, 13]. In contrast, leptin inhibits β-Ala uptake by altering the activity of the H^+-dependent transporter PAT1, most probably through the inhibition of the NHE3 exchanger activity, via a PKA activation pathway, which would decrease the proton gradient required for PAT1 function [13]. Therefore, we may expect that the mechanism of action of leptin acting from the basal membrane here found would be the same. Interestingly, basal leptin inhibition of Gln uptake at 30 min was lower than the inhibition found when it acted from the apical side. On the other hand, leptin did not inhibit β-Ala uptake from the basal side after 5 min. Other authors have reported also in Caco-2 cells that apical leptin (100 nM) increased transport of dipeptides in 15 min, whereas basolateral leptin did not have any effect [5]. These results suggest that the effect of leptin acting from the basolateral membrane is slighter than from the apical membrane, most probably because, in comparison with the apical leptin receptors, the basolateral receptors, and

therefore the intracellular signalling cascade events are located further away from the apical membrane transporters. On the other hand, the basolateral membrane surface exposed to leptin is smaller than the brush border membrane surface.

Surprisingly, preincubation of the cells with basal leptin for 6 h did not increase the degree of inhibition (40 %) indicating that, even though leptin can decrease ASCT2 and B^0AT1 gen expression in rat intestine after pre-exposure of the intestinal lumen to leptin [12], ultimately, leptin would tightly control the amount of these two Gln transporters in the plasma membrane.

We also show that the inhibitory effect of the hormone acting from the apical or basolateral membrane was completely reverted in 15 min after the removal of the hormone from the corresponding compartment. These results are in line with our previous studies in which we demonstrated that leptin inhibition of galactose absorption is reversible in rat intestine *in vivo*, where leptin was perfused through the lumen [18] and also support the involvement of post-translational mechanisms on the regulation of the amino acid transporters by leptin.

That both apical and basal leptin can modulate nutrients absorption in a similar manner would indicate the important role of leptin in the processing of nutrients by the enterocytes. Apical leptin would act during postprandial state [7] whereas basal leptin could be reinforcing apical leptin effect, maybe mainly in obese individuals in which leptin levels are high [16].

In summary, we have demonstrated that leptin acting from the basal compartment decreases amino acids uptake in Caco-2 cells, and that this inhibitory effect is observed both after 5 min of exposure and after long preincubation periods. Moreover, apical and

basal leptin effect is reversible. Taken together, these results contribute to the vision of leptin as an important hormonal signal for the regulation of intestinal absorption of nutrients.

Acknowledgments

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Legends to figures

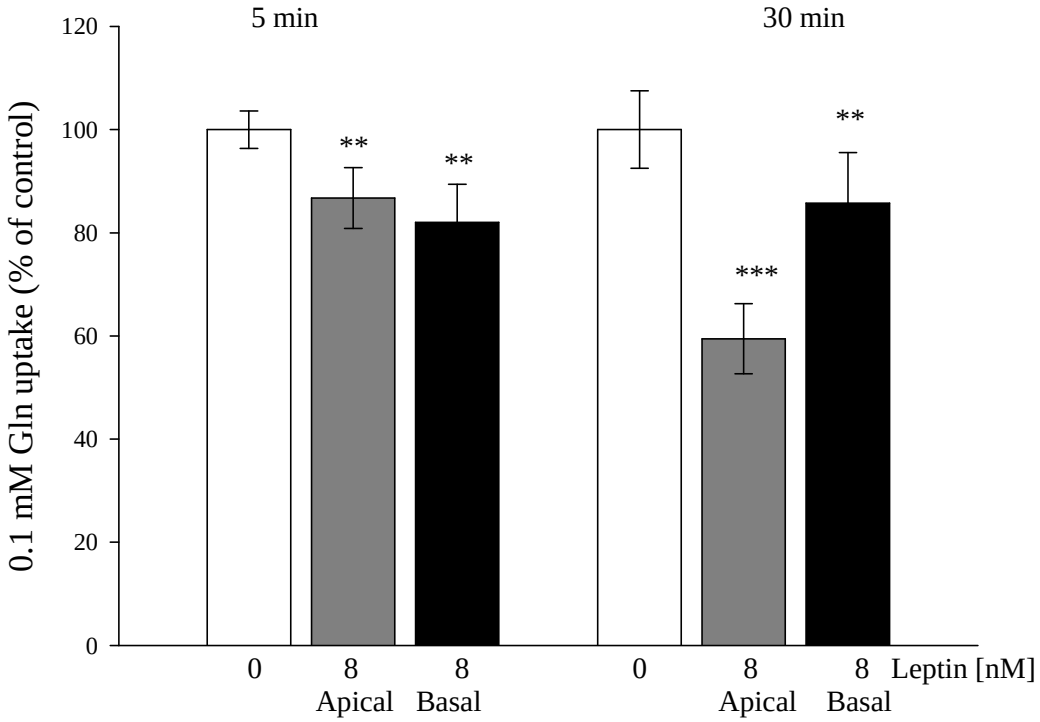
Fig. 1 Effect of apical and basal leptin on Gln and β-Ala uptake by Caco- 2 cells grown on filters. (a) Uptake of 0.1 mM Gln (pH 7.4) and (b) 1 mM β-Ala (pH 6) in Na^+ medium was measured in the absence and in the presence of apical or basal leptin (8 nM), after 5 and 30 min incubation. Data (n = 12–33) are expressed as % (mean ± SEM) of control value (0 leptin). **p < 0.01, *** p< 0.001. Control values for Gln uptake were 0.24 ± 0.009 and 4.15 ± 0.38 nmol mg^{-1} protein at 5 and 30 min respectively. Control values for β-Ala uptake were 0.14 ± 0.015 and 0.61 ± 0.05 nmol mg^{-1} protein at 5 and 30 min respectively.

Fig. 2 Effect of basal leptin preincubation on Gln and β-Ala uptake by Caco- 2 cells grown on filters. Uptake (5 min) of 0.1 mM Gln (pH 7.4) and 1 mM β-Ala (pH 6) in Na^+ medium was determined after 6 h preincubation of the cells with 8 nM leptin present in the basal compartment. Data (n = 18–20) are expressed as % (mean ± SEM) of control value (0 leptin). ***p < 0.001. Control values were 0.93 ± 0.08 and 0.24 ± 0.008 nmol mg^{-1} protein for Gln and β-Ala uptake respectively.

Fig. 3 Reversion of leptin effect on intestinal amino acid uptake by Caco-2 cells. (a) Uptake (15 min) of 0.1 mM Gln (pH 7.5) and 1 mM β-Ala (pH 6) in Na^+ medium was measured in the absence and in the presence of 8 nM apical leptin in Caco-2 cells grown on plates (n = 12-16). (b) Uptake of 0.1 mM Gln (pH 7.5) and 1 mM β-Ala (pH 6) in Na^+ medium was measured in the absence and in the presence of 8 nM basal leptin in Caco-2 cells grown on filters (n = 12). In the leptin group (grew bars) cells were preincubated for 15 min with the hormone before the 15 min uptake in its presence. In the reversion group (black bars) cells were preincubated for 30 min with the hormone before the 15 min uptake in its absence. Data are expressed as % (mean ± SEM) of control value (white bars). * p<0.05, **p < 0.01. (A) Control values for Gln and β-Ala uptake were 0.71 ± 0.014 and 0.37 ± 0.028 nmol mg^{-1} protein, respectively. (b) Control values for Gln and β-Ala uptake were 1.01 ± 0.6 and 0.48 ± 0.05 nmol mg^{-1}, protein respectively.

FIGURE 1

a


5 min
30 min
0.1 mM Gln uptake (% of control)
120
100
80
60
40
20
0
**
**
***
**
0
8
8
Apical
Basal
0
8
8
Leptin [nM]
Apical
Basal

b

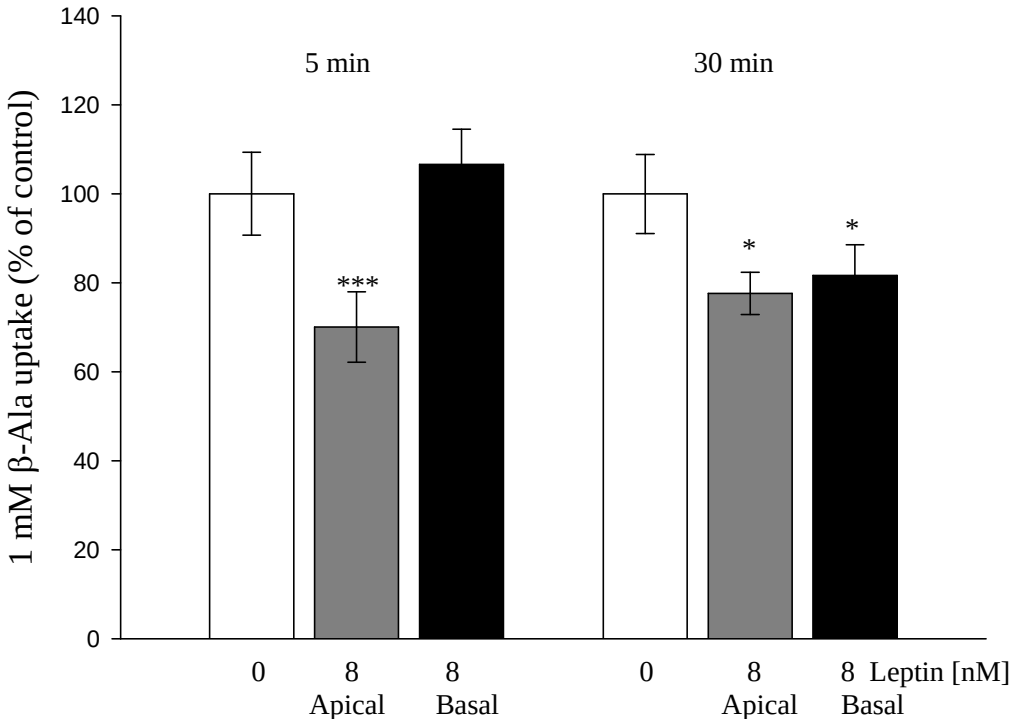

5 min
30 min
1 mM β-Ala uptake (% of control)
140
120
100
80
60
40
20
0
***
*
*
0
8
8
Apical
Basal
0
8
8
Leptin [nM]
Apical
Basal

FIGURE 2

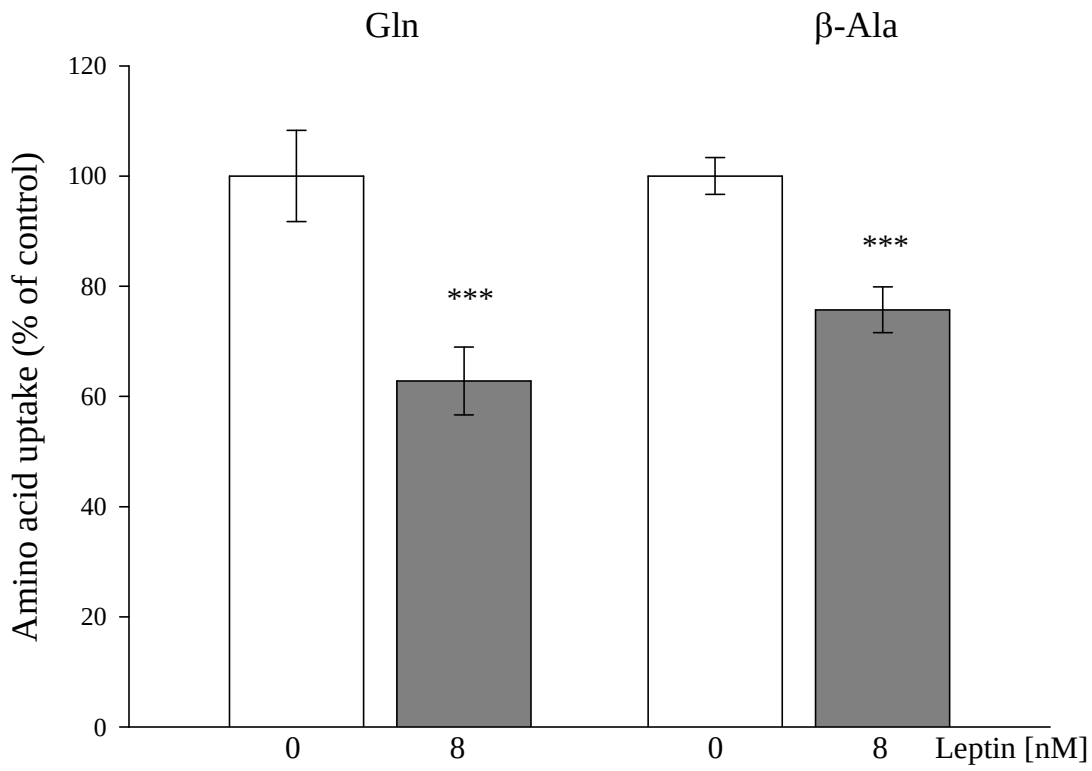
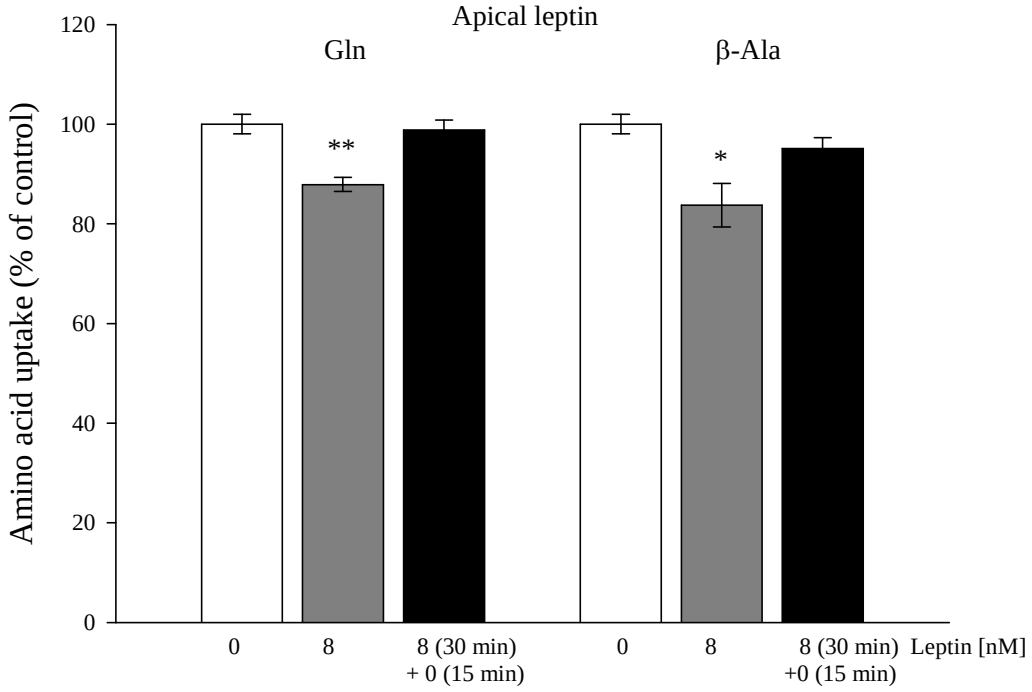
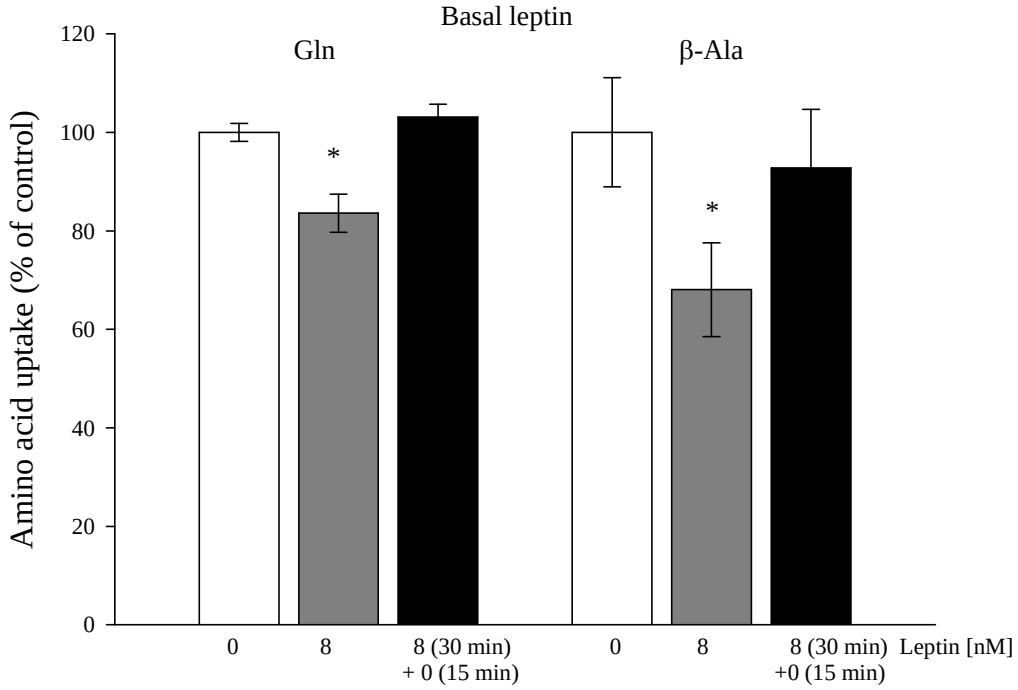

Gln
β-Ala
Amino acid uptake (% of control)
120
100
80
60
40
20
0
***
***
0
8
0
8
Leptin [nM]

FIGURE 3

a


Apical leptin
Gln
β-Ala
Amino acid uptake (% of control)
120
100
80
60
40
20
0
**
*
0
8
8 (30 min)
+ 0 (15 min)
0
8
8 (30 min)
+0 (15 min)
Leptin [nM]

b


Basal leptin
Gln
β-Ala
Amino acid uptake (% of control)
120
100
80
60
40
20
0
*
*
0
8
8 (30 min)
+ 0 (15 min)
0
8
8 (30 min)
+0 (15 min)
Leptin [nM]

Chapter 5:

Glutamate transport mechanisms in rat and human intestine: regulation by leptin

Glutamate transport mechanisms in rat and human intestine: regulation by leptin

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Aim Glutamate uptake by the intestine ismediated by i) a high affinity Na^+-independent transport mechanism, system $x^-_{c;}$ ii) a high affinity Na^+-dependent transport mechanism corresponding to the ubiquitous system $X^-_{A,G;}$ and iii) a low affinity Na^+-dependent transport mechanism, ASC-like system.We have previously demonstrated that apical leptin inhibits sugar and neutral amino acids uptake in rat and Caco-2 cells. In continuation with our work, the aim of the present work was to investigate the transport mechanisms involved in Glutamate uptake in these experimental models and evaluate the possible modulation by leptin.

Methods Glutamate transport in rat intestine and in Caco-2 cells was measured in the presence and in the absence of Na^+ at pH 6 and 7, and was determined by the radioactivity method in jejunal everted rings and Caco-2 cells and by measuring short-circuit current (Isc) in Ussing chambers (rat intestine). The effect of leptin was investigated applying the hormone to the apical medium.

Results Our results show that in rat intestine glutamate uptake is mediated by three different transport mechanisms: i) a high affinity Na^+-dependent mechanism, ii) a Na^+-independent, H^+-dependent mechanisms and iii) a low affinity Na^+-independent, H^+-dependent mechanisms, which was the only one observed in Ussing chambers. In Caco-2 cells, glutamate uptake is mediated by the system $X^-_{A,G,}$ and at acidic pH by the low affinity Na^{+-}dependent transport mechanism, ASC-like system. Leptin is able to modulate all this transport mechanisms by inhibiting (20 %) glutamate uptake by the Na^+-dependent systems and enhancing glutamate uptake (20-50 %) by Na^+-independent ones.

Conclusion In the present work we demonstrate the presence of different glutamate transport systems in rat intestine and Caco-2 cells. All are regulated by leptin which extend our previous findings and contribute to the vision of leptin as a major hormonal signal for the regulation of intestinal absorption of nutrients

Introduction

Glutamate (Glu) is one of the most abundant amino acid in the food that can be present in free form or as peptides and proteins component (Beyrehuther *et al*., 2007). Despite its abundance in food, glutamate concentration in blood is usually low (50 µmol/L) (Adibi *et al*., 1973) mainly because glutamate is extensively oxidized by the small intestine in order to supply the high energy demand of the intestinal epithelium that is in continuous renewal (Blachier *et al*., 2009). Glutamate is the most important intracellular amino acid in the organism reaching concentrations up to 20 mM and is a key regulator of transamination and deamination reactions and protein synthesis. Intracellular glutamate increases the cellular antioxidant defense by the generation of glutathione and participates in kidney gluconeogenesis and in liver urea synthesis (Blachier *et al*., 2009).

Glutamate uptake in intestine occurs by three different transport mechanisms: a high affinity Na^+ independent transport, also carrying cystine, ii) system $x^-_{c;}$ a high affinity Na^+ dependent transport corresponding to the ubiquitous system $X^-_{A,\ G;}$ which members EAAT1 and EAAT3 are expressed in the intestine and iii) a low affinity Na^+ dependent transport. This last component was proposed to be system B^0 (Schultz *et al*., 1970) but other authors (Munck *et al*., 1999) proposed that the low affinity transport system could be mediated by system ASC, a system known to interact with acidic amino acids at low pH (Christensen, 1984).

Leptin is a 167-amino acid peptide first described by Zhang et al in 1994which is implicated in food intake and energy expenditure. Nowadays leptin is known to be not only an adipostatic signal involved in the control of body weight and adiposity but also a multifunctional hormone that can be synthetised by different peripheral tissues such as stomach, salivary glands, placenta and kidney where leptin receptors are also expressed (De Matteis *et al.,* 1998, Barrenetxe *et al.*, 2002, Margetic *et al.,* 2002, Gertler *et al.*, 2009). Previous studies from our laboratory demonstrated, in rat and in Caco-2 cells a short-term down-regulation by luminal leptin of two of the Na^+-dependent transporters involved in glutamine absorption: ASCT2 and B^0AT1 (Ducroc *et al.* 2010; Fanjul *et al*., 2012). In Caco-2 cells leptin also decreased β-Ala absorption. Inthis case, the inhibition was due to a decrease in PAT-1 activity without change in expression in the brush-

border membrane. The hormone was able to indirectly down-regulate the activity of PAT-1 via leptin inhibition of the Na^+/H^+ NHE3 exchanger present in the brush border membrane of the enterocytes (Fanjul *et al*., 2012). In this line, other authors have reported in Caco-2 cells monolayer grown and differentiated on permeable supports that luminal leptin, after 30 min incubation, increased dipeptide uptake mediated by the H^+-dependent transporter PepT1 and that this effect was accompanied by the translocation of the transporter from the intracellular pool to the plasma membrane (Buyse *et al*. 2001).

In this study, we analyse the transport mechanisms of glutamate uptake and its regulation by leptin in isolated rat intestine and in a model of human intestinal cells, the Caco-2 cell line.

Material and Methods

Animals

Male Wistar rats (220–260 g) were obtained from“Janvier, Le Genest St Isle, France”and the “Applied Pharmacology Research Center (CIFA)” of the University of Navarra, Pamplona, Spain. They were caged under standard laboratory conditions (12-h: 12-h light/dark cycle at a temperature of 21–23°C) with free access to tap water and standard chow provided *ad libitum*. Before the beginning of the experiment, rats were fasted for 16–18 h. 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 2, 2004, agreement no. B75-18-02; decision nº. 05/12 established on July 12, 2005 by Prefecture of Police de Paris, France) and from the Animal Research Ethic Committee of the University of Navarra, with the nº. 064-06.

Ussing chambers

Animals were anesthetized by intraperitoneal pentobarbital injection, and the small intestine (jejunum-ileum) was dissected out and rinsed in cold saline solution before sacrifice. The mesenteric border was carefully discarded using forceps and the small intestine opened along this border and gently rinsed in Krebs-Ringer bicarbonate

solution (KRB) without glucose. Four adjacent proximal samples were mounted in modified Ussing chambers (Physiologic

Instruments, 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. KRB solution had the following composition (in mM): NaC1 115.4, KCl5, $MgCl_2$1.2, $NaH_2PO_4$0.6, $NaHCO_3$25, $CaCl_2$1.2, and glucose 10.For sodium-free experiment, the medium had the following composition (in mM): Choline Cl 110, KCl 5, $CaCl_2$ 3.6, $MgCl_2$ 1.2, $KHCO_3$ 26, and glucose (serosal) or mannitol (mucosal) 10. The pH was adjusted to 5.0, 6.0 or 7.0 only with HCl. Each reservoir was gassed with 95% O_2-5% CO_2 and kept at constant temperature of 37°C. Electrogenic ion transport was monitored continuously as short-circuit current (*I*sc) 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 *I*sc (μA/cm^2) or as the difference (Δ*I*sc) between the peak *I*sc after L-glutamate (Sigma-Aldrich) challenge and the basal *I*sc measured just before the addition of the amino acid. Leptin (recombinant rat peptide. (Preprotech EC, London, UK) was added in the mucosal bath 2min before glutamate challenge.

Everted intestinal ring uptake assays

Rats were anesthetized by a 0.5 mL intraperitoneal injection of a mixture (4:1) of ketamine chlorohydrate (IMALGENE 500®; Merial S.A., Barcelona, Spain) and medetomidinechlorohydrate (DOMTOR®; Pfizer Orion, Espoo, Finland), at a dose of 0.25 ml per 100 g body wt. Uptake of 6 mM glutamate by everted jejunal rings was determined as previously described (21). Briefly, rats were anesthetized, and a segment of jejunum (20–25 cm) was quickly excised, rinsed with ice cold saline solution (NaCl 0.9%), everted, and cut into ~30 mg pieces. Groups of six rings were incubated for 15 min at 37°C under continuous shaking and gassed with O_2, in Krebs-Ringer-Tris solution with or without Na^+-containing 6 mM glutamate and 0.1 μCi/ml L-[3H(U)]-glutamate (30mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO), in the absence (control) or in the presence of 0.2 nM recombinant rat leptin. 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 amino acid nanomol glutamate per gram of wet weight (nmol/g wet weight).

Cell culture

The Caco-2 cell line (PD7 clone) was kindly provided by Dr. Edith Brot-Laroche (Inserm, Paris, France).Cells were maintained in a humidified atmosphere of 5% CO2-95% at 37 °C and were grown in Dulbecco‘s Modified Eagles medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin (1000 U .mL^{-1}), 1% streptomycin (1000 g.mL^{-1}) and 1% amphoterycin (250 U. mL^{-1}). Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin-EDTA and subcultured on 25 or 75 cm^2 plastic flasks at a density of 200,000 cells/cm^2. For transport studies, the cells (passage 50-70) were seeded at a 60,000cells/cm^2density in 24-well culture plates.Culture medium was replaced every 2 days. Cell confluence was confirmed by microscopic observance. Experiments were performed 17–21 days post-seeding.

Uptake experiments

Glu (6 mM) and traces of L-[3H(U)]-glutamate (0.25 Ci/mL) were diluted in Krebs modified buffer (also referred as Na+ medium) (in mM): KCl5.4, $CaCl_2$2.8, $MgSO_4$1, $NaH_2PO_4$0.3, NaCl137, $KH_2PO_4$0.3, glucose10 and HEPES/Tris (pH 7.5) or Mes/Tris10 (pH 6). Cells were gently washed with substrate-free medium and the experiment was initiated by adding 0.5 mL uptake buffer in the absence or presence of 0.2nM leptin. After an incubation period of 5 min at 37 °C, uptake was stopped with ice-cold free-substrate buffer. Cells were washed again twice with ice-cold buffer to eliminate non-specific radioactivity fixation and were finally solubilized in 500 µL 1% Triton X-100 in 0.1 N NaOH. Samples (100 µL) were taken to measure radioactivity by liquid scintillation counting. Protein concentration was determined by Bradford method (Bio-Rad Protein Assay; Bio-Rad laboratories, Hercules, CA, USA). Results are expressed as nmol Glu per mg of protein.

Statistical analysis

All results were expressed as means ±SEM. Student’s *t*-test or one-way ANOVA followed by Tukey-Kramer multiple comparison posteriori test were performed using Graph- Pad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA) or SPSS. v.15. The level of significance was set at $p < 0.05$.

Results

Glutamate transport in Ussing chambers

We first studied in Ussing chambers glutamate transport at different concentration from 0.1 to 60 mM. Transepithelial transport was followed as ionic-associated electrogenic short-circuit current (Isc). In the presence of Na^+, glutamate challenge had no effect on basal Isc neither at pH 7 or 6. In contrast, in Na^+-free solution, glutamate induced a concentration-dependent increase in Isc at pH 6. (Fig.1A). Glutamate induced-Isc increased significantly as the pH decreased from 7 to 5 (Fig.1B) the current induced by glutamate was due to H^+ movement across the epithelium.

Taking into account these preliminary observations, we then investigated leptin effect on 6 mM glutamate induced-Isc at pH 6 in the absence of Na^+. As shown in figure 2A leptin, added to the mucosal reservoir 2 min before the amino acid, stimulated by ~50 % glutamate -induced Isc in a biphasic manner. This stimulating effect was dose-dependent being 10^{-10} M (0.1 nM) the most effective concentration. Tissue pre-incubation with a leptin receptor antagonist L39A/D40A (Gertler, 2009) blunted leptin effect on Glu induce-Isc (Fig. 2B), demonstrating that the effect was receptor specific.

All these results show the presence of an electrogenic H^+-dependent Glu transporter in rat intestine whose activity is increased by leptin through a leptin-receptor activation.

Glutamate uptake in evertedjejunal rings

We then investigated leptin effect of glutamate transport in everted intestinal rings using radiolabeled substrate which allows to measure direct uptake. We first characterized glutamate transport mechanisms at pH 7.4 and 6 in the presence and in the absence of Na^+. Figure 3A shows that after 5 min incubation, glutamate uptake was significantly decreased in the absence of Na^+ at pH 7.4 (1.74 ± 0.06 *vs.*0.87± 0.03 nmol/mg of wet weight) while at pH 6, the uptake was not modified (1.76 ± 0.05*vs.*1.57 ± 0.07 nmol/mg of wet weight). On the other hand, in the absence of sodium, glutamate uptake was significantly enhanced at by acidification (0.87 ± 0.03 at pH 7.4 *vs.* 1.57 ± 0.07 at pH 6 $nmol.mg^{-1}.min^{-1}$). Altogether, these data suggest the presence of two transport mechanisms in the rat intestine, one H^+-dependent and another Na^+-dependent.

The effect of leptin on glutamate uptake was further measured after 5 min incubation in two different conditions: Na^+ medium pH 7 and Na^+ free medium at pH 6. Leptin

significantly decreased (~20%) glutamate uptake in Na^+ medium at pH 7 (1.73 *vs.* 1.38 $nmol.mg^{-1}.min^{-1}$) whereas the hormone significantly increased (~20%) glutamate uptake in Na^+-free medium at pH 6 (1.55 *vs.* 1.86 $nmol.mg^{-1}.min^{-1}$) indicating that the two different transport mechanisms present in the intestine are differently regulated by leptin (Fig.3B).

Glutamate uptake in Caco-2 cells

Similar experiments were also carried out in the human intestinal cell line Caco-2. We started with the characterization of the glutamate transport mechanisms. As shown in figure 4, uptake of 0.1 mM glutamate (5 min) was both Na^+ and pH sensitive and that the uptake in the absence of sodium was negligible (0.04 at pH 7 and 0.041 nmol/mg prot at pH 6) in confirmation of the study of Mordrelle *et al*., (2000), showing that glutamate uptake in Caco-2 cells is strictly Na^+ -dependent. In the presence of Na^+, glutamate uptake was pH-dependent, the uptake at pH 6 being significantly higher than the one at pH 7 (0.18 *vs.* 0.14 nmol/ mg, respectively). This could be due to the capacity of ASCT2 to transport glutamate under acidic conditions (Utsunomiya-Tete *et al*., 1996). Accordingly, we further investigated the possible role of ASCT2 in glutamate uptake at pH 6. Figure 4 shows that when Alanine, a specific substrate of ASCT2, was present in the uptake medium (0.1 mM glutamate) at the saturable concentration of 10 mM, only glutamate uptake was significantly decreased compared with control conditions (0.18 vs. 0.14 nmol/mg) and reached uptake levels obtained at pH 7Altogether, these data (Fig. 4) suggest implication of ASCT2 in this process and show that glutamate uptake in Caco-2 cells is both Na^+- and pH-dependent. This pH dependence could be due to ASCT2 implication in glutamate uptake at acidic pH, with the EAAT1 transporter being possibly implicated in glutamate uptake at pH 7.

Leptin effect on glutamate uptake in Caco-2 cells

In order to determine if leptin modulated both transporters activity, the effect of leptin on glutamate uptake in Caco-2 cells at both pH 6 and pH 7, was analyzed. Leptin (0.2 nM) significantly decreased glutamate uptake at pH 7 and pH 6 by a ~30% and ~15% decrease, respectively (Fig.5A). We further investigated leptin effect on glutamate uptake at pH 6 in the presence of saturable concentration of Alanine (10 mM) in order to block ASCT2 transporter activity. As shown in figure 5B, 0.2 nM leptin also

decreased glutamate uptake by ~35%. These results indicated that both EAAT1 and ASCT2 were inhibited by the hormone (Fig.5B).

Discussion

It has been described that transport of glutamate in the small intestine is mediated by i) high affinity Na^+-dependent transporters EAAT1 and EAAT3 which seem to transport protons as well (Kanai & Hediger, 2004); ii) high affinity Na^+-independent exchanger 4F2hc/xCT (Bannai, 1986), and at pH 6 by iii) low affinity Na^+ -dependent transporter ASCT2 (Munck *et al*., 1999; Christensen, 1984). Here, we demonstrated functionally the presence of these transporters in rat small intestine and Caco-2 cells and their regulation by leptin

Our studies on rat jejunum in Ussing chambers established the existence of a Na^+-independent, H^+-dependent glutamate transporter.Since the characteristics of glutamate transport observed did not correspond to any of the transporters described above, we further examined direct glutamate uptake by using reverted intestinal ringsWe found that glutamate uptake at pH 7 decreased in the absence of $Na^+$and was not modified by the pH reduction. Since Auger et al. proposed that glutamate transport does not require binding of extracellular H^+(Auger *et al*., 2000), our data would indicate that glutamate uptake in rat intestine is mediated by the Na^+ dependent EAAT transport family (EAAT1 and EAAT3). Nevertheless, in Na^+-free medium, glutamate transport increases at pH 6. This transport activity could correspond to 4F2hc/xCT because this heterodimeric transporter is located in the intestinal brush-border membrane (Burdo *et al*., 2006) and its activity is increased at acidic pH (Bassi *et al*., 2001). Because 4F2hc/xCT is electroneutral (Bassi*et al*., 2001), it cannot be the transporter described in the Ussing chamber studies.

In Caco-2 cells, Mordrelle *et al*. reported that glutamate uptake is only Na^+ dependent through EAAT1 and EAAT3 (Mordrelle *et al*., 2000). This is in agreement with our results since in the absence of Na+, glutamate uptake was negligible therefore. Nevertheless, in the presence of Na^+ we found that glutamate uptake increased at pH 6, which could be explained by the activity of ASCT2 (Utsunomiya-Tete *et al*., 1996).This was confirmed by competitive experiments in which glutamate uptake at pH 6 in the presence of Ala was the same as the uptake measured at pH 7, which is mediated by

EAATs transporters. Taken together, these data suggest the implication of ASCT2 in glutamate uptake at acidic pH in Caco-2 cells.

Although leptin is secreted mainly by the adipose tissue, there is a significant secretion of leptin by gastric mucosa which contains leptin secreting endocrine and exocrine cells (Bado *et al*., 1998; Cinti *et al*., 2000; Camimissoto *et al*., 2005). Once secreted, the hormone can reach the gastric and duodenal juice (Guilmeau *et al*. 2004) and is able to bind to its specific receptors in the enterocytes (Barrenetxe *et al.* 2002). Nowadays, leptin action in the control of nutrient absorption is well documented (Buyse *et al*. 2002; Barrenetxe *et al*., 2001; Iñigo *et al*., 2004, 2007; Ducroc *et al* 2005; Ducroc *et al* 2010).

In rat intestine leptin inhibits in a short-term manner glutamate uptake mediated by the EAATs. In this regard, we have previously shown that leptin also inhibits two of the Na^+-dependent transporters involved in glutamine absorption, ASCT2 and B^0AT1, by decreasing their expression in the apical membrane of rat enterocytes (Ducroc *et al.* 2010). These data suggest a post-transcriptional regulation of these glutamate transporters by leptin. In contrast, in the absence of Na^+ at pH 6, leptin increases glutamate uptake. This enhancing effect is also found in Ussing chambers where dose-response can be studied on a large scale. We found that leptin produces a biphasic stimulatory effect. This nonmonotonic effect of leptin is actually documented (Vandenberg *et al*., 2012).

These data are in accordance with observations from other authors that described stimulatory effect of leptin on peptide uptake through regulation of the expression of the H^+-dependent transporter PepT1, in the apical membrane of rat enterocytes (Buyse *et al*., 2001). By contrast, in Caco-2 cells, leptin reduced glutamate uptake mediated by the two Na^+-dependent transporters EAATs and ASCT2. Like in rat intestine, we recently demonstrated in Caco-2 cells that leptin inhibits the Na^+-dependent ASCT2 and B^0AT1 transporters activity by decreasing the proteins amount in the apical membrane of the cells (Fanjul *et al*., 2012).

Altogether these data indicate that in rat intestine leptin would decrease Na^+-dependent amino acid transport, and on the contrary would increase Na^+-independent transport.

In summary, in rat intestine glutamate transport can be mediated by a Na^+-dependent and Na^+-independent, H^+-dependent system, whereas in Caco-2 cells the amino acid uptake is strictly Na^+-dependent. In both experimental models, leptin is able to modulate glutamate uptake but further investigations are needed to identify the intracellular mechanisms responsible for this regulation. Overall, these results contribute to the view of leptin as a key hormone in the control of nutrient absorption in the small intestine.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Legends to figures

Figure 1. Glutamate -induced short-circuit current (Isc) studied in Ussing chambers. (A) glutamate -induced Isc in a dose-dependent manner. Experiments were performed in the presence orthe absence of Na^+. (B) Proton-dependence of 6 mM glutamate -induced Isc in Na^+ free medium. The values are represented as the mean ± SEM of 3-5 non cumulative values from individual experiments.

Figure 2. Leptin effect on glutamate -induced Isc in Ussing chambers. (A) Dose-dependent effect of mucosal leptin on glutamate -induced Isc. Leptin was added in the mucosal bath 2 min before tissue was challenged with 6 mM Glu. Each point represents the mean ± SEM of 4-6 non-cumulative values from individual experiments. ***** p<0,05.(B) Leptin action is receptor specific: Effect of 10^{-13} M leptin was blunted by the leptin antagonist L39A/D40A. Leptin antagonist was added to the mucosal bath 5 min before the addition of leptin and 2 min later, tissue was challenged by6 mM glutamatein the mucosal side. The values are represented as the mean ± SEM of 2-9 non-cumulative values from separate experiments.

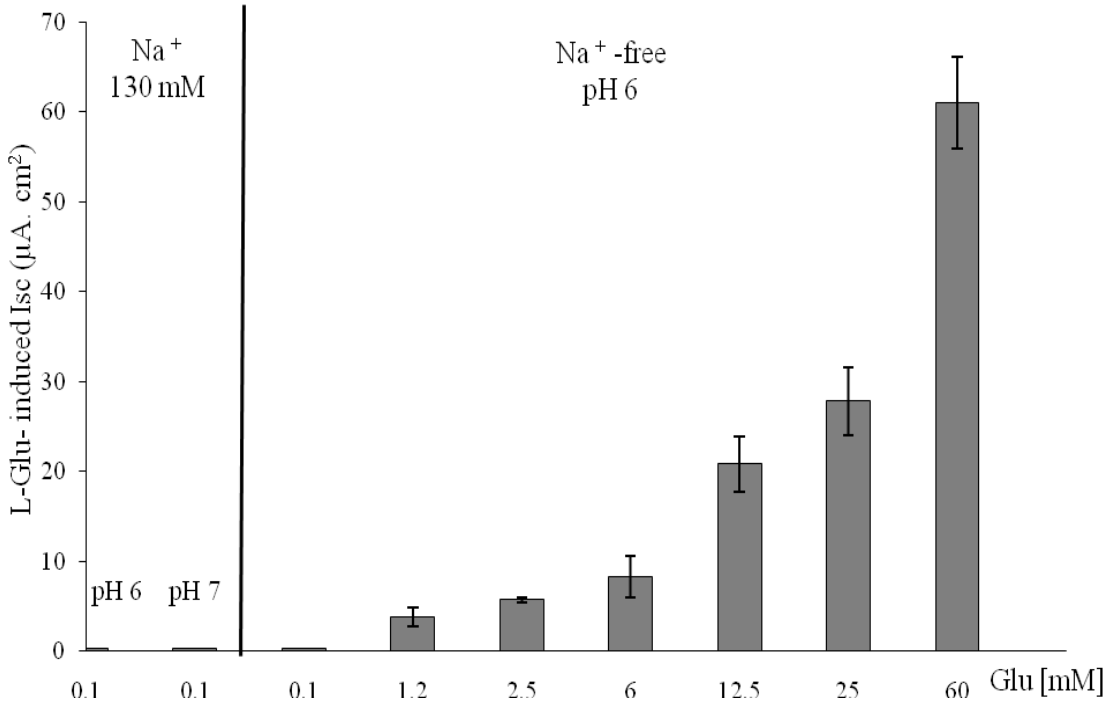
Figure 3. Glu uptake by everted intestinal rings. Amino acid uptake was measured during 5 min incubation. (A) Na^+ and pH dependence of 6 mM glutamate uptake. ***p< 0.001(Na^+ medium *vs*. Na^+ free medium, at pH 7);### p< 0.001 (Na^+ free pH 7.4 *vs* Na^+ free pH 6); n=18–37. (B) Leptin effect on 6 mM glutamate uptake in the presence of Na^+ at pH 7 and in the absence of Na^+ at pH 6. *** p< 0.001, control (0 nM) *vs.* leptin (0.2 nM) at pH 7.4. ** p<0.01, control (0 nM) 6 *vs.* leptin (8 nM) at pH 6. The results are expressed as means ±SEM; n= 17-18.

Figure 4. Glu uptake by Caco-2 cells. Uptake of 0.1 mM glutamate uptake (5 min) was measured in the presence and in the absence of Na^+ at pH 7.4 and pH 6. In Na^+ medium, Glu uptake was also measured in the presence of 10 mM Ala at both pHs. ### p< 0.001(presence*vs*. absence of Na^+); $$ p< 0.01(Na^+ pH 7.4 *vs*. Na^+ pH 6). ** p < 0.01 (Na^+*vs*. Na^+ + 10 mMAla at pH 6). The results are expressed as means ±SEM; n= 12-24.

Figure 5. Leptin effect on Glu uptake by Caco-2 cells. (A) Uptake of 0.1 mM Glu in Na^+ medium was measured for 5 min in the presence and in the absence of leptin at pH 7 and pH 6. *** p< 0.001, control (0 nM) *vs.* leptin (0.2 nM), at pH 7. ** p< 0.01, control (0 nM) *vs*. leptin (8nM) at pH 6. ## p< 0.01 (pH 7 vs. pH 6). (B) Uptake of 0.1 mMglutamate was measured in Na^+medium at pH 6 in the presence of 10 mM Alanine without and with leptin. *** p< 0.001, control (0 nM) *vs.* leptin (0.2 nM). The results are expressed as means ± SEM; n= 24-40.

FIGURE 1

A


Na+ 130 mM
Na+ -free pH 6
L-Glu- induced Isc (µA. cm²)
70
60
50
40
30
20
10
0
pH 6
pH 7
0.1
0.1
0.1
1.2
2.5
6
12.5
25
60
Glu [mM]

B

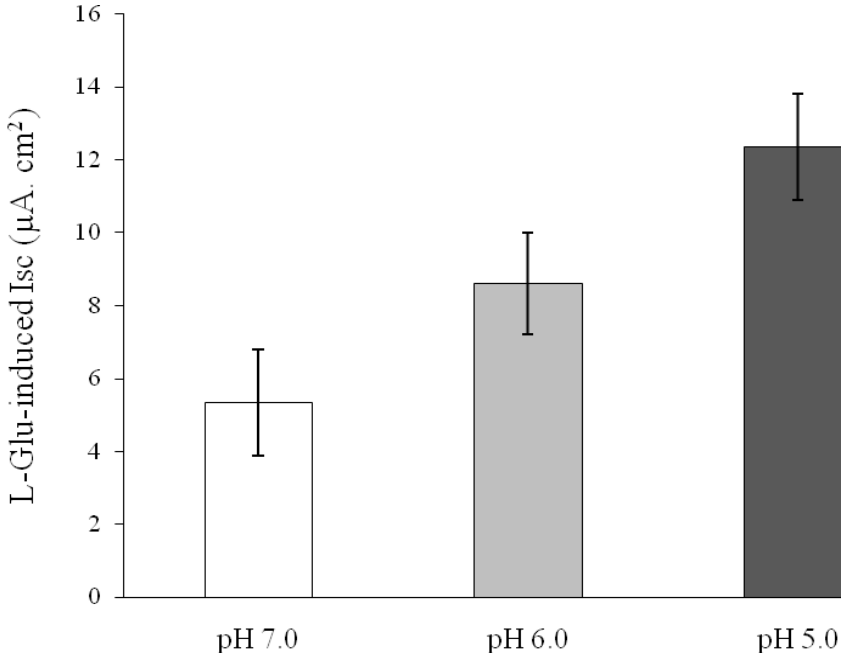
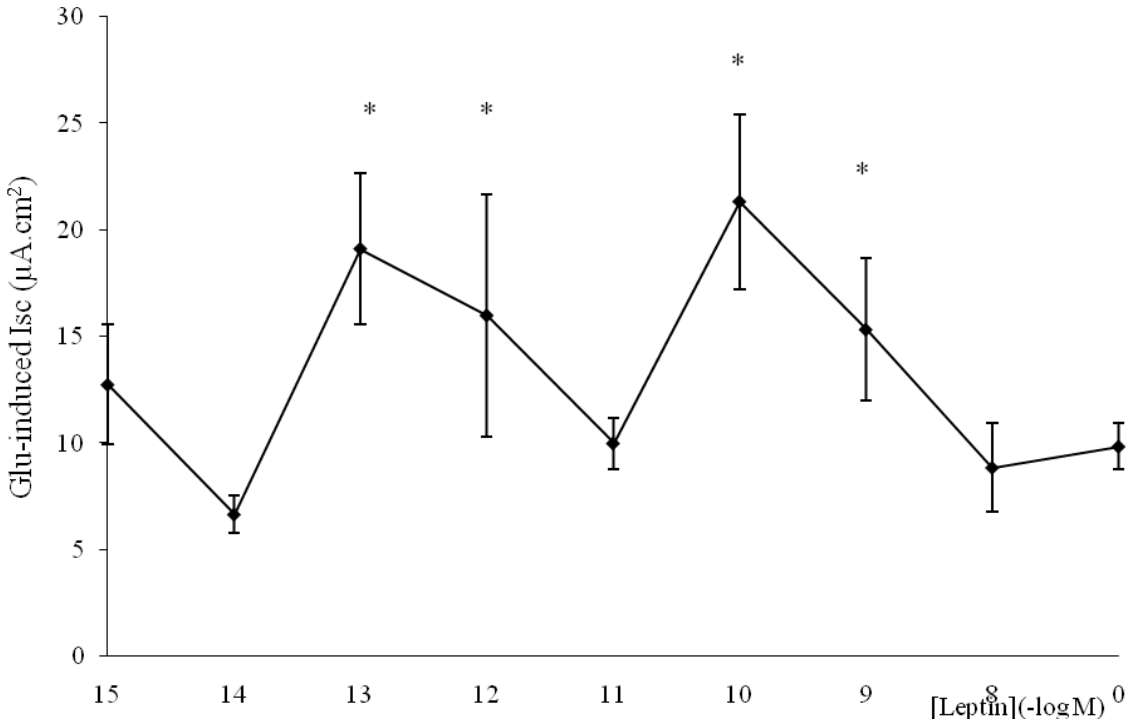

L-Glu-induced Isc (µA. cm²)
16
14
12
10
8
6
4
2
0
pH 7.0
pH 6.0
pH 5.0

FIGURE 2

A


Glu-induced Isc (µA.cm²)
30
25
20
15
10
5
0
*
*
*
*
15
14
13
12
11
10
9
8
0
[Leptin](-logM)

B

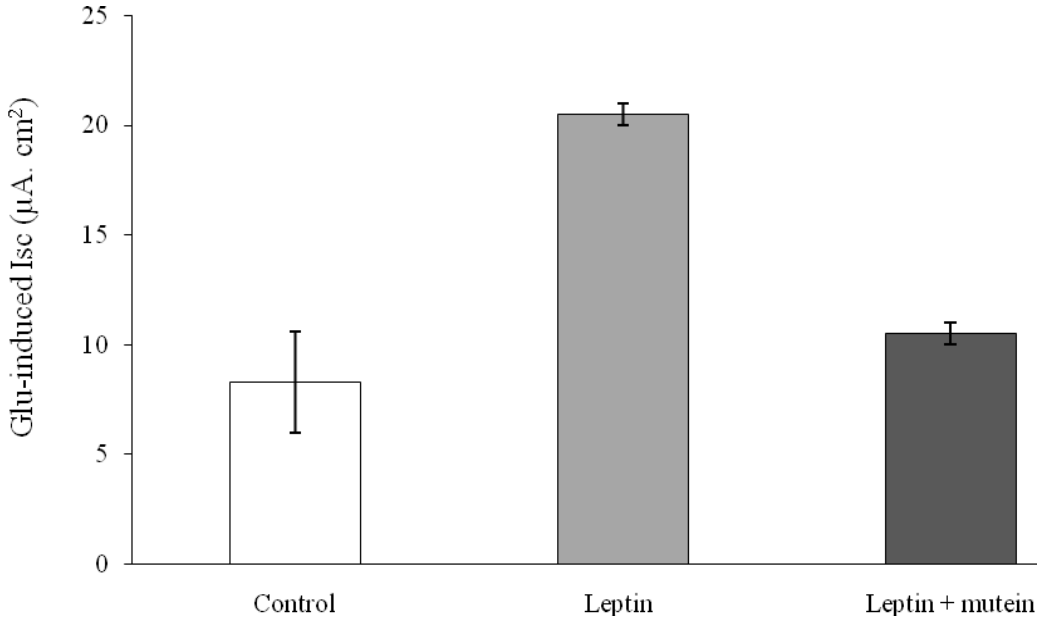
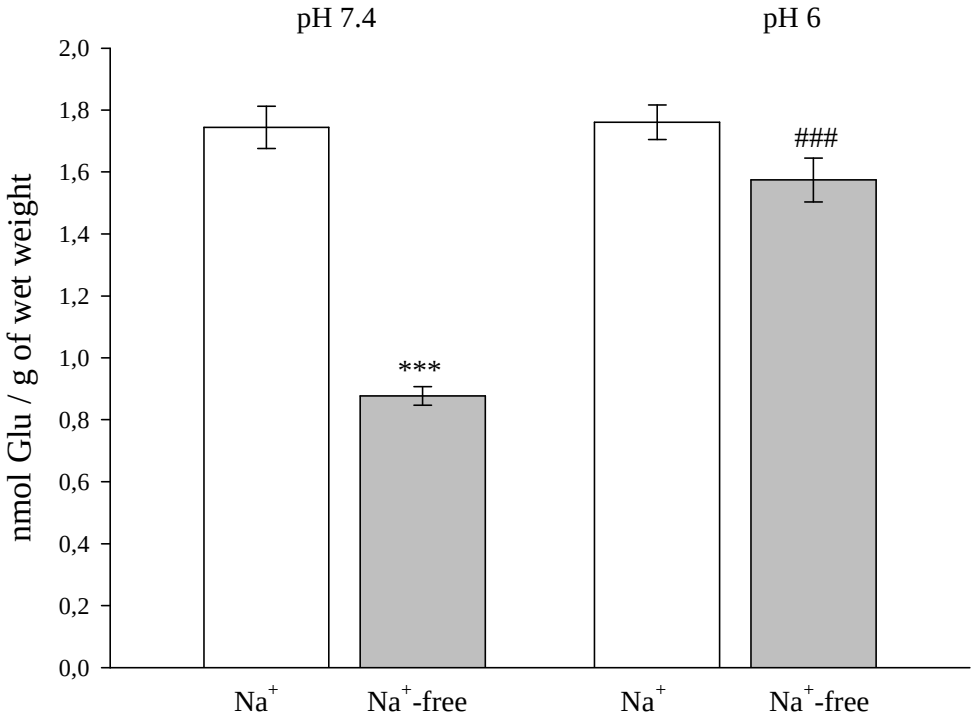

Glu-induced Isc (µA. cm²)
25
20
15
10
5
0
Control
Leptin
Leptin + mutein

FIGURE 3

A


pH 7.4
pH 6
nmol Glu / g of wet weight
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
***
###
Na+
Na+-free
Na+
Na+-free

B

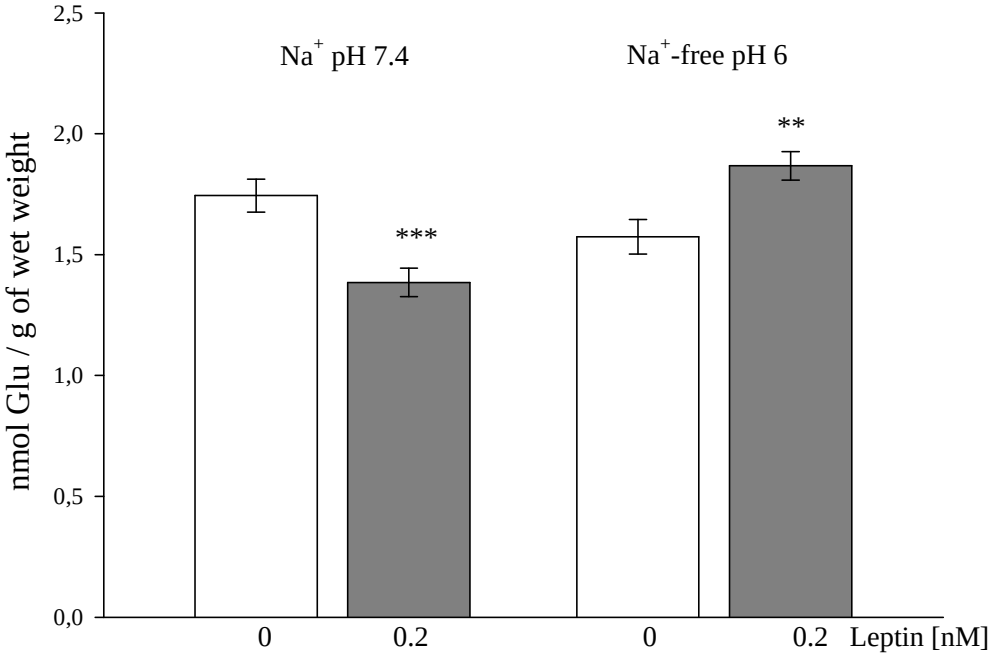

Na+ pH 7.4
Na+-free pH 6
nmol Glu / g of wet weight
2,5
2,0
1,5
1,0
0,5
0,0
***
**
0
0.2
0
0.2
Leptin [nM]

FIGURE 4

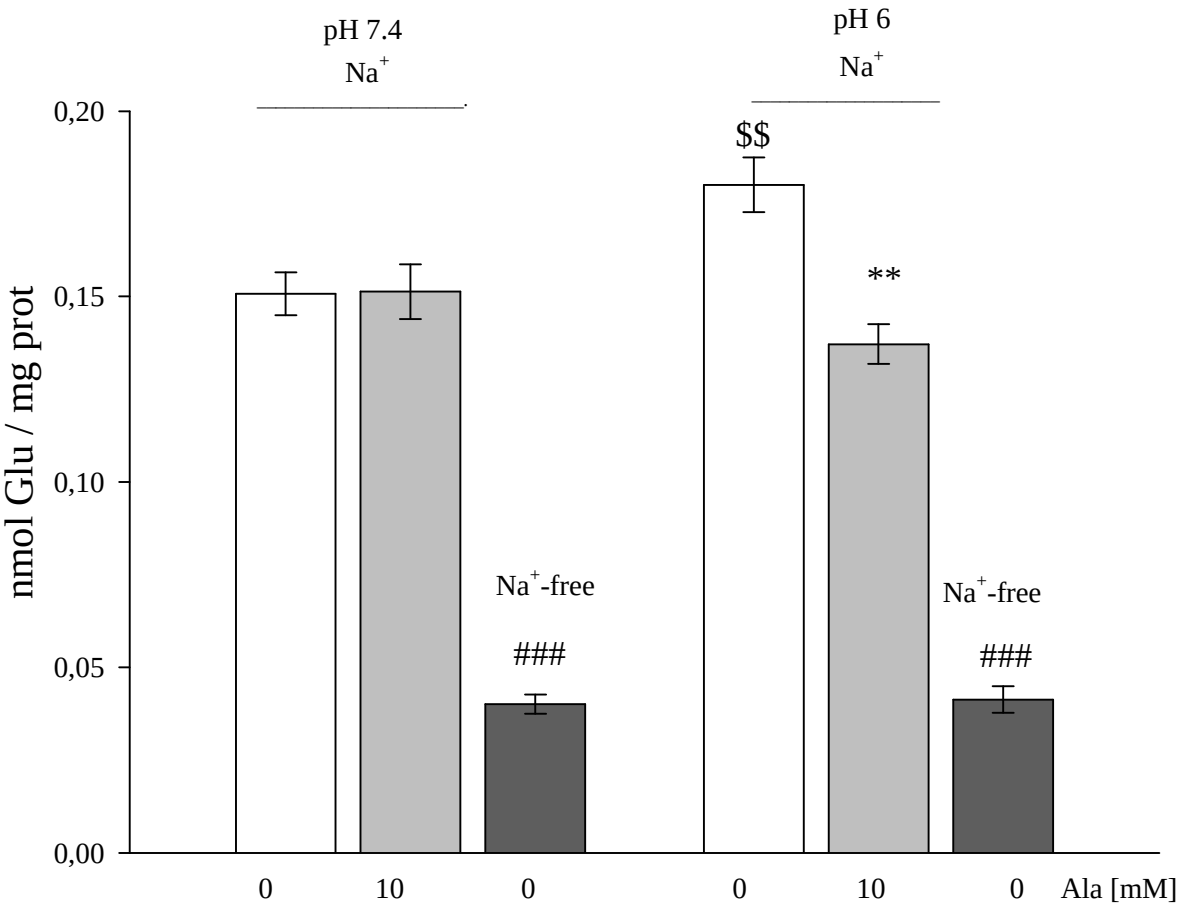
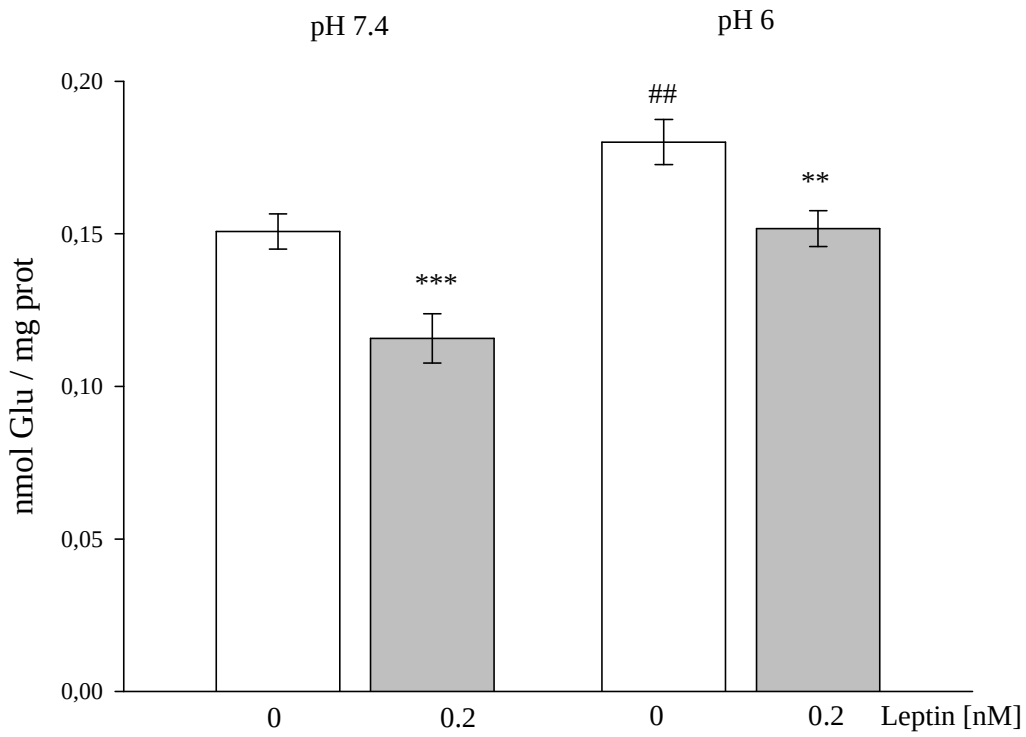
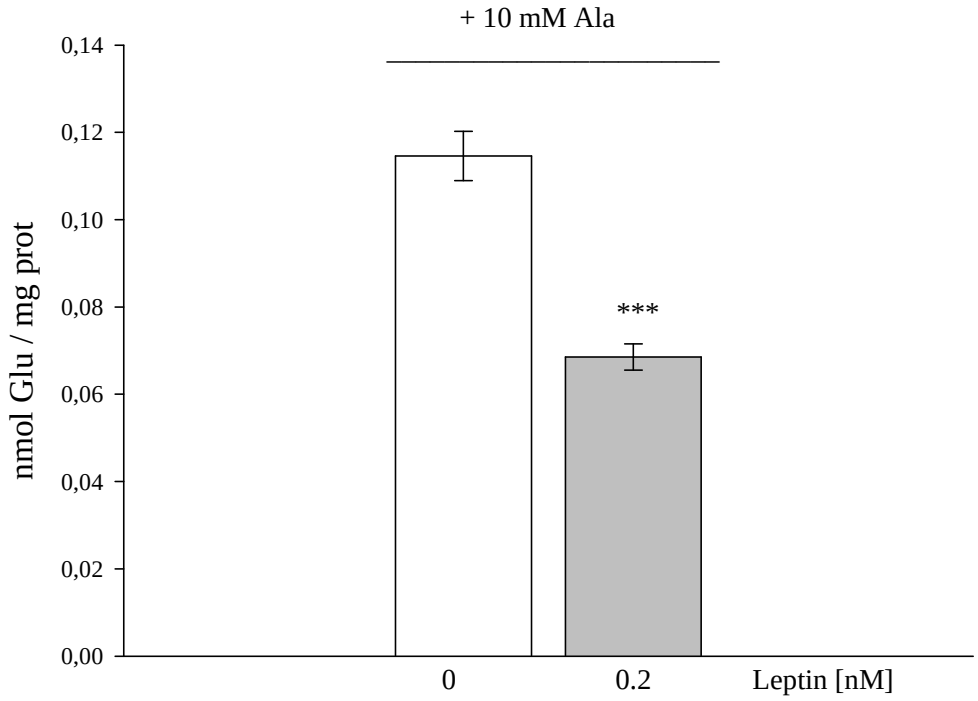

pH 7.4
Na+
pH 6
Na+
$$
**
Na+-free
###
Na+-free
###
nmol Glu / mg prot
0,20
0,15
0,10
0,05
0,00
0
10
0
0
10
0
Ala [mM]

FIGURE 5

A


pH 7.4
pH 6
0,20
0,15
0,10
0,05
0,00
nmol Glu / mg prot
##
**
***
0
0.2
0
0.2
Leptin [nM]

B


+ 10 mM Ala
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
nmol Glu / mg prot
***
0
0.2
Leptin [nM]

General discussion

Although all the results obtained in the present work have been discussed in detail in each chapter, a general discussion is here presented.

Hormone regulation of digestive secretions and gastrointestinal tract motility is well established, nevertheless, little is known about endocrine regulation of nutrients intestinal absorption and information on this regard is not included in the text books yet. For example, serotonin and CCK inhibit sugar absorption (Arruebo *et al*., 1989, Hirsh *et al*., 1996; Barber *et al*., 1997) whereas glucagon-37 shows a stimulatory effect (Stümpel *et al*., 1998). Serotonin also decreases leucine absorption (Salvador *et al*., 1996).

Shortly after leptin identification, we demonstrated that leptin inhibits sugar absorption in rat intestine *in vitro* by short-term regulation of the Na^+/glucose cotransporter SGLT1 (Lostao *et al.* 1998). In the same year, another group reported that the stomach chief cells secrete leptin into the gastric lumen after a meal (Bado *et al.* 1998). We later described that leptin receptors are expressed in both apical and basolateral membrane of human and murine enterocytes (Barrenetxe *et al.* 2002). This study was followed by the demonstration that the chief cells also secrete the leptin soluble receptor (Cammisotto *et al.* 2006), and that leptin remains stable in the gastric juice because the binding to this receptor protects it from the acidic pH and the proteolytic activity of the stomach, and favours its arrival to the small intestine (Guilmeau *et al.* 2004). All these data supported our initial discovery about the role of the luminal leptin as regulator of intestinal sugar absorption, and were followed by further studies on leptin modulation of sugars transport (Barrenetxe *et al.* 2001, 2004, Iñigo *et al.* 2004, 2007, Ducroc *et al.* 2005, Sakar *et al.* 2009).

In the present work, we decided to extend our studies and investigate the possible effect of leptin on amino acids intestinal absorption in rat and in Caco-2 cells, using *in vivo* and *in vitro* techniques. Taken together, the observations from these studies show that leptin, acting from the apical membrane, is able to modulate the uptake of amino acids in a short-term manner, and that this effect is rapidly reverted once the hormone is removed from the uptake medium. Moreover, the effect is never higher than ~ 40 % and in Caco-2 cells is also observed when the hormone is acting from the basolateral membrane

.In the small intestine, the amino acids can be transported by Na^+-dependent and Na^+-independent, H^+-dependent transport mechanisms. Our data demonstrate that leptin inhibits glutamine, phenylalanine and glutamate transport, which mainly occurs by Na^+-dependent mechanisms. On the contrary, the hormone enhances glutamate uptake when the transporter involved is Na^+-independent, most probably the heterodymeric transporter 4f2hc/xCT which has been described to be H^+-dependent (Bassi *et al*., 2001). In line with these results, other authors have reported that leptin increases peptides and butyrate absorption by enhancing the expression in the apical membrane of the H^+-dependent, Na^+-independent transporters PepT1 and CD147/MCT-1 respectively (Buyse *et al*., 2001; 2002). Interestingly, we also have demonstrated that leptin inhibits proline and β-alanine transport through PAT-1, another H^+-dependent, Na^+-independent transporter, without modifying its expression in the brush border membrane. In this case, the mechanism implicated would be indirect, most probably by alteration of the H^+ gradient through modification of the Na^+/H^+ exchanger activity, as discussed in chapter 3. For PepT1 and the CD147/MCT-1 transporters, the reduction of the H^+ gradient would be overcompensated by the increase on the amount of the transporters in the apical membrane.

On the view of this data, we could suggest that the decrease on the activity of the Na^+-dependent transporters due to leptin would be a way for the enterocyte to economize the energy expenditure required by the Na^+/K^+-ATPase to maintain the Na^+ gradient during the absorptive period, which is energetically very demanding. Thus, leptin would slow down the transport of some nutrients to allow the enterocyte to process them and restore the energy. Later on, this effect would disappear (leptin effect is reversible) so the remaining nutrients in the lumen of the intestine would be completely absorbed. In line with this hypothesis, it has been found that leptin also delays gastric emptying and transit activity in the jejunum (Martinez *et al*., 1999; Kiely *et al*., 2005).

In summary, leptin acting from the apical membrane of the enterocytes modulates, in a short-term manner *in vivo* and *in vitro,* amino acids transport in rat intestine and human Caco-2 cells. The mechanisms implicated include direct effects on transporters expression in the apical membrane and indirect processes that leads to modulation of the activity of the implicated transporters. Leptin also rapidly inhibits amino acids uptake acting from the basolateral membrane. In both conditions, the inhibition is reversible.

Interestingly, the activity of Na^+-independent glutamate transporters was increased by leptin. Taken together, these results contribute to the vision of leptin as an important hormonal signal for the regulation of intestinal absorption of nutrients.

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Conclusions

Rat intestine

1. Leptin acting from the apical membrane of the enterocytes inhibits, in a short-time manner, intestinal glutamine and phenylalanine uptake *in vitro* by reducing the expression of ASCT2 and B^0AT1 in the brush border membrane.

2. Leptin acting from the apical membrane of the enterocytes inhibits, in a short-time manner, glutamate uptake *in vitro* in the presence of Na^+ at pH 7.4, whereas in the absence of Na^+ at pH 6, the hormone enhances the amino acid uptake. Therefore, leptin is able to modulate the two different mechanism of glutamate transport present in rat intestine, the Na^+-dependent and the Na^+-independent one.

3. Luminal leptin (gastric origin) inhibits *in vivo* intestinal glutamine, proline and β-alanine absorption. This effect is observed within minutes and is rapidly reversed by the removal of the hormone from the intestinal lumen.

4. Luminal leptin (gastric origin) inhibits *in vivo* the successive absorption of galactose and proline, indicating that the hormone is able to regulate separately different intestinal transporters.

Human intestine: Caco-2 cell line

5. Glutamate transport is a strictly Na^+-dependent process which is increased at pH 6 due to ASCT2 activity.

6. Apical leptin inhibits glutamine, β-alanine and glutamate uptake. This inhibition is rapid and reversible. Glutamine inhibition is due to a reduction in the apical expression of ASCT2 and B^0AT1 whereas leptin indirectly inhibits β-alanine uptake most probably by reducing NHE3 exchanger activity. Moreover, PKA is implicated in leptin inhibition of glutamine and β-alanine uptake.

7. Basal leptin inhibits glutamine and β-alanine uptake in Caco-2 cells grown on permeable supports. The inhibition is observed at short time incubation periods and after long pre-incubation time and is of the same magnitude. This inhibition disappears in few minutes upon removal of the hormone from the incubation medium.

General conclusion

8. In summary, leptin acting from the apical membrane of the enterocytes modulates, in a short-term manner *in vivo* and *in vitro*, amino acids transport in rat intestine and human Caco-2 cells. The mechanisms implicated include direct effects on transporters expression in the apical membrane and indirect processes that leads to modulation of the activity of the implicated transporters. Leptin also inhibits rapidly amino acids uptake acting from the basolateral membrane. In both conditions, the inhibition is reversible. Interestingly, the activity of Na^+-independent glutamate transporters was increased by leptin. Taken together, these results contribute to the vision of leptin as an important hormonal signal for the regulation of intestinal absorption of nutrients.