THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 11, pp. 6801–6808, March 13, 2009
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Reduced Intestinal Absorption of Dipeptides via PepT1 in Mice with Diet-induced Obesity Is Associated with Leptin Receptor Down-regulation*

Received for publication, July 21, 2008, and in revised form, December 26, 2008 Published, JBC Papers in Press, January 14, 2009, DOI 10.1074/jbc.M805564200

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Leptin is a major determinant of energy homeostasis, acting both centrally and in the gastrointestinal tract. We previously reported that acute leptin treatment enhances the absorption of di- and tripeptides via the proton-dependent PepT1 transporter. In this study, we investigated the long term effect of leptin on PepT1 levels and activity in Caco2 cell monolayers *in vitro*. We then assessed the significance of the regulation of PepT1 *in vivo* in a model of diet-induced obesity. We demonstrated that 1) leptin regulated PepT1 at the transcriptional level, via the MAPK pathway, and at the translational level, via ribosomal protein S6 activation, in Caco2 cells and 2) this activation was systematically followed by a time- and concentration-dependent loss of leptin action reflecting desensitization. Deciphering this desensitization, we demonstrated that leptin induced a down-regulation of its own receptor protein and mRNA expression. More importantly, we showed, in mice with diet-induced obesity, that a 4-week hypercaloric diet resulted in a 46% decrease in PepT1-specific transport, because of a 30% decrease in PepT1 protein and a 50% decrease in PepT1 mRNA levels. As shown in Caco2 cells, these changes in PepT1 were supported by a parallel 2-fold decrease in leptin receptor expression in mice. Taken together, these results indicate that during induction of obesity, leptin resistance may also occur peripherally in the gastrointestinal tract, disrupting the absorption of oligopeptides and peptidomimetic drugs.

Leptin, the *ob* gene product, was first described as an adipocyte-derived hormone involved in fat and energy storage (1). Subsequent studies have shown that other tissues, such as the placenta, brain, bone marrow, and stomach produce leptin (2). This hormone produced by multiple sites is now thought to have pleiotropic functions, controlling not only food intake, but also immunity, the autonomic nervous system, or tissue remodeling and growth (reviewed in Ref. 3). In addition, several lines of evidence indicate that leptin is closely associated with intestinal functions, with potential indirect effects on energy balance. Indeed, leptin can be secreted by the gastric mucosa and is rapidly released into the intestine following the ingestion of a meal as an active protein, which may be free or bound to its receptor (4–7). Furthermore, leptin receptors are found on both the apical and basolateral sides of the enterocyte, facilitating the action of both adipocyte-derived leptin (on the basolateral side) and gastric leptin (on the apical side) (8–10). In this view, leptin has been shown to regulate the secretion of glucagon-like peptide 1 and cholecystokinin by enteroendocrine cells (6, 11) and to induce mucin secretion in the large intestine (12).

Leptin directly modulates nutrient absorption by decreasing carbohydrate absorption (via its action on the Na^+/glucose cotransporter 1) and cholesterol absorption (13–15), enhancing butyrate uptake via its action on the CD147-monocarboxylate transporter 1 complex (16), and fatty acid uptake and transport by triggering the expression of intestinal fatty acid-binding protein (17).

In their studies of the role of leptin in the intestinal absorption of proteins and peptides, Kiely *et al.* (18) showed that the activity of the jejunal aminopeptidase and dipeptidylpeptidase IV, cleaving mono- and dipeptides, respectively, were lower in leptin-deficient mice than in wild-type mice. The relationship between leptin and PepT1 (H^+-coupled peptide cotransporter 1), which transports most of the di- and tripeptides in the intestine, together with peptidomimetic drugs, has been the matter of studies. Gastric leptin has been shown to increase PepT1 activity within minutes by recruiting the preformed intracellular pool of the transporter to the brush border of the enterocytes, without modifying PepT1 mRNA levels (8). Interestingly, we and others have reported that leptin is capable of increasing the mRNA levels for this transporter, thereby reconstituting the pool of PepT1 after long term challenge *in vitro* and *in vivo* (19, 20). Building on these findings, this study was designed to investigate the regulation of PepT1 in a model of diet-induced obesity in mice characterized by progressive long term hyperleptinemia. It has been suggested that central densensitization to leptin is associated with obesity in this model. However, it was unknown whether such densensiti-

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zation also occurred in the small intestine. We found that PepT1 regulation was dependent on the duration of leptin treatment and diet and investigated the possible mechanisms underlying this phenomenon.

EXPERIMENTAL PROCEDURES

Cell Culture—Caco2 cells were cultured in Dulbecco's modified Eagles medium (Invitrogen, Cergy Pontoise, France) supplemented with 20% fetal bovine serum (Invitrogen), 1% nonessential amino acids, and 1% penicillin/streptomycin in an environment containing 5% CO_2, with 95% humidity, at 37 °C. The cells were seeded on Costar Transwell® membrane inserts with 0.4-μm pores (Corning, NY) at a density of 5×10^4 cells/cm^2. The experiments were conducted on the 17th day of culture, after treatment in both the apical and basal sides with leptin (R & D Systems, Minneapolis, MN) and/or with one of the mitogen-activated protein kinases (MAPK)[2] inhibitors U0126 (Cayman Chemicals, SPI-BIO, Montigny le Bretonneux, France) and PD98059 (Sigma-Aldrich, Saint Quentin Fallavier, France) or the mTOR (mammalian target of rapamycin) inhibitor rapamycin (Cayman Chemicals).

Animals—The experiments were conducted in male wild-type C57BL/6J mice (Janvier, Le Genest Saint Isle, France). The animals were housed in a room maintained at 21 °C, with 12-h light/12-h dark cycles and free access to water (accreditation number A92-01901). They were fed with standard laboratory chow (SC, control mice) (A04 biscuits; UAR, Villemoisson, France) or a high fat diet (referred to as the hypercaloric (HC) diet; purchased from SAFE, Augy, France). The standard chow diet provides 2,820 kcal/kg of food and contains 3% fat (270 kcal/kg, accounting for 9.6% of the kilocalories), 48% complex carbohydrates (1,910 kcal/kg, 67.7% kcal, primarily starch), and 16% protein (640 kcal/kg, 22.7% kcal). The HC diet provides 5,320 kcal/kg and includes 36% fat (3,220 kcal/kg, 60.5% kcal, primarily lard), 35% simple carbohydrates (1,440 kcal/kg, 26.3% kcal, mainly saccharose), and 18% protein (700 kcal/kg, 13.2% kcal). Actual food consumption was measured for both diets. All of the experiments were performed in accordance with European Committee Standards concerning the care and use of laboratory animals and were approved by the head of the staff responsible for laboratory animal care.

PepT1 Activity in Caco2 Cells—PepT1 activity was assessed by following the transport of cephalexin (Sigma-Aldrich), a PepT1 substrate, across the Caco2 monolayer. All of the procedures were as previously described (19). Briefly, the apical compartments of the Transwell® apparatus were filled with 0.5 ml of 1 μM cephalexin. Basolateral compartments were sampled at $t = 0$, 5, 10, 15, 20, 25, and 30 min, and the cephalexin concentration was calculated after high pressure liquid chromatography determination. The apparent permeability (P_{app}) was used to assess cephalexin transport across the monolayer, according to the following equation $P_{app} = (dQ/dt) \cdot (V/Q_0 \cdot A)$, where V is the volume of the basolateral compartment, Q_0 is the total amount of cephalexin in the apical compartment, A is the surface area of the membrane, and dQ/dt is the permeability rate (slope of plot of the concentration in the basolateral compartment against time).

Transport of [^{3}H]Gly-Sar in the Mouse Jejunal Loop ex Vivo Model—The animals were killed, and the transport of Gly-Sar, a specific PepT1 substrate, was monitored in the *ex vivo* jejunal loop model. Briefly, a 6-cm segment of jejunum was filled with 100 μl/cm Krebs modified buffer, pH 6, containing [^{3}H]Gly-Sar (1 μmol/liter [^{3}H]Gly-Sar Isobio, Fleurus, Belgium; specific activity, 0.5 Ci/mmol), 20 μmol/liter Gly-Sar (Sigma-Aldrich), and 500 mg/liter phenol red, to assess paracellular permeability. The segment was placed in a 37 °C thermostat-controlled bath of Krebs modified buffer at pH 7.4, through which a 95:5 mixture of O_2:CO_2 was continually bubbled. The samples were withdrawn from the bath at $t = 5$, 10, 15, 20, 25, and 30 min, and radioactivity was measured with a β counter. Apparent permeability to Gly-Sar was estimated as follows: $P_{app} = (dQ/dt) \cdot (V/Q_0 \cdot A)$, where V is the volume of the bath, A is the area of the loop, Q_0 is the total amount of radiolabeled Gly-Sar introduced into the loop, and dQ/dt is the flux across the intestinal loop.

Protein Extraction—All of the procedures were carried out at 4 °C to inhibit proteolysis. For total protein extraction, Caco2 cells or samples scraped from the jejunum were homogenized in TENTS lysis buffer (containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 126 mM NaCl, 1% Triton X-100 (v/v), 0.1% SDS (v/v), and protease inhibitors) and incubated for 15 min. The homogenates were then centrifuged at 12,000 × *g* for 20 min. This supernatant corresponded to a total protein extract. For the study of protein phosphorylation, the cells were homogenized in lysis buffer (containing 90 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100 (v/v), protease inhibitors, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 2 mM sodium orthovanadate as phosphatase inhibitors) and incubated for 30 min. The solution was then centrifuged at 12,000 × *g* for 15 min. The phosphorylated proteins were present in the supernatant.

Western Blot Analysis—The proteins (20–25 μg) were separated by SDS-PAGE in gels containing 8–12% acrylamide. The proteins were transferred to nitrocellulose membranes and subjected to immunoblotting. The dilutions of primary antibodies used were: 1:1000 for PepT1 (gift from Dr Merlin for *in vitro* studies or from Prof. Kapel for *in vivo* studies), 1:100 for Ob-R (H-300, Santa Cruz Biotechnology, Santa Cruz, CA), 1:5000 for phosphorylated signal transducer and activator of transcription (STAT) 3 (Tyr^{694}) and STAT5 (Tyr^{705}) (Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France), 1:1000 for total STAT3 and STAT5 (C-20 and C-17; Santa Cruz Biotechnology), 1:1000 for phosphorylated p44/42 MAPK (Thr^{202}/Tyr^{204}) (ERK1/2) (Cell Signaling Technology), and total ERK (K-23; Santa Cruz Biotechnology), 1:1000 for phosphorylated S6 ribosomal protein (Ser$^{235/236}$) (Cell Signaling Technology), 1:5,000 for β-actin (clone AC74; Sigma-Aldrich). Peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) were used at a dilution of 1:10,000, and the membranes were probed with ECL (PerkinElmer Life Sciences). The intensity of the bands was quantified with Scion Image (National Institutes of Health, Scion Corporation,

[2] The abbreviations used are: MAPK, mitogen-activated protein kinases; Gly-Sar, glycyl-sarcosine; HC, hypercaloric chow; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinases; SOCS, suppressor of cytokine signaling.

TABLE 1
Characteristics of the primers used for the reverse transcription-PCR studies

Gene	Access number	Primer sequences (5′ → 3′)	Size
			bp
hPepT1	NM_005073	F-GTTGGCAACATCATTGTGCT R-TCCGCTGGGTTGATGTAAGT	149
hGAPDH	NM_002046	F-GTGAAGGTCGGAGTCAACG R-TGAGGTCAATGAAGGGGTC	112
hOb-Ra	NM_002303	F-TCACACCAAAGAATGAAAAAGC R-TGCTTGATAAAAAGATGCTCAAAC	119
hOb-Rb	NM_001003679	F-CCTGGGCACAAGGACTTAAT R-CCTTCTTCAAAATGTAAAAATCTTCT	137
hSOCS3	NM_003955	F-CAAGGACGGAGACTTCGATT R-AACTTGCTGTGGGTGACCAT	137
hPTP1B	NM_002827	F-TTCATCATGGGGGACTCTTC R-ATTGTGTGGCTCCAGGATTC	126
mPepT1	NM_053079	F-CGTGCACGTAGCACTGTCCAT R-GGCTTGATTCCTCCTGTACCA	66
mOb-Rb	NM_146146	F-CGCTTCCCTTGTGAATTTTAA R-ACAGTGTCCAGGAAAGGATGA	118
mS14	NM_020600	F-CAGGACCAAGACCCCTGGA R-ATCTTCATCCCAGAGCGAGC	69

Bethesda, MD). For the study of the expression of phosphorylated protein, the membranes were blotted with antibodies specific for phospho-STATs or phospho-ERK and then scraped and reblotted with antibodies recognizing total STATs or total ERK protein.

Real Time PCR Analysis—Total RNA was isolated by the guanidine thiocyanate method, with RNAble (Eurobio, Les Ulis, France), used according to the manufacturer's instructions. The first strand cDNA was synthesized by reverse transcription from 5 μg of total RNA, with SuperScript II reverse transcriptase (Invitrogen).

We quantified cDNA with the Light Cycler system (Roche Applied Science), used according to the manufacturers instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or S14 was used as a housekeeping gene. The primers were designed with Primer3 software (Table 1).

Statistical Analysis—All of the values are expressed as the means ± S.E. Mann-Whitney tests (or Student's *t* tests, as appropriate) were used to compare pairs of means, and Kruskal-Wallis tests were used to compare groups of more than two means. Statistical analysis was performed with Graph Pad Prism (Graph Pad software, San Diego, CA). The values of $p <$ 0.05 were considered statistically significant for all analyses.

RESULTS

Leptin Induces a Time- and Concentration-dependent Regulation of PepT1 Levels and Activity—Challenging Caco2 cells with 0.2 nM leptin (corresponding to a normoleptinemia) for 7 days induced a significant 2.3-fold increase in cephalexin transport across the Caco2 monolayer, consistent with an increase in PepT1 activity (Fig. 1*A*). The increase in PepT1 activity was associated with a parallel 3.2-fold increase in protein levels (Fig. 1*B*). Surprisingly, in Caco2 cells treated for 7 days with 1 nM (corresponding to hyperleptinemia), the increase in PepT1 protein levels and activity was no longer observed, indicating resistance to the administered leptin.

We investigated this phenomenon further by carrying out a time course study of the effect of leptin. As expected, the lower concentration (0.2 nM) of leptin, which increased PepT1 activity and total protein levels after a 7-day challenge, increased PepT1 protein levels in a time-dependent manner (Fig. 2). Indeed, 0.2 nM leptin induced a gradual increase in PepT1 levels, which peaked after 7 days of treatment. Interestingly, the higher concentration (1 nM) of leptin rapidly induced the expression of total PepT1 protein (2-fold increase) at 24 h, but this effect was transient and completely disappeared after 72-h treatment, reflecting desensitization.

We then investigated whether the change in PepT1 protein levels could be explained by changes in mRNA levels. For both

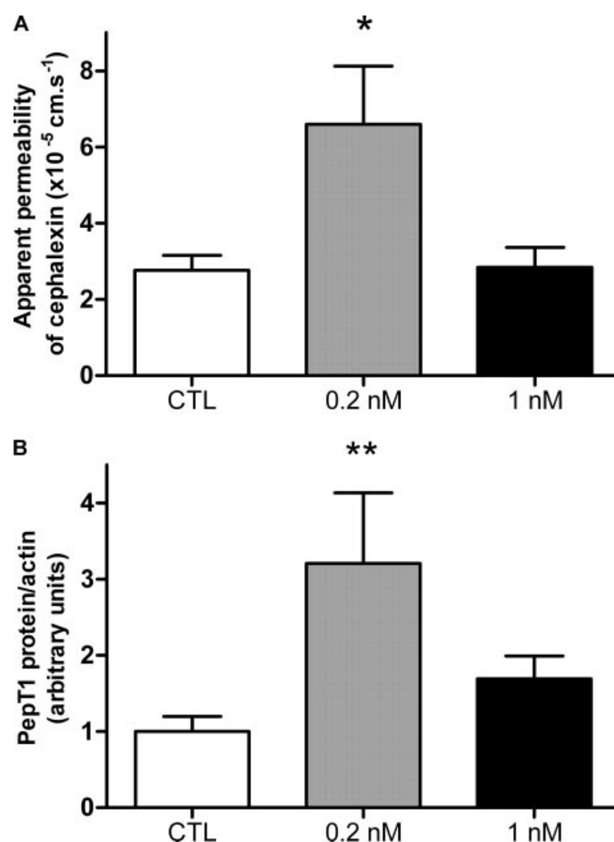

FIGURE 1. **Effects of chronic leptin treatment on PepT1 activity and expression *in vitro*.** Caco2 cells were treated daily for 7 days with 0.2 nM or 1 nM leptin. *A*, PepT1 activity in leptin-treated Caco2 cells. The apparent permeability coefficient of cephalexin across Transwell® membranes was measured over a period of 30 min (n = 8–15; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*)). All of the data are the means ± S.E. *B*, densitometric analysis of PepT1 protein levels normalized on the basis of β-actin levels in leptin-treated Caco2 cells (n = 6–8; Kruskal-Wallis test; **, $p < 0.05$ *versus* control). All of the data are the means ± S.E.

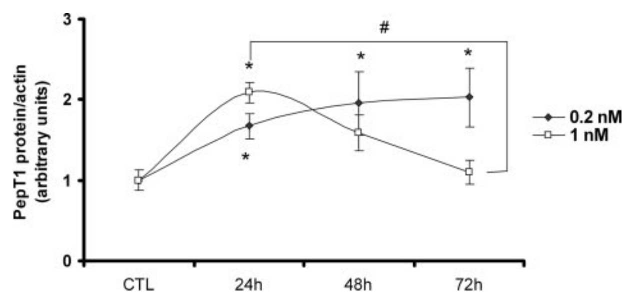

FIGURE 2. **The time-dependent effect of leptin treatment on PepT1 total protein levels.** Densitometric analysis of PepT1 protein levels normalized as a function of β-actin levels in leptin-treated Caco2 cells. Caco2 cells were treated for 24, 48, or 72 h with 0.2 or 1 nM leptin (n = 4; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*); #, $p < 0.05$ *versus* 24 h, 1 nM leptin-treated cells). All of the data are the means ± S.E.

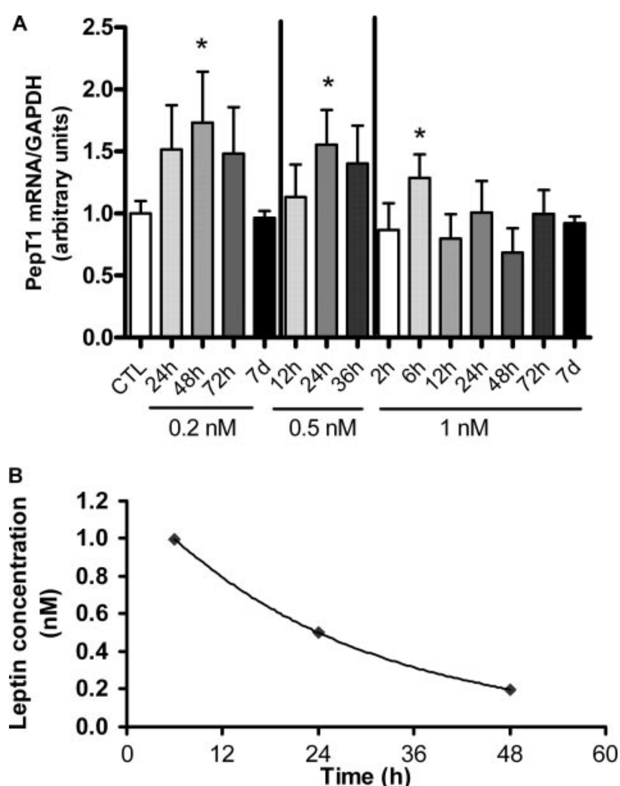

FIGURE 3. **Time- and concentration-dependent effects of leptin on PepT1 mRNA levels.** *A*, relative PepT1 mRNA levels, normalized with respect to GAPDH mRNA levels. Caco2 cells were treated for the indicated times with 0.2, 0.5, or 1 nM leptin ($n = 4–10$; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*)). All of the data are the means ± S.E. *B*, correlation between time taken to reach PepT1 mRNA levels and leptin concentration ($n = 4–10$).

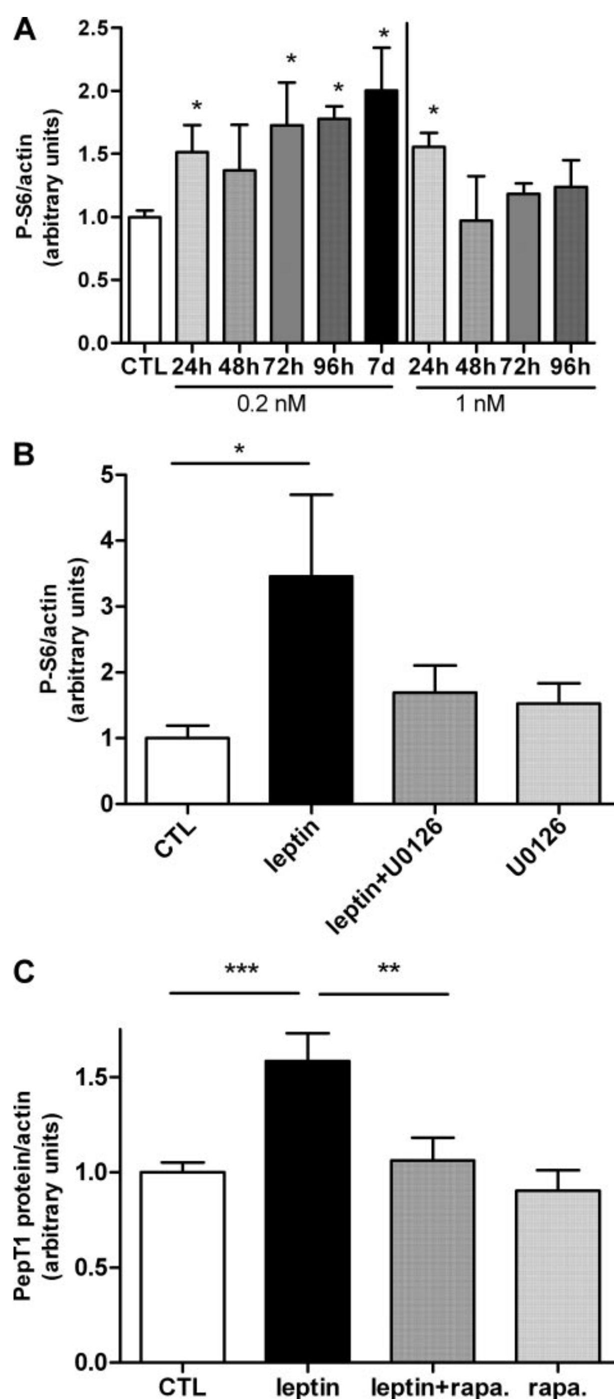

FIGURE 4. **Effect of leptin on PepT1 protein expression.** *A*, densitometric analysis of phospho-S6 protein expression normalized to β-actin expression ($n = 7–8$; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*)). *B*, densitometric analysis of phospho-S6 protein expression normalized to β-actin expression. Caco2 cells were treated for 24 h with 0.2 nM leptin. U0126 (20 μM) was added in the culture medium 30 min before leptin treatment and during the leptin treatment ($n = 6$; Kruskal-Wallis test; *, $p < 0.05$ *versus* control). *C*, densitometric analysis of PepT1 protein expression normalized to β-actin expression. Caco2 cells were treated for 7 days with leptin 0.2 nM. Rapamycin (*rapa.*, 20 nM) was added in the culture medium 30 min before leptin treatment and during the leptin treatment ($n = 12$; Kruskal-Wallis test; ***, $p < 0.001$; **, $p < 0.01$ *versus* control). All of the data are the means ± S.E.

concentrations tested, leptin up-regulated PepT1 mRNA levels (×1.7 and 1.4 for 0.2 and 1 nM leptin, respectively; Fig. 3*A*), but this effect was only transient, with PepT1 mRNA levels returning to basal levels after treatment. More importantly, we found that mRNA levels peaked after only 6 h for the 1 nM treatment, whereas the peak was reached later (48 h) with the 0.2 nM treatment. Thus, the time at which mRNA levels peaked also appeared to depend on leptin concentration. Indeed, with a 0.5 nM leptin challenge, peak mRNA levels were reached at 24 h of treatment, and there was an inverse correlation between the time taken to reach peak PepT1 levels and leptin concentration (Fig. 3*B*).

Leptin Increases PepT1 Protein Levels by Enhancing Its Translation—We then investigated the apparent discrepancy between the sustained levels of PepT1 protein at 7 days with the 0.2 nM treatment and the peak in mRNA levels observed after only 48 h of treatment. We hypothesized that an enhancement of translation might account for this pattern. We therefore analyzed phosphorylation of the ribosomal protein S6, which is involved in the activation of translation, in a time course experiment. Densitometric analysis of the ribosomal protein S6 showed that treatment with 0.2 nM leptin was associated with a significant increase in S6 phosphorylation at 24 h treatment, with higher levels of phosphorylation persisting for a further 7 days (Fig. 4*A*). By contrast, when cells were treated with 1 nM leptin, ribosomal protein S6 phosphorylation occurred earlier and was transient, peaking after 24 h of treatment and returning to basal level thereafter. Fig. 4*B* showed that the activation of S6 by leptin was, at least in part, mediated by the MAPK pathway as the inhibitor U0126 reversed the action of leptin on S6 phos-

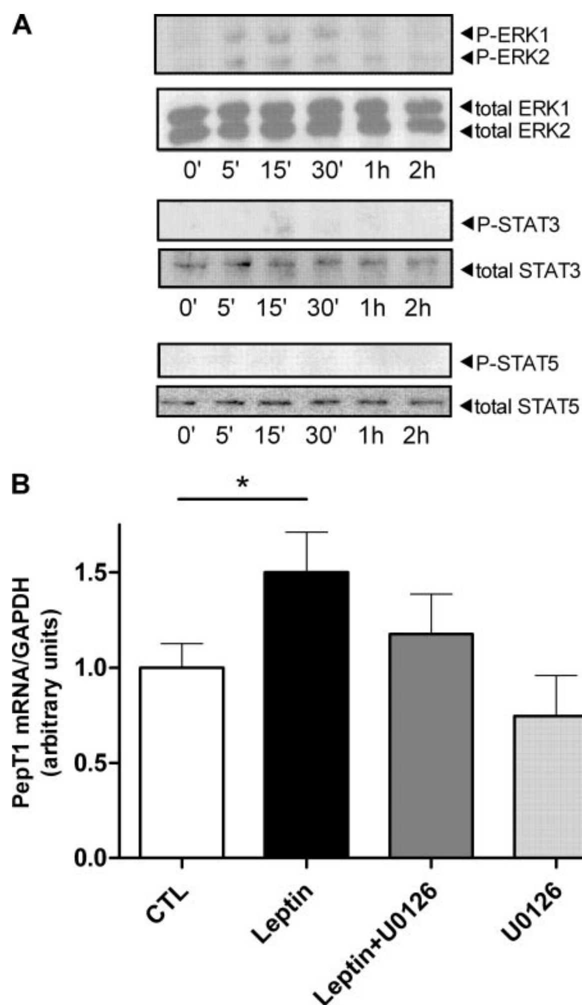

FIGURE 5. **Involvement of ERK1/2, STAT3, and STAT5 in the activation of PepT1 expression.** *A*, phosphorylation of ERK1/2, STAT3, and STAT5 upon leptin challenge. Caco2 cells were treated at the indicated times with 0.2 nM leptin, and immunoblot analysis was carried out with the antibodies indicated on the *right*. The blots are representative of three independent experiments. *B*, relative quantification of PepT1 mRNA normalized as a function of GAPDH mRNA levels. Caco2 cells were treated for 6 h with 1 nM leptin. U0126 (20 μM) was added and incubated with the culture medium for 30 min before leptin treatment and during the leptin treatment ($n = 4-10$; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*)). All of the data are the means ± S.E.

phorylation. Moreover, the induction of PepT1 protein expression by leptin after 7 days of treatment was totally abolished by the mTOR inhibitor rapamycin that blocks the S6 kinase action (Fig. 4*C*).

Leptin Activates the Production of PepT1 mRNA through the ERK1/2 Pathway, but Not by STAT3 or STAT5 Activation—We then focused on intracellular events that might account for the effect of leptin on PepT1 mRNA levels. Interestingly, no phosphorylation of either STAT3 or STAT5 was observed under our conditions. However, a rapid and transient activation of extracellular signal-regulated kinases 1/2 (ERK1/2) occurred after 0.2 nM leptin treatment, corresponding to the phosphorylation of the two immunoreactive bands at 42 and 44 kDa (Fig. 5*A*). In addition, the effect of leptin was partially reversed by the MAPK/ERK1/2 kinase (MEK1/2) inhibitors U0126 and PD98059 (Fig. 5*B* and data not shown).

Leptin Does Not Modify SOCS3 or Protein-tyrosine Phosphatase 1B Activation—We investigated the origin of the desensitization observed after activation by assessing the transcription of two genes encoding known inhibitors of leptin signaling: SOCS3 and protein-tyrosine phosphatase 1B. No significant change in mRNA levels was observed under the conditions in which Caco2 cell desensitization occurred (data not shown). Thus, Caco2 cell desensitization is unlikely to have been caused by these inhibitors in our conditions.

Leptin Regulates Its Own Receptors, Ob-Ra and Ob-Rb—Alternatively, the desensitization of PepT1 expression and activity may reflect changes in leptin receptor expression. We observed the same pattern of expression for leptin receptor as the one seen for PepT1; leptin induced an increase of leptin receptor protein and mRNA levels, followed by a large decrease in both concentrations. Indeed, receptor protein or mRNA levels did not return to basal expression but diminished further and displayed a 2–4-fold decrease (Fig. 6). Moreover, the time at which peak expression occurred was again concentration-dependent. Indeed, 1 nM leptin treatment rapidly induced mRNA Ob-Rb and Ob-Ra (long and short form leptin receptors) expression, with levels peaking after 12 h of treatment, whereas the action of 0.2 nM leptin was more gradual with maximal Ob-Rb and Ob-Ra mRNA levels after 24–48 h of incubation (Fig. 6, *B* and *C*).

Mice Fed a HC Diet Display Lower Levels of PepT1 Expression and Activity and of Ob-R Expression—We then investigated whether the changes in PepT1 and leptin receptors triggered by leptin *in vitro* could be observed in an *in vivo* model displaying hyperleptinemia (*i.e.* mice fed an HC diet). As expected, 4 weeks on the HC diet led to increases in weight gain, plasma leptin and insulin concentrations, and glycemia. In addition, this diet also resulted in a significantly higher daily caloric intake, with no significant effect on protein intake (Table 2). The HC diet induced a 46% decrease in PepT1-specific Gly-Sar transport (Fig. 7*A*), with no change in paracellular transport, as monitored by red phenol flux (data not shown). The modification in PepT1 activity was supported by a 30% decrease in PepT1 protein levels and a 50% decrease in PepT1 mRNA levels (Fig. 7, *B* and *C*). Moreover, leptin receptor expression was reduced by 40% by 4 weeks on the HC diet (Fig. 7*D*). In our previous study, we induced hyperleptinemia in rats by subcutaneously implanting an Alzet® pump delivering 1 μg of leptin/g/day for 7 days. By contrast to the decrease in leptin receptor levels observed in mice fed the HC diet, hyperleptinemia in leptin-treated rats induced no change in leptin receptor mRNA production (data not shown).

DISCUSSION

We provide here the first demonstration that the induction of obesity provokes a time-dependent loss of responsiveness in intestinal target genes. Indeed, diet-induced obesity is associated with a dramatic decrease in the PepT1-mediated transport of oligopeptides in the intestine, related to down-regulation of leptin receptor expression.

As shown in our previous study, leptin-deficient *ob/ob* mice display reduced levels of PepT1 activity and expression; furthermore, hyperleptinemia induced for 7 days in rats, associ-

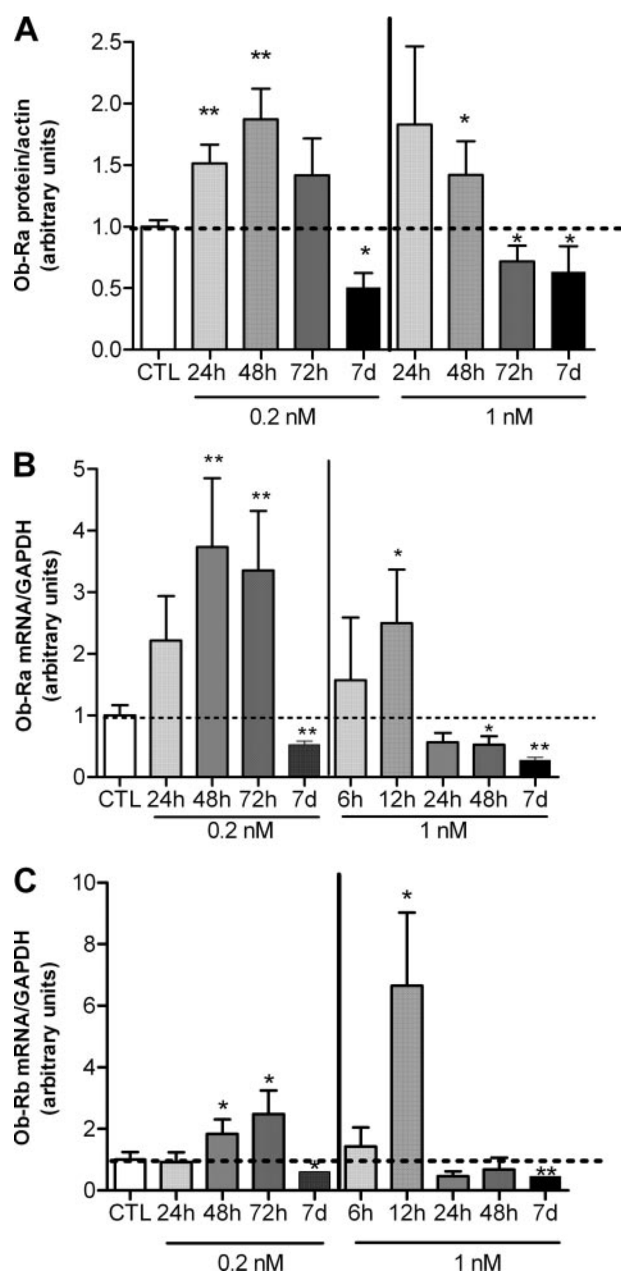

FIGURE 6. **Effect of leptin challenge on Ob-R protein and mRNA levels.** *A*, densitometric analysis of Ob-Ra protein levels normalized with respect to β-actin levels ($n = 5$–10; Kruskal-Wallis test; *, $p < 0.05$ *versus* control (*CTL*); **, $p < 0.01$ *versus* control). All of the data are the means ± S.E. *B* and *C*, relative quantification of Ob-Ra or Ob-Rb mRNA levels normalized as a function of GAPDH mRNA levels ($n = 3$–11; Kruskal-Wallis test; *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control). All of the data are means ± S.E.

TABLE 2

Plasma glucose, insulin and leptin concentrations, weight gain, and daily food, calorie, and protein intake after 4 weeks on the HC or standard chow diet ($n = 7$ Student's *t* test)

	Standard chow-fed control mice ($n = 6$)	Hypercaloric diet-fed mice ($n = 6$)
Plasma glucose (mmol/liter)	9.76 ± 0.14	13.20 ± 0.82[a]
Plasma insulin (ng/ml)	0.31 ± 0.04	0.71 ± 0.15[a]
Plasma leptin (ng/ml)	0.80 ± 0.17	1.62 ± 0.19[a]
4-week weight gain (g)	2.63 ± 0.20	4.20 ± 0.21[a]
Daily food intake (g)	4.51 ± 0.50	3.86 ± 0.41[a]
Daily caloric intake (kcal)	12.69 ± 1.41	20.53 ± 2.18[a]
Daily protein intake (g)	0.72 ± 0.08	0.69 ± 0.08

[a] $p < 0.05$ *versus* control mice.

ated with a decrease in food intake, induces an increase in PepT1 expression and oligopeptide transport (19). In addition, in the rat model of hyperleptinemia, we observed no change in leptin receptor expression in the small intestine (data not shown). Surprisingly, in the present study, we observed desensitization of the leptin response to continuous hyperleptinemia, because PepT1 levels ceased to increase after long term treatment. There may be several reasons for this desensitization. First, desensitization may be mediated by the induction of well described leptin signaling pathway inhibitors, such as SOCS3 and protein-tyrosine phosphatase 1B (21). However, these two inhibitors are unlikely to play a role in our conditions, because no transcriptional activation was observed.

Alternatively, leptin may contribute to the intestinal regulation of the gene encoding its receptor. Ob-R down-regulation was evident in the animals fed the HC diet for 4 weeks, in which plasma leptin concentration increased steadily. A shorter duration of hyperleptinemia and/or the difference in the model may explain why such a regulation was not observed in the 7-day leptin-treated rats. A similar pattern of regulation was also observed following prolonged leptin treatment in Caco2 cells. The close association between the levels of Ob-R and PepT1 in both Caco2 cells and mouse jejunum suggests that the regulation of PepT1 levels depends on receptor abundance and the resulting level of signaling. Indeed, treatment *in vitro* with 0.2 or 1 nM leptin rapidly induced an increase in Ob-R expression and may induce PepT1 expression. However, prolonged leptin treatment *in vitro* or four weeks of the HC diet *in vivo* may down-regulate expression of the receptor, leading to a net decrease in leptin signaling within enterocytes and the abolition of leptin effects on PepT1. Such a regulation has already been described in the central nervous system. Consistent with this, some authors showed, in neuroblastoma cells, that leptin down-regulates its own receptors (22) and that hypothalamic leptin receptors were down-regulated in hyperleptinemic rodents, leading to leptin resistance (23, 24).

We found that leptin treatment *in vitro* induced a transient increase in Ob-R and PepT1 mRNA levels, followed by a normalization of PepT1 levels or a larger decrease in leptin receptor expression. The pattern of change in expression was clearly time- and concentration-dependent. Indeed, the time required to reach peak PepT1 mRNA levels was inversely correlated with leptin concentration, with higher concentrations resulting in faster effects on PepT1 transcription.

A few studies have focused on the activation of PepT1 transcription. For example, the transcriptional factors Sp1 and peroxisome proliferator-activated receptor α have been shown to be involved in PepT1 induction (25, 26). Similarly, the authors of these studies demonstrated that PepT1 may also be induced by caudal-related homeobox 2 (27). Nduati *et al.* (20) identified a link between the induction of PepT1 by caudal-related homeobox 2 and leptin, because leptin was shown to increase cAMP levels, consequently activating caudal-related homeobox 2. We investigated the role of the MAPK pathway in PepT1 activation in our model. Leptin has been shown to activate ERK1/2 in the hypothalamus (28, 29) and many other tissues, including the colon and intestinal cells (12, 30, 31) and STC-1 enteroendocrine cells (6). Gong *et al.* (32)

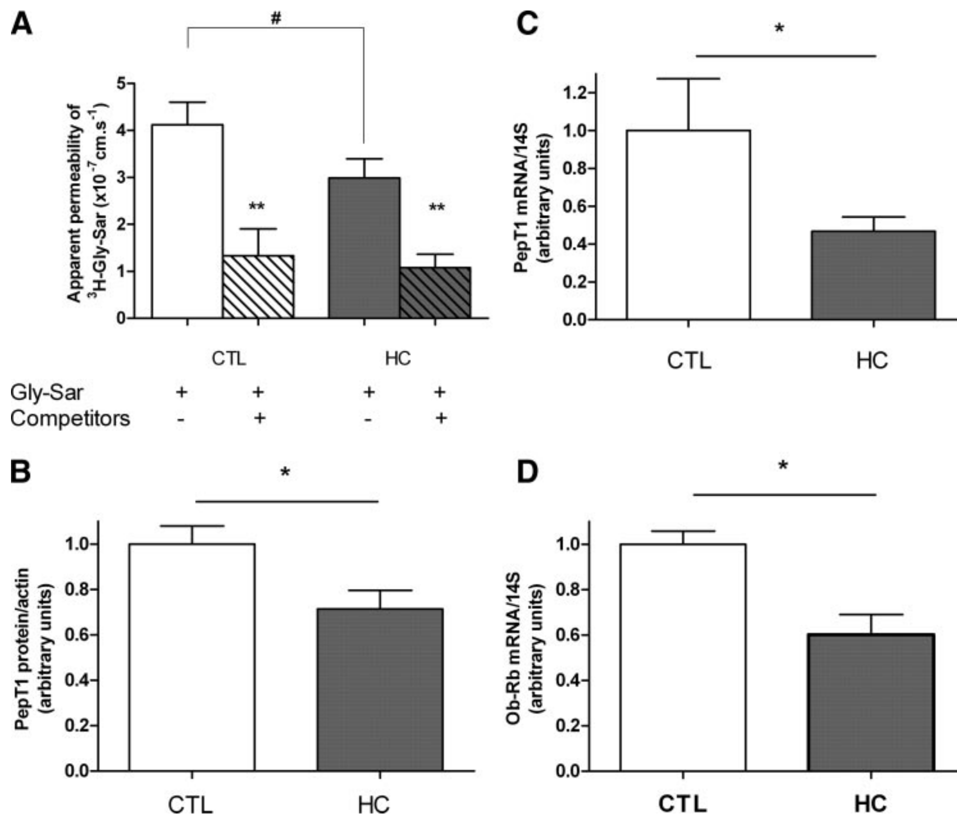

FIGURE 7. **PepT1 levels and activity and Ob-Rb expression after 4 weeks of the HC or standard chow diet in mice.** *A*, Gly-Sar transport was monitored by the *ex vivo* jejunal loop method. Gly-Gly and Gly-Pro were used as competitors, to assess specific transport via PepT1 ($n = 5$ in each group; Kruskal-Wallis test; #, $p < 0.05$ *versus* control (*CTL*) mice; **, $p < 0.01$ *versus* transport without competitors in each group). All of the data are the means ± S.E. *B*, densitometric analysis of PepT1 protein levels normalized as a function of β-actin levels ($n = 5$ in each group; Mann-Whitney test; *, $p < 0.05$ *versus* control mice). All of the data are the means ± S.E. *C*, relative quantification of PepT1 mRNA levels normalized with respect to 14 S ($n = 4$ in each group; Mann-Whitney test; *, $p < 0.05$ *versus* control mice). *D*, relative quantification of Ob-Rb mRNA normalized with respect to 14 S ($n =$ 6–7; Mann-Whitney test; *, $p < 0.05$ *versus* control mice).

showed that, in the hypothalamic arcuate nucleus in mice or in cultured transfected cells, leptin activated the ERK1/2 cascade by phosphorylating the Tyr^{985} residue of the intracellular domain of Ob-Rb. We show here, using inhibitors of the MAPK pathway U0126 and PD98059, that this ERK1/2 phosphorylation contributes to the stimulatory effect of leptin on PepT1 mRNA production.

Surprisingly, the STAT3 and STAT5 pathways were not activated with our physiological concentrations of leptin. This lack of STAT activation is particularly striking, because these transducers are thought to play a key role in leptin signaling (33). Moreover, Morton *et al.* (34) showed that leptin could activate STAT3 and STAT5 in Caco2 cells after treatment with concentrations of 20–200 nM, much higher than the concentrations used in our study. However, the absence of downstream SOCS3 transcription activation provides further evidence that the STAT3 pathway was not activated in our conditions, because SOCS3 expression is dependent on STAT3 activation (28). Valerio *et al.* (35) obtained similar data for leptin signaling pathways in mouse cortical neurons, in which leptin is unable to activate STAT3 and to induce the subsequent SOCS3 protein expression. However, we cannot rule out the possibility that a weak expression of phosphoSTAT3 and phosphoSTAT5 occurs, below the detection threshold of Western blotting.

We investigated the basis of the sustained effect of leptin on PepT1 protein levels, whereas the effect on mRNA is only transient by studying the action of leptin on the ribosomal protein S6, which has been implicated in mRNA translation.

It has been shown that S6 and its kinase (ribosomal S6 kinase) are phosphorylated via the MAPK pathway (32, 35, 36), which we demonstrated to be activated in our *in vitro* model. In Caco2 cells, we confirmed the leptin-stimulated phosphorylation of S6 via the ERK1/2 pathway using U0126. Moreover, we showed that the PepT1 sustained protein expression is induced by the activation of S6 by leptin.

Taken together, these data show, *in vitro*, that leptin regulates PepT1 levels and activity at the transcriptional level, via the MAPK pathway, and at the translational level, via ribosomal protein S6 activation. We have also shown, *in vivo*, that PepT1 levels and activity are down-regulated in hyperleptinemic states of leptin resistance, such as obesity. Thus, obesity may be associated with decreases in oligopeptide absorption and the bioavailability of peptidomimetic drugs. These findings are of major interest because protein intake can induce satiety. Protein-induced satiety is supported by the aminostatic hypothesis developed by Mellinkoff *et al.* (37) in 1956 and has since been clearly demonstrated (38). Consistent with this hypothesis, Darcel *et al.* (39) showed that the activation of the vagal afferents known to induce satiety depends on PepT1. Further studies are now required to investigate the potential effects of PepT1 regulation on food intake in obesity.

Acknowledgment—We thank Lisa McErlean for careful reading of the manuscript.

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