

Basal leptin regulates amino acid uptake in polarized 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 whether leptin acting from the basolateral side could also regulate amino acids uptake.

Methods Tritiated Gln and β -Ala were used to measure uptake into Caco-2 cells grown on filters, in the presence of basal leptin at short incubation times (5 and 30 min) and after 6 h of pre-incubation with the hormone. In order to compare apical and basal leptin effect, Gln and β -Ala uptake was measured in the presence of leptin acting from the apical membrane also in cells grown on filters.

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 exposure, and the effect was maintained after long pre-incubation 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 [26] which regulates food intake and energy expenditure by providing afferent signals to the hypothalamus [23, 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, 21, 7]. Leptin can be also produced by different peripheral tissues such as stomach, salivary glands and kidney [10, 3, 22, 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 its different regulation pattern have been described in Caco-2 cells [8]. All this information explains the well documented physiological role of the 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 co-transporter (SGLT-1) in the brush border membrane [2, 11, 13, 18-20]. Similarly, leptin decreases neutral amino acid transport by the regulation of ASCT2, B⁰AT1 and PAT1 transporters [12, 13]. Interestingly, uptake of other nutrients such as dipeptides, butyrate or fructose is increased by the hormone acting from the apical membrane [4, 5, 24].

Since leptin receptors are expressed in the basolateral membrane of the enterocytes [25, 3] systemic leptin may also have a role on nutrients absorption, however, the studies are scarce and no effect has been found [4]. Therefore, the aim of the present work was to

extend our previous studies to investigate whether leptin acting from the basolateral plasma membrane can regulate amino acid uptake by the enterocytes.

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% CO²-95% at 37 °C. Cells (passages 50-70) were grown in Dulbecco's Modified Eagles medium (DMEM) (Gibco Invitrogen, Paisley, UK) supplemented with 10% fetal 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 cm² 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 post-seeding.

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 β-[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 CaCl₂, 1mM MgSO₄, 0.3 mM NaH₂PO₄, 137 mM NaCl, 0.3 mM KH₂PO₄, 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 starting the uptake experiment. In some assays, cells were pre-incubated

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 [17] and was used in our previous studies [13, 20]. After an incubation period of 5 or 30 min at 37 °C, uptake was stopped with ice-cold free-substrate buffer. Then, cells were 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^+ -containing buffer (Krebs modified buffer) at pH 6, which is the optimal condition for PAT1 transporter activity [13]. Glutamine presence in the culture medium did not affect the uptake of the analysed substrates [13].

Statistical analysis

Results of transport experiments are expressed as $\text{nmol}\cdot\text{mg}^{-1}$ 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]. To investigate 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 than those grown on filters and, therefore, could differently express membrane proteins. In order to compare apical and basal leptin effect on the amino acids uptake, we studied 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. When the incubation time was 30 min, the inhibitory effect increased up to ~40 % in those cells in which leptin was acting on the apical membrane, and remained at 20% when the hormone was present in the basal compartment. In the case of 1mM β -Ala, the magnitude of the inhibition was also ~20 % in all conditions except when leptin was present in the basal compartment for 5 min, where no effect was observed (figure 1b).

These results demonstrated that leptin inhibits in a short-time manner Gln and β -Ala uptake acting from the apical or basal membrane of Caco-2 cells grown on filters, although the effect from the basal membrane in some experimental conditions seemed to be less powerful than the effect from the apical membrane. 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 a similar expression of the implicated proteins.

Since the basolateral membrane of the enterocytes is continuously exposed to the circulating leptin while, exposure of the brush border membrane to gastric leptin is shorter (systemic vs. postprandial leptin), we decided to investigate leptin effect on the amino acids uptake after 6 h pre-incubation of the cells with 8 nM leptin present in the basal compartment. Figure 2 shows that leptin again inhibited uptake of 0.1 mM Gln and 1 mM β -Ala (5 min) 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 when leptin was removed from the intestinal lumen [19]. 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 pre-incubated for 15 min in the absence (control) or in the presence of 8 nM leptin present 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 15 min. As shown in figure 3, leptin inhibited the uptake of both amino acids by ~15 % acting 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 pre-incubated 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 of the enterocyte was rapidly 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 decrease on the export of triglycerides to the basolateral medium and reduced the output of *de novo*-synthesized apolipoproteins [25]. 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 Gln and β -Ala uptake. The magnitude of the inhibition is similar to that obtained with apical leptin [13], and lower than 40 %, which is the maximum percentage of inhibition of nutrients transport by leptin found [2, 12, 13, 18-20].

Apical leptin diminishes Gln uptake by decreasing the amount of the Gln transporters, ASCT2 and B⁰AT1, in the apical membrane of the absorptive cells with the involvement of a PKA-dependent pathway [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 action of leptin acting from the basal membrane would also involve short-term post-translational mechanisms.

Interestingly, basal leptin inhibition of Gln uptake at 30 min was lower than the inhibition found when leptin was present in the apical side. On the other hand, leptin from the basal side did not inhibit β -Ala uptake after 5 min. Other authors have reported also in Caco-2 cells that apical leptin (100 nM) increased transport of dipeptides within 15 min, whereas basolateral leptin did not have any effect, suggesting the activation of different intracellular pathways [5]. The differences of apical and basal leptin could be related to differences on the regulation of the leptin receptors in each membrane. In this regard, a recent study has demonstrated in Caco-2 cells that the same stimuli (nutrients, hormones or inflammatory agents) added to the apical or basolateral medium have opposite effects on leptin receptors gene and protein expression [8]. The authors point out that these differences could be explained by the distinct characteristics of the apical and basolateral membrane such as the lipid composition, physicochemical properties and fluidity [16]. Moreover, they suggest that the origin of leptin could also be determinant in the response to the hormone, since the luminal exocrine leptin comes

from gastric Chief cells while the basal leptin is mainly synthesized in the adipose tissue [8]. Thus, *in vivo*, the apical membrane is exposed to leptin only during the post-prandial periods, whereas the basolateral membrane is continuously exposed to plasma leptin, which may regulate leptin receptors expression in a different way. Finally, in comparison with the apical receptors basolateral receptors, and therefore the intracellular signalling cascade is located further away from the apical membrane where the target transporters are located. .

On the other hand, studies with whole intestinal tissue in Ussing chambers have demonstrated that the inhibition of glucose uptake observed after leptin addition to the serosal compartment, requires the presence of active CCK-2 receptors, indicating that basal leptin action on sugar absorption needs the implication of an intermediate molecule. [11]. More studies are needed to understand in physiological conditions whether leptin, acting from the basolateral membrane, needs or not the presence of intermediary molecules to inhibit intestinal amino acid absorption.

Surprisingly, pre-incubation of the cells with basal leptin for 6 h did not increase the degree of inhibition (40 %) indicating that, although leptin can decrease ASCT2 and B⁰AT1 gene 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 in rat intestine *in vivo* that leptin inhibition

of galactose absorption is reversible [19] and support the involvement of post-translational mechanisms on the regulation of the amino acid transporters by leptin.

The fact that both, apical and basal leptin, can modulate nutrients absorption in a similar manner would suggest that the role of leptin in the processing of nutrients by the enterocytes is essential for the organism. Apical leptin would act during post-prandial state [7] whereas basal leptin could be reinforcing apical leptin effect, maybe mainly in obese individuals in which leptin levels are high [17].

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 exposure and after long pre-incubation 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.

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Disclosures

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

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Legends to figures

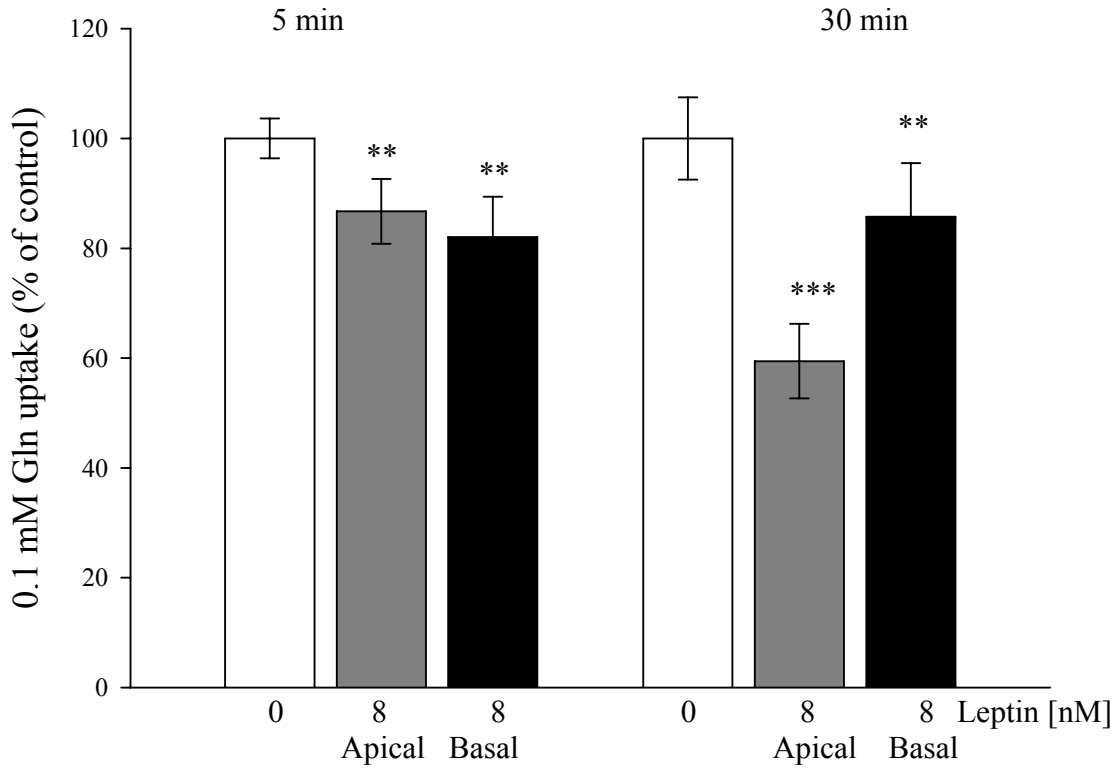
Fig. 1 Effect of apical and basal leptin on Gln and β -Ala uptake by Caco-2 cells grown on filters. (a) Uptake of 0.1 mM Gln (pH 7.4) and (b) 1 mM β -Ala (pH 6) in Na^+ medium was measured in the absence and in the presence of apical or basal leptin (8 nM), after 5 and 30 min incubation. Data (n = 12–33) are expressed as % (mean \pm 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 pre-incubation 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 pre-incubation of the cells with 8 nM leptin present in the basal compartment. Data (n = 18–20) are expressed as % (mean \pm 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 (grey bars) cells were pre-incubated for 15 min with the hormone before the 15 min uptake in its presence. In the reversion group (black bars) cells were pre-incubated for 30 min with the hormone before performing the 15 min uptake in its absence. Data are expressed as % (mean \pm 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



b

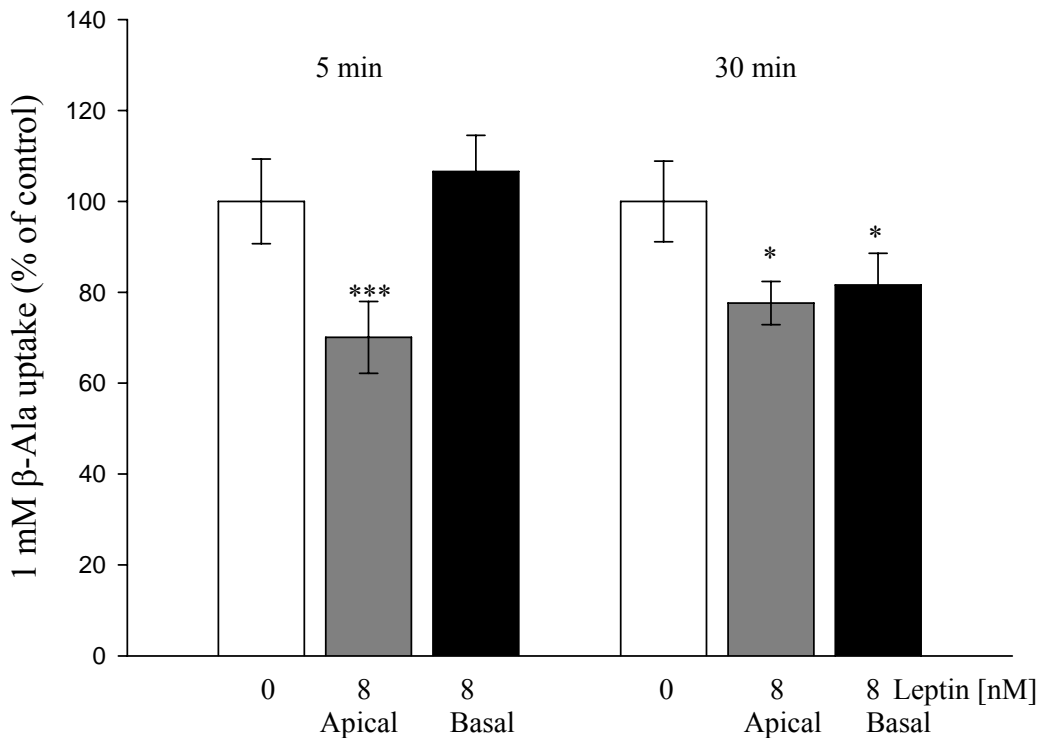


FIGURE 2

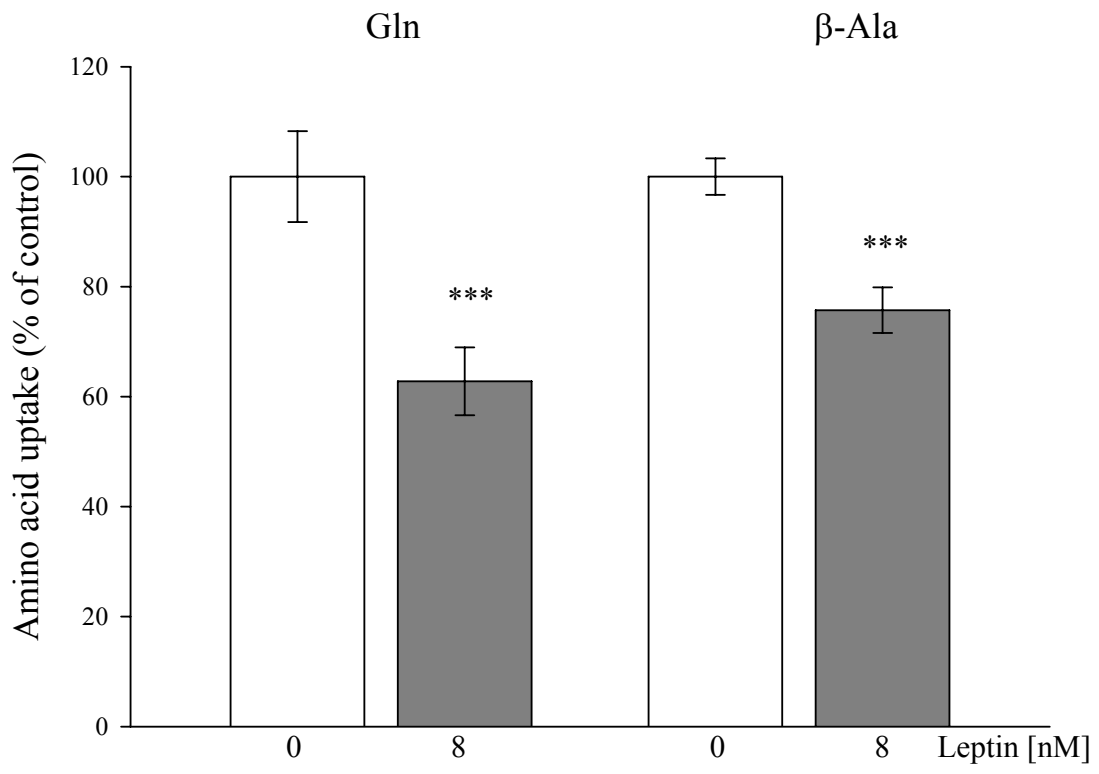


FIGURE 3

