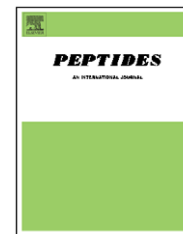


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Differential BBB interactions of three ingestive peptides: Obestatin, ghrelin, and adiponectin

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ABSTRACT

Endogenous compounds, including ingestive peptides, can interact with the blood-brain barrier (BBB) in different ways. Here we used in vivo and in vitro techniques to examine the BBB permeation of the newly described satiety peptide obestatin. The fate of obestatin in blood and at the BBB was contrasted with that of adiponectin. By the sensitive multiple time-regression method, obestatin appeared to have an extremely fast influx rate to the brain whereas adiponectin did not cross the BBB. HPLC analysis, however, showed the obestatin result to be spurious, reflecting rapid degradation. Absence of BBB permeation by obestatin and adiponectin was in contrast to the saturable transport of human ghrelin reported previously. As a positive control, ghrelin showed saturable binding and endocytosis in RBE4 cerebral microvessel endothelial cells. By comparison, obestatin lacked specific binding and endocytosis, and the small amount internalized showed rapid intracellular degradation before the radioactivity was released by exocytosis. The differential interactions of obestatin, adiponectin, and ghrelin with the BBB illustrate their distinctive physiological interactions with the CNS.

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1. Introduction

Among ingestive peptides that decrease feeding, some readily cross the blood-brain barrier (BBB) by a saturable transport system. These include insulin [1], leptin [2], activated urocortin [11], mahogany (1377-1428) [6], and galanin-like peptide (GALP) [8]. This is in contrast to most ingestive peptides that increase feeding (with the exception of ghrelin discussed below), which do not cross the BBB by a saturable transport system. These include neuropeptide Y (NPY) [4], orexin [5], agouti-related protein (AgRP) (83-132) [7], melanin-concentrating hormone (MCH) [9], and glucagon-like peptide (GLP)-1 [10]. If a structural function relationship in the permeation of these peptides across the BBB could be identified, this would facilitate the design of synthetic analogs for therapeutic intervention of feeding behavior.

The 23 amino acid peptide obestatin is the newest member of the endogenous ingestive peptide family [21]. The primary structure of obestatin is a homologous sequence conserved among species in preproghrelin, downstream to the appetite-stimulating peptide ghrelin [21]. Purified from the rat stomach, obestatin binds to the orphan G-protein coupled receptor GPR39, which is present in the brain [14]. Obestatin decreases food ingestion after peripheral administration, as opposed to ghrelin, which increases feeding. As the original paper focused more on biochemical assays rather than behavioral studies, it is not certain whether obestatin acts mainly in the hypothalamus, reducing the drive for ingestion, or in the area postrema and other regions, affecting taste aversion. The hypothalamus is separated from peripheral circulation by the BBB whereas the area postrema is not [19].

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In this study, we determined the pharmacokinetic features of obestatin and adiponectin, another peptide that suppresses feeding, in adult mice. Ghrelin, which has previously been shown to penetrate the BBB *in vivo* [3], was used for additional experiments. Adiponectin and ghrelin have opposite effects on feeding behavior [15,20]. We also tested for the presence of a possible specific transport system for obestatin in cerebral microvessel endothelial cells in culture. The findings should determine whether obestatin and adiponectin, the newly appreciated ingestive peptides from the periphery, exert significant CNS actions by permeating the BBB.

2. Materials and methods

C57 mice, 5–6 weeks old, were bred in our facility and used according to the protocol approved by the Institutional Animal Care and Use Committee. The mice were studied after anesthesia induced by intraperitoneal injection of ketamine and xylazine. Mouse/rat obestatin and human ghrelin were obtained from Phoenix Pharmaceuticals (Belmont, CA), and mouse adiponectin from Apotech (San Diego, CA). All other reagents were from Sigma (St. Louis, MO). Obestatin was radioactively labeled (radiolabeled) with ^{125}I by the chloramine-T method, the reaction being stopped at 1 min by addition of sodium metabisulfite. The specific activity of ^{125}I -obestatin was about 90–130 Ci/g for different experiments. Ghrelin and bovine serum albumin were radiolabeled with ^{131}I by the chloramine-T method with specific activities of about 20 Ci/g. Mouse adiponectin was radiolabeled with ^{125}I by the chloramine-T method with a specific activity of about 120 Ci/g. These peptides were purified by elution on Sephadex G-10 columns, and the purity was verified by both acid precipitation and reversed phase high performance liquid chromatography (HPLC).

2.1. Multiple time-regression analyses

To determine whether obestatin enters the mouse brain and whether it does so by saturable transport, the following four groups of mice were studied (8 mice/group): (1) ^{125}I -obestatin only; (2) ^{125}I -obestatin with the inclusion of 1 μg /mouse of unlabeled obestatin; (3) ^{125}I -obestatin with the inclusion of 1 μg /mouse of unlabeled human ghrelin; and (4) ^{125}I -obestatin with the inclusion of 1 μg /mouse of unlabeled leptin. About 30,000 cpm/ μl of ^{125}I -obestatin was delivered to the isolated left jugular vein in 100 μl of lactated Ringer's with 1% BSA (LR/BSA) at time 0. At various times between 1 and 20 min, blood was collected by dissection of the right common carotid artery. The mouse was decapitated immediately afterward. The radioactivity was measured in the whole brain and 50 μl of serum, and the brain/serum ratio of ^{125}I -obestatin and ^{131}I -albumin in each gram of brain was calculated separately. Based on the exponential decay pattern of serum radioactivity, the exposure time was calculated for each time point. The exposure time is the integral of serum radioactivity over time divided by the serum radioactivity at a given time [12]. The linear regression correlation between the brain/serum ratio and exposure time was determined with Prism GraphPad Statistical Software (San Diego, CA). The unidirectional influx

transfer rate (K_i) was determined from the slope of the linear regression line, and the initial volume of distribution (V_i) was determined from the intercept. Differences in regression lines between the groups were compared by the least square method with the GraphPad program.

2.2. Capillary depletion

Two groups of mice were studied ($n = 4/\text{group}$): those receiving ^{125}I -obestatin and ^{131}I -albumin *i.v.*, with blood collection from the abdominal aorta and decapitation 10 min later, and those receiving ^{125}I -obestatin and ^{131}I -albumin *i.v.*, with blood collection after intracardial perfusion of 30 ml of PBS and decapitation at 10 min. The cerebral cortex of the mouse was dissected, weighed, and homogenized in capillary buffer followed by thorough mixing with 26% dextran as described previously [16]. The homogenate was centrifuged at $9000 \times g$ for 30 min at 4°C to separate the capillary and brain parenchyma. Radioactivity was measured in the total cortex, parenchyma, and capillaries, and that in the cerebral vasculature was calculated by comparison of the values of perfused and non-perfused brains.

2.3. Degradation assays of mouse samples by HPLC

Each mouse received an *i.v.* injection of 3–4 μCi of ^{125}I -obestatin in 100 μl of injection at time 0. At 10 or 20 min, arterial blood and brain were obtained and processed on ice. The brain was homogenized in 1 ml of LR/BSA containing Complete Protease Inhibitor cocktail (Sigma). To correct for *in vivo* degradation, a processing control was generated by adding ^{125}I -obestatin into the blood-collection tube and brain homogenate. About 30,000 cpm of brain supernatant or serum was used for reversed phase HPLC. The positive control consisted of ^{125}I -obestatin stock solution immediately after radiolabeling. The mobile phase of HPLC was acetonitrile with 0.1% trifluoroacetic acid increasing from 10 to 70% over 40 min. One milliliter fractions were collected.

2.4. Binding and endocytosis assays in RBE4 cerebral microvessel endothelial cells

RBE4 cells (kind gift from Dr. Pierre-Olivier Couraud, Institute of Cochin, Paris, France) were grown to confluency in collagen-coated 12-well plates as described previously. Triplicates of wells were used for each time point in each group. For binding assays, the cells were kept on ice during the entire course of the study (3 h). For endocytosis assays, the cells were pre-equilibrated in transport buffer ($\alpha\text{MEM} + \text{F10}$ with 20 mM of HEPES and 1% of bovine serum albumin, pH 7.4) 30 min before the radiotracers were added. At time 0, ^{125}I -obestatin and ^{131}I -ghrelin (about 200,000 cpm/ml each) were added in 0.38 ml of transport buffer. The plates were incubated in a shaking water bath at 37°C for the desired time points (0, 5, 10, 20, 30, and 60 min). For both types of assays, the radiotracer was quickly removed at the end of incubation, and the cells were washed with cold PBS to remove the unbound ligands. Cell surface binding was determined by the use of stop/strip buffer, and the amount of ligands internalized was determined after cell lysis and collection of the lysis buffer, as previously described

[18]. In two of the three groups, more than a 200-fold excess of unlabeled obestatin or ghrelin were included to determine potential saturable binding and endocytosis.

2.5. Intracellular degradation and release of endocytosed intact peptides into cell culture medium

RBE4 cells were allowed to internalize ^{125}I -obestatin and ^{131}I -ghrelin for 30 min under the same conditions as specified above. The cells were then quickly washed by PBS and a mild acid buffer (pH 5.0) and two quick washes with PBS which effectively removed cell-surface bound ligands [13]. Groups of cells were then incubated with transport buffer for different durations between 2.5 and 60 min. At the end of the study, the transport buffer was collected for acid precipitation and measurement of radioactivity. The amount of ^{125}I -obestatin and ^{131}I -ghrelin remaining inside the cells was determined after incubation of cells with lysis buffer. The percent peptide exocytosed was determined by the radioactivity recovered from the transport buffer divided by the total radioactivity from the transport buffer and cell lysate. The percent intact peptide in the exocytosed fraction was determined from the acid precipitable radioactivity in the transport buffer. Acid precipitation involved the addition of an equal amount (1 ml) of 30% NaCl-over-saturated trichloroacetic acid to the buffer. The vigorously mixed solution was kept on ice for 15 min and centrifuged at $3500 \times g$ for 15 min at 4°C . The precipitate and supernatant were carefully separated and the radioactivity measured.

2.6. Statistical analyses

For multiple-time regression analysis, the differences among regression lines were determined with the least-squares method from Prism GraphPad software. For single-time measures, group means were expressed with standard errors, and the overall difference among groups was determined by analysis of variance, followed by Tukey's post-hoc test where applicable.

3. Results

3.1. Apparent influx of ^{125}I -obestatin from blood to brain contrasted with adiponectin

After i.v. injection of ^{125}I -obestatin, the influx rate of radioactivity from blood to brain was linear up to 20 min, at which time the exposure time was 28.7 min, due to the short plasma half-life of 2 min. The influx transfer constant was $1.86 \pm 0.15 \mu\text{l/g min}$ and the initial volume of distribution was $18.53 \pm 3.17 \mu\text{l/g}$. Addition of $1 \mu\text{g}/\text{mouse}$ of unlabeled obestatin (over 100-fold excess) to the ^{125}I -obestatin injection solution did not significantly affect the rate of entry. Similarly, addition of $1 \mu\text{g}/\text{mouse}$ of unlabeled human ghrelin or mouse leptin to the ^{125}I -obestatin injection solution did not significantly affect the rate of entry (Fig. 1).

The rapid rate of uptake of radioactive material in mice receiving ^{125}I -obestatin was in contrast with the minimal entry of ^{125}I -adiponectin, which showed an influx rate of $0.016 \pm 0.079 \mu\text{l/g min}$. Excess unlabeled adiponectin failed to

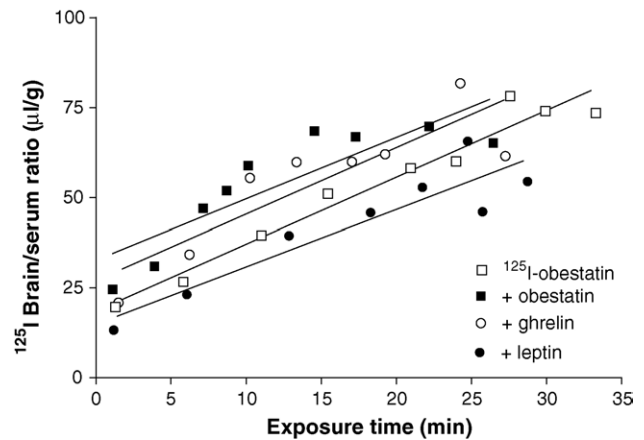


Fig. 1 – The influx rate of radioactivity from blood to the brain was determined by sampling mice 1–20 min after i.v. injection of ^{125}I -obestatin. The brain/serum ratio showed a linear regression correlation with the exposure time corresponding to the theoretical steady-state value calculated for each time point. The influx was rapid and not modulated by unlabeled obestatin, ghrelin, or leptin co-administered.

modulate this insignificant influx (Fig. 2). However, ^{125}I -adiponectin was extremely stable in the circulation and more than 97% was acid precipitable at 30 min after i.v. injection. There was no apparent redistribution of adiponectin with a serum half-life of over 5 min, shown by flat disappearance curves either in the absence or presence of excess unlabeled adiponectin (Fig. 2, inset).

3.2. Differentiation of the amount of radioactivity taken up by brain tissue from that trapped in the cerebral vasculature

At 10 min after i.v. injection of ^{125}I -obestatin and ^{131}I -albumin, most of the ^{125}I radioactivity was present in the parenchyma of

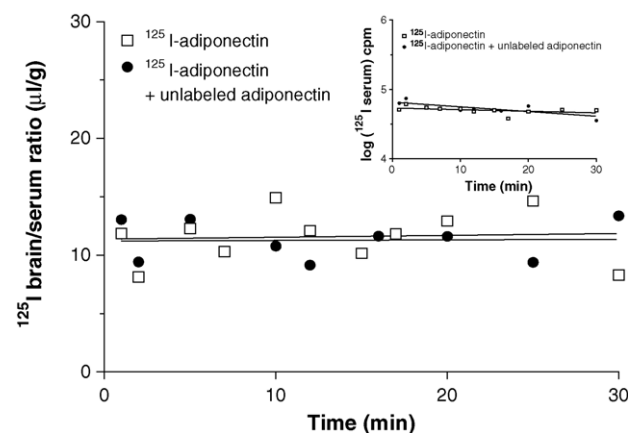


Fig. 2 – Adiponectin showed no significant entry into the brain with minimal volume of distribution and near-zero influx rate. Excess unlabeled adiponectin had no effect on this minimal influx. There was a long serum half-life in both groups with acid precipitable radioactivity being greater than 97% at 30 min (inset).

the cerebral cortex, over 40 times more than that of ^{131}I in the perfused brain. Intracardial perfusion did not affect the amount of ^{125}I in either brain parenchyma or capillary fraction. This was in contrast to the six-fold decrease of ^{131}I which indicated that most of the ^{131}I remained in the lumen of the microvessels (Fig. 3).

3.3. Degradation assay of mouse samples by HPLC

Ten minutes after i.v. injection, HPLC showed that intact ^{125}I -obestatin accounted for less than 15% of the total radioactivity in serum, similar to that in the serum of the processing control in which ^{125}I -obestatin was mixed with blood in the test tube (Fig. 4A). There was no intact ^{125}I -obestatin remaining in the serum samples 20 min after i.v. injection (Fig. 4B). The results indicate rapid degradation of circulating obestatin which obscured its possible permeation across the BBB to act in the brain.

3.4. Binding and endocytosis of obestatin and ghrelin in RBE4 cerebral microvessel endothelial cells

There was no substantial binding or endocytosis of ^{125}I -obestatin in RBE4 cells, and a 200-fold excess of unlabeled obestatin did not inhibit the minimal binding and uptake of ^{125}I -obestatin. There was no significant change in endocytosis over time. This is in contrast with ^{131}I -ghrelin in the same experiment, which had saturable binding and endocytosis as shown by the inhibitory effect of 200-fold excess unlabeled ghrelin (Fig. 5A and B).

3.5. Intracellular degradation and potential exocytosis

The radioactivity released into cell culture medium after endocytosis of ^{125}I -obestatin had significant degradation at each time point tested. This is in contrast with ^{131}I -ghrelin which mainly exited the cells in intact form (Fig. 6).

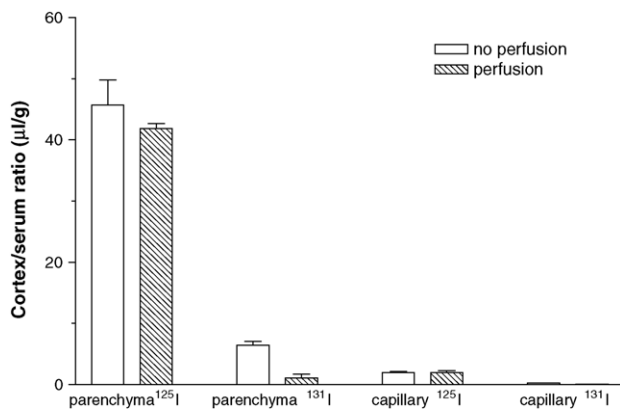


Fig. 3 – Capillary depletion performed 10 min after i.v. injection of ^{125}I -obestatin and ^{131}I -albumin showed that the majority of ^{125}I was recovered from brain parenchyma rather than retained in the capillary fraction, and that vascular perfusion did not cause a significant change in the distribution. This is in contrast to the low amount of ^{131}I in either compartment.

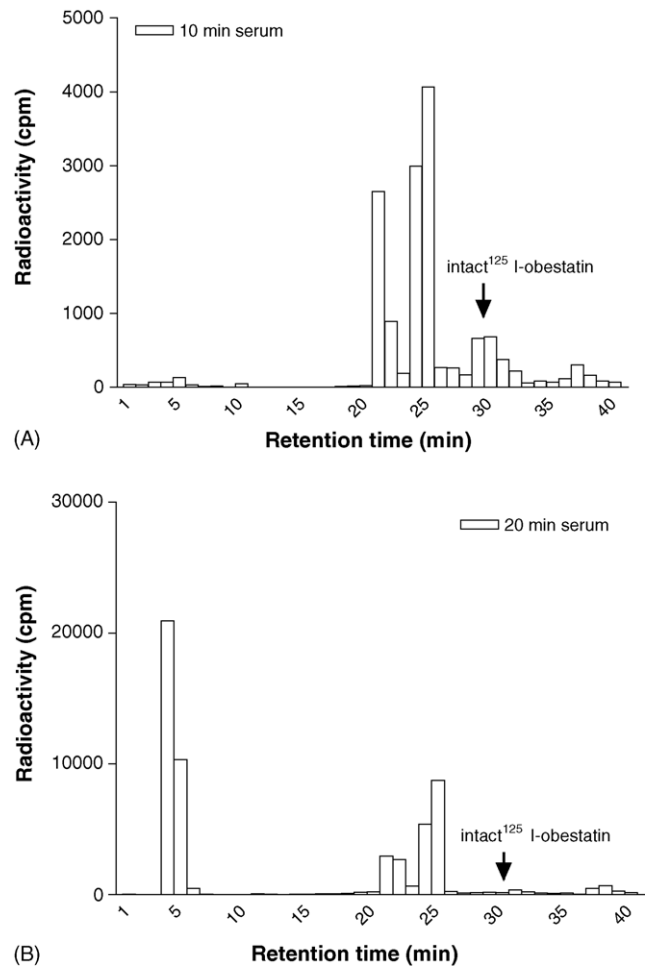


Fig. 4 – HPLC results showed that intact ^{125}I -obestatin accounted for only 14.2% of the total radioactivity recovered 10 min after i.v. injection (A), and nearly 0 at 20 min (B).

4. Discussion

Obestatin has a profound effect in reducing food intake and body weight after peripheral administration, although no greater than that of urocortin [21]. Therefore, it is possible that circulating obestatin acts on CNS targets by crossing the BBB. Serum obestatin concentrations are more than 0.3 ng/ml and not affected by fasting or refeeding [21]. Thus, we first tested whether circulating obestatin can cross the BBB by use of radiolabeled obestatin delivered by intravenous bolus injection.

When the amount of radioactivity in the brain was measured, ^{125}I -obestatin had a high apparent influx rate. The uptake by parenchyma was more than 20-times higher than that retained in the capillaries, and more than seven-times higher than that of the co-administered ^{131}I -albumin. The influx rate, $1.86 \pm 0.15 \mu\text{l/g min}$, was higher than that of most peptides and proteins we have tested [17]. However, this influx was not mediated by a specific transport system, since excess unlabeled obestatin failed to reduce the influx, and the vascular perfusion procedure used in the capillary depletion

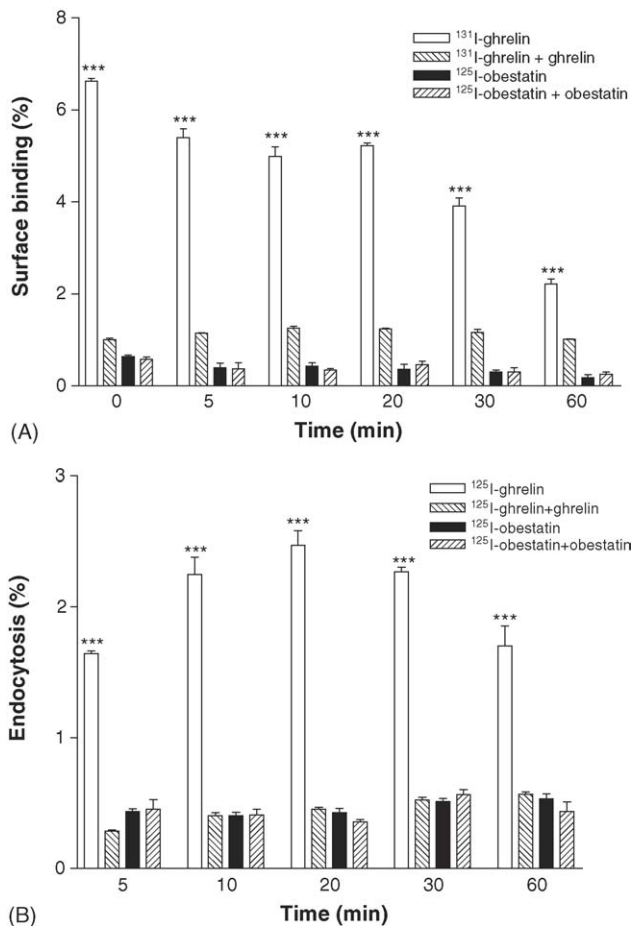


Fig. 5 – (A) Cell surface binding of ^{131}I -ghrelin was 6.6% at time 0 and was reduced to 2.2% 60 min after initiation of endocytosis. Excess unlabeled ghrelin (200-fold) caused significant ($p < 0.005$) reduction of surface binding at each time tested. The binding of ^{125}I -obestatin was negligible and not modified by a 200-fold excess of unlabeled obestatin. (B) About 2% of ^{131}I -ghrelin could be endocytosed at the times studied, and such endocytosis was significantly ($p < 0.005$) inhibited in the presence of 200-fold excess of unlabeled ghrelin. ^{125}I -obestatin showed minimal endocytosis and lack of inhibition by excess unlabeled obestatin.

studies did not decrease the amount of radioactivity in the brain. When the stability of the peptide was further tested by HPLC, it was shown that most of the ^{125}I -obestatin was degraded to smaller peptide fragments 10 min after i.v. injection into the circulation. At 20 min, the majority of radioactivity recovered from blood was free ^{125}I .

This is in contrast to the extreme stability of adiponectin in blood. The longer half-life and greater stability of adiponectin were consistent with its blood concentration being many times higher than that of obestatin. In our experiments, the serum level of adiponectin was $24.8 \pm 1.25 \mu\text{g/ml}$, whereas the reported value for obestatin is about 0.32 ng/ml [21]. However, we could not show that obestatin or adiponectin crossed the BBB in mice. By contrast, human ghrelin possesses a moderate but specific saturable transport system to permeate the BBB in

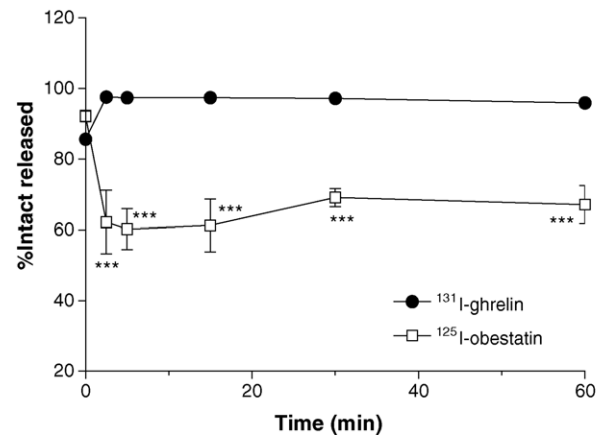


Fig. 6 – While only intact ^{131}I -ghrelin was internalized as shown by the nearly 100% acid precipitability of the radioactivity exocytosed over a time course of 2.5–60 min, ^{125}I -obestatin showed significant intracellular degradation compared with the stock solution added before the 30 min endocytosis ($p < 0.005$).

mice [3]. The different behaviors of these ingestive peptides in their interactions with the BBB illustrate the various mechanisms of action of feeding-related signals.

Since the instability of obestatin in blood does not exclude the presence of a saturable transport system, we performed further studies on RBE4 cerebral microvessel endothelial cells. Human ghrelin, which crosses the rodent BBB by a specific transport system [3], was used as a positive control. About 6.6% of ^{131}I -ghrelin showed specific binding to RBE4 cells, and the maximal potential internalization was highest at 20 min of endocytosis (7.6%). After this time there was decrease of available ghrelin from the cell surface for internalization, suggesting a rapid receptor turnover. Both binding and endocytosis of ^{131}I -ghrelin were inhibited in the presence of 200-fold excess unlabeled ghrelin, indicating the presence of a saturable system. By contrast, ^{125}I -obestatin had less than 1% cell surface binding; endocytosis was not increased from the minimal baseline at 37°C . Neither process showed a change after addition of more than 200-fold excess of unlabeled obestatin. This is consistent with the findings in mice that obestatin is rapidly degraded and does not have specific uptake by the endothelial cells composing the BBB.

In summary, neither obestatin nor adiponectin crosses the BBB by a specific transport system. Obestatin was rapidly degraded in the circulation, but adiponectin was very stable. Human ghrelin exhibited saturable binding and endocytosis in the RBE4 rat cerebral microvessel endothelial cell line, consistent with its specific transport across the mouse BBB as previously described by Banks et al. [3]. These pharmacokinetic experiments illustrate three different mechanisms of CNS interactions by three different ingestive peptides.

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