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Obestatin reduces food intake and suppresses body weight gain in rodents

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Abstract

Obestatin was recently described as a bioactive peptide encoded for by the ghrelin gene as ghrelin but with opposite actions on food intake. Although some groups have confirmed these findings others find no effect. We investigated the effect of obestatin on feeding in rodents over a wide range of doses. Acute administration of obestatin inhibited feeding at doses of 10–100 nmol/kg i.p. in mice and 100–300 nmol/kg i.p. in lean and Zucker fatty rats. Interestingly, the dose–response relationship was U-shaped such that both low and high doses were without effect in either species. Treatment of mice with obestatin over a 7-day period decreased body weight gain and food consumption. Overall, obestatin suppressed food intake and body weight gain in rodent and an unusual dose–response relationship was found. These findings may explain the difficulties reproducing the effects of obestatin on feeding reported by some groups.

Keywords: Obestatin; Ghrelin; Feeding; Body weight; Obesity; Endocrine

Obestatin is a recently identified 23 amino-acid peptide, which is derived from the preproghrelin gene [1]. This peptide was designated obestatin due to its inhibitory effects on feeding and, thus, it displays opposite actions to ghrelin. Obestatin was originally extracted from rat stomach and has subsequently been shown to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and growth hormone secretion [2]. In addition, obestatin levels are reduced in rats in which micro-gastric bypass surgery was performed, demonstrating that the stomach is a major source of obestatin [3].

In addition to effects on feeding and weight gain, obestatin was also shown to cause a sustained reduction in gastric emptying and reduced spontaneous contractile activity in the rat jejunum [1]. The in vitro and in vivo actions of obestatin on gut motility and feeding have also been re-investigated and, with the exception of two reports [4,5], no positive findings were described [6,9]. During this period, additional effects of obestatin have been described and it has been shown that obestatin causes a dose-dependent proliferation of human retinal pigment epithelial cell [10], has effects on memory and anxiety [5], elevates cytosolic calcium concentrations in populations of cortical neurons [11], centrally acts to inhibit thirst [12], alters sleep patterns in rats [13] and partially, but not completely, inhibits ghrelin stimulated food intake and ghrelin stimulation of growth hormone release in rats [2]. It should be noted that while the first description of obestatin suggested that GPR39 was the cognate receptor for obestatin [1] other groups have been unable to repeat this observation and, as such, the molecular mechanism for obestatin's effects in vivo remains controversial [14–17]. Therefore it seems that obestatin is a functionally active, circulating peptide, although its precise role has not been completely defined.

The aim of the studies described here was to investigate the actions of obestatin on feeding and body weight regulation in rodents. The protocols used included the thor-

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ough acclimatization of the animals prior to experimentation and utilization of a wide range of doses. The data obtained show that acute peripheral administration of obestatin produced a reproducible and significant decrease in food intake and reduces body weight gain after repeated administration. The inhibitory effects on feeding were observed in lean mice and rats, and also in Zucker fatty rats. These studies demonstrate that obestatin is a bioactive peptide that modulates feeding and body weight regulation over a narrow range of doses in vivo.

Materials and methods

Drugs and doses. Human obestatin was synthesized by California peptide Research, Inc. (Napa, CA, USA) and purified by reverse phase high performance liquid chromatography. The peptide sequences were verified by amino acid analysis and mass spectrometry. Obestatin stock solution was first dissolved in water and stored at −80 °C until use. Immediately before the experiment it was diluted in physiological saline to achieve the desired dose. The solubility of obestatin in the dosing solution was verified by HPLC and found to be between 95% and 99%. Fenfluramine was used as a positive control for anorexigenic activity. The doses of fenfluramine were selected based on previous reports [18,19] and our own in-house pilot studies. Ghrelin (Bachem, Torrance, CA, USA) was included to further evaluate the robustness of the feeding response and to allow evaluation of any functional antagonism of obestatin-induced responses. Doses of ghrelin were selected from the literature [20,21] and in-house pilot studies.

Animals. Experiments were conducted on adult male Sprague–Dawley rats weighing 250–300 g (age ~80 days), fatty Zucker rats (fa/fa) weighing 355–530 g and male lean mice (CD-1(ICR)BR) weighing 25–30 g (~35 days of age). Sprague–Dawley rats were obtained from Harlan (Livermore, CA, USA). CD-1(ICR)BR mice and Zucker (fa/fa) rats were obtained from Charles River (Hollister, CA, USA). Animals were housed at 21–23 °C under reverse light cycle conditions (lights off 7:00 AM–on 7:00 PM). A standard rodent chow and water were available ad libitum. Animals were handled daily for a period of 1 week by the same individuals that conducted the experiments for at least 10 days prior to the experiment. Animals were also accustomed to the fasting process and to the intraperitoneal injection (i.p.) procedure by administration of saline (0.1 mL for mice and 0.5 mL for rats) for 2 days prior to the initial study [22]. Animals were grouped housed for all feeding studies. All procedures and experiments were carried out in accordance with the internationally accepted guidelines for the care and the use of the laboratory animals in research and were approved by the local IACUC.

Experimental protocol for acute studies. Fifteen minutes after treatment a pre-weighed amount of rodent chow was made available to the animals. The food remaining at 1, 3 and 5 h later was weighed. The amount of food spilled by the animals was also collected by weighing the crumbs collected at the bottom of the animal cage. The weight of crumbs was subtracted to get a more accurate measurement of the animal's food consumption. This correction was applied only to the final 5 h time point data. In all cases, food consumption was expressed as the amount of food consumed per animal. Two dose–response studies were conducted in mice. The first was done in an un-blinded fashion while the second study was conducted in a blinded fashion such that the investigators did not know which animals received which treatment.

A dose–response relationship for ghrelin was evaluated in mice using the same methods described above. Mice and rats were fasted overnight and received one of the following treatments: 100 nmol/kg ghrelin i.p., 100 nmol/kg obestatin, or the combination of ghrelin and obestatin (both 100 nmol/kg; two i.p. injections at 2 min interval, first ghrelin followed by obestatin). The food consumption was measured 1, 3 and 5 h later.

Experimental protocol for repeated administration of obestatin. The effects of obestatin on food consumption and body weight in lean mice were evaluated after repeated injections of obestatin. Mice were fed ad libitum and each cage of 5 mice was randomly assigned to the following treatment groups: vehicle, obestatin (10 and 100 nmol/kg i.p.) and fenfluramine (18 μmol/kg i.p.). Animals were dosed i.p. three times per day (approximately every 8 h) for 7 days after which animals were monitored for a further 4 days before they were euthanized. Body weight and food intake were measured daily at the end of the animals' sleep cycle.

Statistical analysis. All values reported are mean ± SEM. Results were analyzed for statistically significant difference using ANOVA, followed by a Bonferroni post-test. Differences were considered as significant when p values $\leqslant 0.05$.

Results

Obestatin inhibits food intake in mice and rats

Two series of studies were conducted to evaluate the effects of obestatin in mice. In an initial study, intraperitoneal injection of 10 and 100 nmol/kg significantly reduced food intake over a 5 h period in mice that were fasted overnight. These mice consumed on average 2.9 ± 0.2 g and 2.9 ± 0.1 g (respectively) vs. 3.9 ± 0.1 g in vehicle controls ($p < 0.05$). However, 1 nmol/kg dose of obestatin was without effect (3.5 ± 0.2 g, ns vs. control). As a result of this unexpected finding a second study was conducted in a blinded fashion using a wide range of obestatin doses. As in the first study, 10 and 100 nmol/kg doses of obestatin reduced food intake by ~25% (Fig. 1A). Lower (0.01–1 nmol/kg) and higher (1–3 μmol/kg) doses of obestatin did not reduce food intake relative to vehicle controls. Thus, the resulting dose–response curve was a U-shaped. Fenfluramine at a dose of 18 μmol/kg i.p. produced the expected reduction in food intake (31.2 ± 1.3%, $p < 0.05$ vs. vehicle control, Fig. 1A).

To investigate whether the effect of obestatin on food intake occurred across species, food intake was assessed in rats at doses that were found to be effective in mice. Obestatin reduced food intake at doses of 100 and 300 nmol/kg by 19.8 ± 3.5% and 19.6 ± 2.8%, respectively, when compared to vehicle-treated control rats (Fig. 1B, $p < 0.05$). However, 1 and 3 μmol/kg dose of obestatin were without effect compared to vehicle control (Fig. 1B).

Ghrelin and obestatin exhibit functional antagonism for effects on food intake in mice and rats

The effect of ghrelin (10–300 nmol/kg) on food intake was investigated using the same methods in mice and rats. As expected, ghrelin increased food intake in a dose-dependent fashion in both species (Fig. 2). A dose of 100 nmol/kg i.p. ghrelin increased food intake from 3.1 ± 0.1 g to 4.1 ± 0.3 g in mice and from 13.0 ± 0.3 g to 15.5 ± 0.6 g in rats (both $p < 0.05$). This dose was used for further studies.

When administered together in a cross-over experiment in mice and rats, ghrelin (100 nmol/kg) and obestatin (100 nmol/kg) counteracted each other's action such that no difference relative to vehicle control was observed

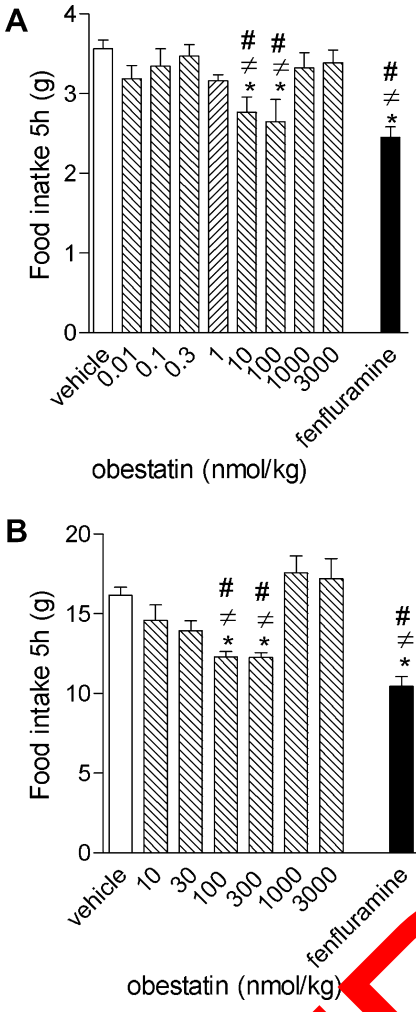

Fig. 1. Dose–response relationship for obestatin's effect on food intake in mice and rats. Food intake over 5 h was measured and expressed as means ± SEM. (A) Food intake in mice vehicle-treated (first column) or mice treated with different doses i.p. of obestatin (0.1 nmol/kg to 3 μmol/kg). The final column shows the response to 18 μmol/kg fenfluramine i.p. $n = 4$–5. (B) Food intake in rats in vehicle-treated rats (first column) or rats treated with 10 nmol/kg^{-3} μmol/kg obestatin i.p. $n = 6$–12. The final column shows the response to 18 μmol/kg fenfluramine i.p. $n = 3$. $^{*}p < 0.05$ compared to vehicle control; $^{\neq}p < 0.05$ compared to 1 μmol/kg dose of obestatin; $^{\#}p < 0.05$ compared to 3 μmol/kg dose of obestatin.

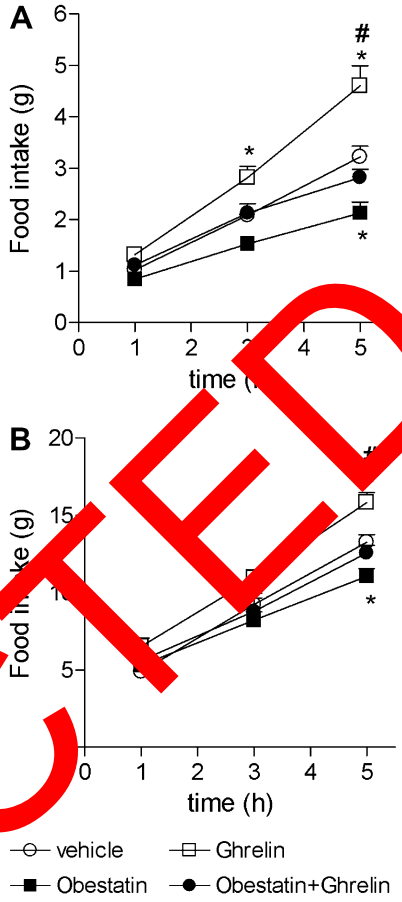

Fig. 2. Effect of equal molar doses of obestatin and ghrelin in mice and rats. Food intake was measured 1, 3 and 5 h after administration of vehicle control, 100 nmol/kg obestatin, 100 nmol/kg ghrelin in mice (A, $n = 8$) and rats (B, $n = 8$–10). Values are mean ± SEM. $^{*}p < 0.05$ compared to vehicle control; $^{\#}p < 0.05$ compared to the combination of ghrelin and obestatin.

($p > 0.05$, [illegible]). When administered alone in this experiment obestatin and ghrelin had the actions previously described.

Acute effect of obestatin in obese Zucker falfa rats

This experiment was conducted to investigate whether obestatin inhibited food intake in a rat model of obesity. Zucker *falfa* rats were fasted overnight and administered a single 300 nmol/kg i.p. dose of obestatin or vehicle control. Food intake was measure over 5 h. Food intake over this time period was significantly reduced (obestatin-treated: 8.7 ± 0.3 g vs. control group: 9.8 ± 0.4 g, $n = 11$, $p < 0.05$).

Effect of administration of repeated doses of obestatin on food intake and body weight in mice

To evaluate the effect of obestatin on food consumption and body weight after repeated administration, obestatin (10 and 100 nmol/kg) was administered three times a day for 7 days. A once daily dose of fenfluramine (18 μmol/kg i.p.) was used as a positive control. These animals were administered vehicle control for the other two daily doses. Both doses of obestatin reduced food consumption and body weight gain over the course of the 7-day study (Fig. 3). On day 4 of the study, food intake was significantly reduced by obestatin (control, fenfluramine, 10 and 100 nmol/kg obestatin: 5.2 ± 0.3, 3.4 ± 0.3, 3.5 ± 0.1 and 3.0 ± 0.2 g, respectively, all $p < 0.05$ vs. control). After discontinuing treatment with obestatin and fenfluramine, animals progressively gained weight back to the vehicle-treated control level over the course of the 4-day monitoring period. This was associated with an increase in their food consumption. On day 10, mice in their respective groups consumed 4.9 ± 0.1, 4.3 ± 0.4, 4.1 ± 0.2 and

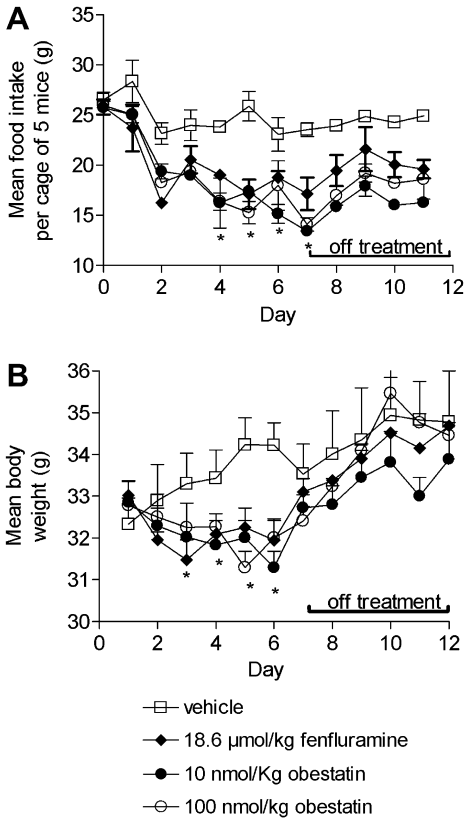

Fig. 3. Effect of repeated administration of obestatin over 7 days in mice. Mice were administered either 10 or 100 nmol/kg doses of obestatin i.p. three times a day for 7 days, a single daily dose of 18 μmol/kg fenfluramine (with vehicle control twice a day) or vehicle control 3 times a day for 7 days. Three cages of mice were included in each group. Values are mean ± SEM. $^{*}p < 0.05$ compared to vehicle control.

4.0 ± 0.2 g (control, fenfluramine, 10 and 100 nmol/kg obestatin), i.e., found constipation was no longer suppressed.

Discussion

Obestatin was recently identified as a bioactive peptide by an evolutionary genomics screening approach [1]. This peptide was originally reported as having effects on feeding in vivo, however a number of groups have recently reported that they are unable to reproduce the initial findings [10–13]. In the present work, we showed that the peripheral administration of obestatin reduced food intake and opposes the action of ghrelin in both mice and rats. In addition, chronic administration of obestatin reduced food consumption and body weight gain over 7 days of treatment. This magnitude of effect was similar to that induced by fenfluramine. Interestingly, peripheral administration of obestatin also reduced food intake in Zucker fatty rats.

A wide range of obestatin doses were examined for effects on feeding behavior in a fasted mouse model and an U-shaped dose–response relationship was found. Thus, low doses (0.01–3 nmol/kg) and high doses (1–3 μmol/kg) of obestatin were ineffective. However, a robust and reproducible reduction in food intake was observed at doses of 10 and 100 nmol/kg in mice. This result was confirmed in a subsequent experiment, which was conducted in a blinded fashion. These results are similar to those of Zhang et al. [1] but with an important difference in the effective dose. Zhang et al. found 1 μmol/kg to be an effective dose while in our hands it was not. The discrepancy of the effective dose between these two studies is not clear, it is conceivable that differences of the purity of the peptide batches initially used might affect the actual dose of obestatin administered [7]. Therefore, due to the nature of the dose–response curve, our data is consistent with reports describing no effect of 1 μmol/kg obestatin on feeding [11,23]. Bassil et al. [7] also reported a similarly unusual dose–response relationship for obestatin's effect on EFS-evoked, nerve-mediated contraction of the isolated rat forestomach. Our results also showed that in rat, the 100 nmol/kg dose reduced significantly food intake in comparison to vehicle control and 3 μmol/kg does not. These data corroborate the "U-shaped" dose–response relationship for obestatin in a second species. This type of dose–response relationship highlights the importance of evaluating a wide range of doses and may also explain the published difficulties in reproducing the effects of obestatin on feeding. Other factors such as the timing of its administration relative to presentation of the food might influence feeding behavior.

The effects of repeated administration of obestatin were examined in mice. Administration of 10 and 100 nmol/kg doses of obestatin three times a day was found to significantly decrease food intake and body weight gain over the course of a 7 day treatment period. Fenfluramine was used at a dose that in our hands caused an acute reduction in food intake by ~30%. In repeated dose studies with this compound, body weight was reduced by ~10% at a maximum dose of 20 mg/kg [18,19]. Again our positive findings may reflect the choice of dose of obestatin given. It is important to note that lower doses of obestatin were used in the present studies than were used by Zhang et al. [1]. Furthermore, a recent study [10] showing a lack of effect of obestatin on body weight changes utilized once daily injection of obestatin or a mini-pump delivery protocol (thus far we have also been unable to identify an effective mini-pump formulation, unpublished observation).

Given the evolutionary approach used to discover obestatin one would expect it to have similar actions across species. For this reason, further studies were conducted in the rat. Doses of 100 and 300 nmol/kg obestatin were also found to reduce food intake in lean and Zucker fatty rats while no effect was observed at high doses (1 and 3 μmol/kg). The results with obestatin in lean rats are similar to those previously reported with a ~127 nmol/kg dose i.p. [8]. Therefore, it is conceivable that the experimental conditions are also important to obtain positive responses with this peptide. Although we have not evaluated the influence of our experimental protocols on the results obtained, it may be important that the animals were group caged, thor-

oughly acclimatized by handling and to the fasting-feeding procedures and also given vehicle injections i.p prior to the initiation of these studies. Thus, the situation with obestatin is not dissimilar to that with PYY3-36 where some investigators find significant effects in rodents [24] while others do not [25]. The fact that PYY3-36 was ultimately shown to inhibit food intake in healthy volunteers [24] and in obese human subjects [26] highlights the need to conduct further studies with obestatin in humans subjects.

Functional antagonism between obestatin and ghrelin was assessed in feeding studies in both species using the doses found to be effective in our hands. Obestatin reduced food intake over 5 h (as discussed above) and ghrelin produced the expected increase in food intake. When the peptides were administered together in a cross-over study in both species no difference was found from vehicle controls. This indicates that these two products of the ghrelin gene can functionally antagonize each other's actions. Similar results were reported by Zhang et al. [1] and more recently by Zizzari and co-workers who, showed that obestatin, partially, but did not completely, inhibited ghrelin-induced food intake and also partially inhibited ghrelin stimulated growth hormone secretion [2].

In conclusion, our experiments have demonstrated that acute, peripheral administration of obestatin reduced food intake in lean mice and rats and that obestatin reversed ghrelin-induced increases in food intake. The reduction in food intake in mice was maintained after repeated administration of obestatin and mice treated three times daily with effective doses of obestatin lost weight relative to vehicle-treated animals. While it is not clear why our results differ from those obtained by other investigators, one issue that deserves further attention is the complex dose–response relationship for obestatin effects on food intake.

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