Impact of intracerebroventricular obestatin on plasma acyl ghrelin, des-acyl ghrelin and nesfatin-1 levels, and on gastric emptying in rats

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Abstract. Obestatin, which is a putative 23-amino-acid peptide, is derived from the C-terminal part of the mammalian preproghrelin gene. Nesfatin-1 mRNA is co-expressed with ghrelin in gastric endocrine X/A-like cells; therefore, nesfatin-1 may also interact with preproghrelin gene products in the stomach. In this study, we investigated the impact of obestatin on the plasma levels of acyl ghrelin, des-acyl ghrelin and nesfatin-1, and on the gastric emptying of a solid nutrient meal 2 h after an intracerebroventricular (ICV) injection in conscious, fasted rats. The rats were implanted with ICV catheters. Plasma levels of acyl ghrelin, des-acyl ghrelin and nesfatin-1, expected to be co-expressed with obestatin, were measured, whereas the human/rat corticotropin-releasing factor (h/rCRF) was applied as an inhibitor of gastric emptying. The ICV administration of obestatin (0.1, 0.3 and 1.0 nmol/rat) did not modify the plasma acyl ghrelin and des-acyl ghrelin levels, the acyl ghrelin/des-acyl ghrelin ratio and nesfatin-1 concentrations. The ICV acute administration of obestatin had no influence on the 2-h rate of gastric emptying of a solid nutrient meal, but the ICV h/rCRF injection delayed it. The weight of food ingested 1 h before ICV injection significantly, but negatively correlated with the gastric emptying of a solid nutrient meal. Our study indicates that the ICV injection of obestatin does not change the 2-h rate of gastric emptying of a solid nutrient meal and the relatively weak interrelationships between ghrelin gene products and nesfatin-1. However, the weight of the ingested food negatively affects the gastric emptying of a solid nutrient meal in conscious, fasted rats.

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Introduction

Obestatin, a putative 23-amino-acid peptide, is derived from the C-terminal part of the mammalian preproghrelin gene via a bioinformatics approach (1). Originally projected as a rival of acyl ghrelin to counteract acyl ghrelin-induced orexigenic effects on food intake (1), obestatin has been suggested to bind to an orphan G-protein coupled receptor (GPR), termed GPR39, and increase intracellular cAMP levels (1). High levels of GPR39 mRNA have been found in the amygdala, hippocampus and auditory cortex, but not in the hypothalamus (2,3). Previous studies have indicated that obestatin is not the endogenous cognate ligand for GPR39 (4-7), whereas only one study has suggested that a specific iodinated form of obestatin binds to GRP39 (8). If obestatin exists as an endogenous hormone, it most likely does not act through GPR39. The fact that the mRNA transcript of GPR39 has not been observed in the brain and that the antisense gene, LYDC, is highly expressed in the brain, suggests that GPR39 is critical for the functions of the liver, gastrointestinal tract, pancreas and white adipose tissue, but not for those of the brain (9). To date, the effects of obestatin on food intake remain debatable (10).

Obestatin has manifested various biological functions in the brain, e.g., the *in vivo* inhibition of thirst (11), increased non-rapid eye movement sleep episodes and decreased sleep latency (12), and improved memory retention and reduction of anxiety (13) in rats, as well as the *in vitro* activation of cortical neurons (14) and the stimulation of retinal pigment epithelial cell proliferation (15). Moreover, plasma obestatin levels are lower in obese (16), but higher in lean individuals (17), suggesting that obestatin may influence appetite and body composition in humans. Recent reports have revealed that plasma obestatin levels are lower in uremic patients than in healthy subjects, indicating that obestatin is implicated in the inflammatory state and in disturbances of the calcium/phosphate metabolism of hemodialysis patients (17,18). Taken together, these emerging data imply that obestatin may play a role in metabolism (19). In addition, *c-fos* expression in the paraventricular nucleus has been shown to increase after the peripheral injection of obestatin in brain slides (20). Thus, in order to validate the evidence for and against the existence

of obestatin as an endogenous hormone, further investigation is required. As feeding and gut motility are closely associated (21), a better understanding of the role of obestatin in gastric motility may help to elucidate its controversial function in feeding. Unfortunately, little information is available regarding the intracerebroventricular (ICV) influence on gastric emptying.

Aside from acyl ghrelin and des-acyl ghrelin, nesfatin-1 may also play a role in the stomach and may participate in active interactions with obestatin. Nesfatin-1/nucleobindin-2 mRNAs have been shown to be co-expressed with ghrelin in gastric endocrine cells (22). The interplay between either acyl ghrelin, des-acyl ghrelin or nesfatin-1 and obestatin is intriguing; however, the influence of ICV obestatin administration on the plasma levels of acyl ghrelin and des-acyl ghrelin, the acyl ghrelin/des-acyl ghrelin ratio and nesfatin-1 remains unknown. In the present study, we aimed to explore the ICV effects of obestatin on the plasma concentrations of acyl ghrelin, des-acyl ghrelin and nesfatin-1, and on the gastric emptying of a solid nutrient meal in conscious, fasted rats.

Materials and methods

Animals. Male Sprague-Dawley rats (National Laboratory Animal Center, Taipei, Taiwan), weighing 250-320 g at the initial period of the experiment, were used in group cages under controlled illumination (light cycle 08:00-20:00), humidity and temperature (22.5±1.5°C) with free access to water and laboratory chow pellets (LabDiet®, Brentwood, MO, USA). All experiments were initiated at 8 a.m. in freely moving conscious, fed rats, according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the Taipei Veterans General Hospital, Taipei, Taiwan.

Surgery. ICV catheter implantation was performed as described previously (23,24). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Nembutal; Abbott Laboratories, Abbott Park, IL, USA), placed in a stereotaxic apparatus and implanted with a guide cannula (25-gauge; Eicom, Kyoto, Japan) that reached the right lateral ventricle. Stereotaxic coordinates were 0.8 mm posterior to the bregma, 1.4 mm lateral to the midline and 4.5 mm below the outer surface of the skull using a stereotaxic frame (Benchmark™, myNeuroLab, St. Louis, MO, USA), with the incisor bar set at the horizontal plane passing through the bregma and lambda. The guide cannula was secured with dental cement anchored by two stainless steel screws fixed on the dorsal surface of the skull.

After surgery, a dummy cannula (Eicom) was inserted into each guide cannula. A screw cap (Eicom) was placed on the guide cannula to prevent blockage. Correctness of the ICV cannula placement was verified by injecting 100 µl dye (0.05% cresyl violet; Sigma) into the right lateral ventricle by the brain sections at the end of the experiments after euthanasia (25). Before beginning all food intake tests, the animals receiving an implantation of ICV catheters were allowed 7 days for recovery. All ICV injections were performed over a period of 60 sec in 5 µl using the AMI-5 (Eicom).

Preparation of drugs. Rat obestatin (Peptides International, Inc., Louisville, KY, USA) and human/rat corticotropin-releasing factor (h/rCRF) (American Peptide Company, Sunnyvale, CA, USA), were kept in powder form at -20°C and dissolved in sterile, pyrogen-free 0.9% saline (Otsuka, Tokyo, Japan) immediately before use.

Measurement of plasma acyl ghrelin, des-acyl ghrelin and nesfatin-1. Blood samples were obtained following decapitation 2 h after the ICV injection. Each blood sample was immediately transferred to a chilled glass tube containing disodium ethylenediaminetetraacetic acid (EDTA) (1 mg/ml) and aprotinin (500 U/ml), and stored in an ice bath. After centrifugation at 300 x g, the plasma was separated, dispensed into polypropylene tubes in aliquots and stored at -20°C until analysis. Enzyme immunoassays (EIA) for plasma acyl ghrelin (SPI-Bio, Montigny le Bretonneux, France), des-acyl ghrelin (SPI-Bio) and nesfatin-1 (Phoenix Pharmaceuticals, Burlingame, CA, USA) were conducted in a single batch run and in a blinded fashion. The assay protocol was carried out as previously described, and the only difference was the primary antibody (26).

Gastric emptying of a solid nutrient meal. A gastric motility test was conducted as previously described (27). Light-phase experiments for the fasted rats were initiated at 8 a.m. Before the experiments, the rats were deprived of food for 16 h with free access to water. The fasted rats were given access to pre-weighed pellets (350 kcal/100 g) for 1 h. One of the following was then injected ICV: vehicle (10 µl of saline), obestatin (0.1, 0.3 or 1 nmol/rat) or h/rCRF (1 nmol/rat). After ICV injection, the rats were fasted for 2 h. Food intake was calculated by subtracting the weight of the uneaten pellets from the weight of the pellets initially provided, and then split at the end of each time-point (23,28).

The rats were decapitated 3 h after the start of the experiment. The stomach and attached small intestine were then immediately exposed by laparotomy. After ligating the esophago-gastric and gastro-duodenal junctions, the whole stomach was carefully mobilized. The gastric contents were dried using a vacuum freeze-drying system and weighed. Gastric emptying was calculated as: [1 - (dry weight of food in the stomach/weight of food ingested)] x 100%. The weight of food emptied into the duodenum was calculated as: weight of food ingested - dry weight of food in the stomach.

Statistical analysis. All results are expressed as the means ± SEM. Correlations between gastric emptying, weight of food ingested and weight of food emptied into the duodenum were analyzed using Pearson's correlation test. One-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test were used to analyze the differences among the groups. Differences were considered statistically significant at $P<0.05$.

Results

Effects of ICV obestatin injection on plasma acyl ghrelin, des-acyl ghrelin, acyl ghrelin/des-acyl ghrelin ratio and nesfatin-1. Obestatin at 3 different doses (0.1, 0.3 and 1 nmol/rat) did not change the plasma levels of acyl ghrelin and des-acyl ghrelin and the acyl ghrelin/des-acyl ghrelin ratio compared to the vehicle-injected controls 2 h after ICV injection (Fig. 1A-C).
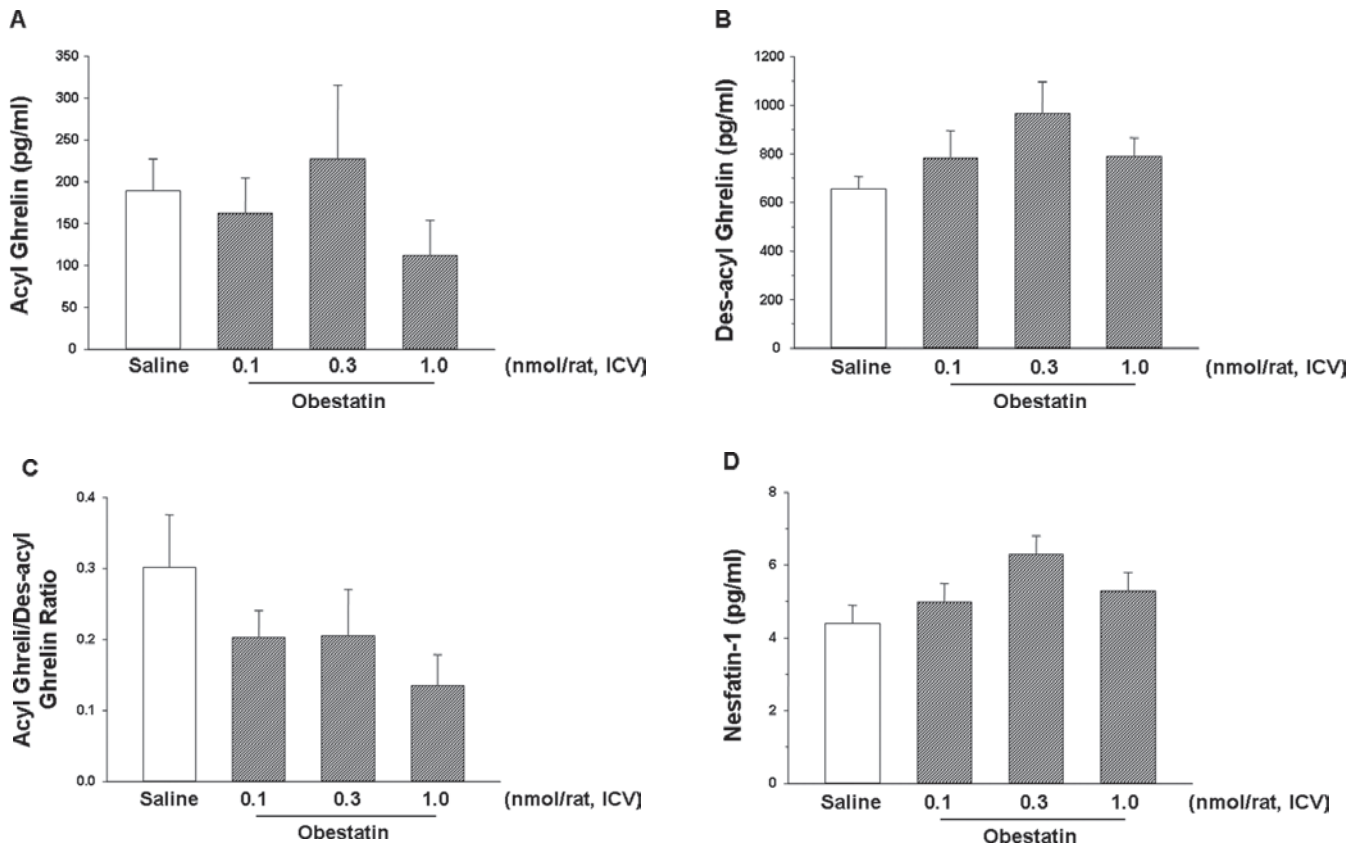
Figure 1. The influence of intracerebroventricular (ICV) injection of obestatin (0.1, 0.3 and 1.0 nmol/rat) on plasma levels of (A) acyl ghrelin, (B) des-acyl ghrelin, (C) acyl ghrelin/des-acyl ghrelin ratio and (D) nesfatin-1 levels; n=6 rats in each group.
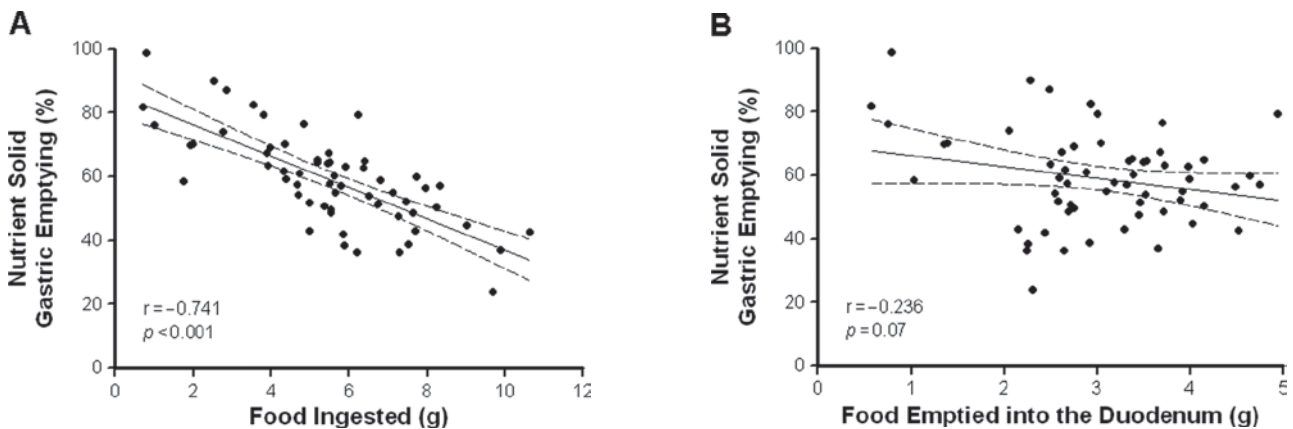

Figure 2. There was (A) a significant negative correlation between the gastric emptying of a solid nutrient meal and the weight of food ingested at 1 h before intracerebroventricular (ICV) injection (r=-0.741, P<0.001), and (B) a tendency of negative correlation observed between the gastric emptying of a solid nutrient meal and the weight of food emptied into the duodenum 2 h after ICV injection (r=-0.236, P=0.07); n=60 rats total.

Obestatin at these doses did not affect the plasma nesfatin-1 concentration 2 h after ICV injection (Fig. 1D).

Effects of the weight of food ingested and that of food emptied into the duodenum on gastric emptying of a solid nutrient meal. Our results showed that there was a significant, but negative correlation between the gastric emptying of a solid nutrient meal and the weight of food ingested (Fig. 2A). The gastric emptying of a solid nutrient meal revealed only a tendency for correlation with the weight of food emptied

into the duodenum 2 h after ICV injection (Fig. 2B). In addition, the 2-h rate of gastric emptying of a solid nutrient meal significantly, but negatively correlated with the amount of calories ingested (kcal/h) (r=-0.741, P<0.001).

Effects of ICV obestatin and h/rCRF on gastric emptying of a solid nutrient meal. Obestatin (0.1, 0.3 and 1 nmol/rat) did not accelerate or delay the gastric emptying of a solid nutrient meal compared to the vehicle-injected controls 2 h after ICV injection in conscious, fasted rats (Fig. 3A). By contrast, h/rCRF (1 nmol/
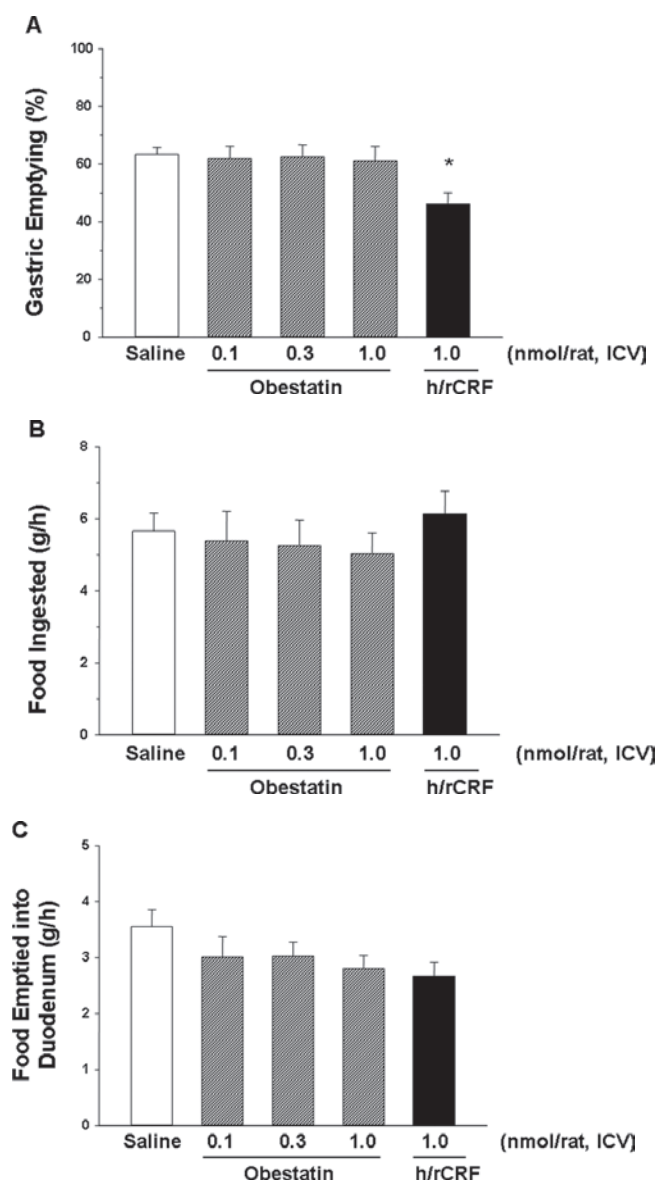
Figure 3. The gastric emptying of a solid nutrient meal, food ingested and food emptied into the duodenum in groups treated with intracerebroventricular (ICV) injection of either obestatin, or human/rat corticotropin-releasing factor (h/rCRF). (A) ICV obestatin (0.1, 0.3 and 1.0 nmol/rat) did not modify the gastric emptying of a solid nutrient meal, whereas h/rCRF (1.0 nmol/rat) significantly delayed it (*P<0.05 compared to all the other groups, n=12 rats in each group). (B) Ingested food and (C) food emptied into the duodenum were comparable among the different treatment groups (n=12 rats in each treatment group).

rat) significantly delayed the gastric emptying of a solid nutrient meal compared to the vehicle-injected controls (Fig. 3A). However, the weight of food ingested 1 h before and that of food emptied into the duodenum 2 h after ICV injection was comparable among the different groups (Fig. 3B and C).

Discussion

Previous studies have only measured the effects of obestatin on pituitary hormones and hypothalamic neuropeptides. For instance, obestatin does not affect the secretion of growth hormone, thyroid-stimulating hormone and adrenocorticotropic hormone, or the mRNA expression of neuropeptide Y,

agouti-related protein, cocaine- and amphetamine-regulated transcript, and pro-opiomelanocortin, through either intravenous or ICV administration (29-31).

On the one hand, the influence of obestatin on other plasma hormones, such as acyl ghrelin, des-acyl ghrelin and nesfatin-1, has not yet been well addressed. Des-acyl ghrelin is the major molecular form circulating in the blood (32-34) that can be converted to acyl ghrelin through ghrelin *O*-acyltransferase in the stomach, intestine and pancreas (35,36). Both the acyl and des-acyl forms of ghrelin are actively involved in the regulation of appetite, gut motility and energy homeostasis (21).

On the other hand, nesfatin-1 is a novel 82-amino-acid peptide, identified as a satiety signal encoded by nucleobindin-2 gene (37). Recent studies have indicated that nesfatin-1 is co-localized with ghrelin in rat gastric oxyntic mucosa. It is postulated to interact with ghrelin gene products to modulate food intake and body weight (22,37). The interplay between obestatin and acyl ghrelin, des-acyl ghrelin, as well as nesfatin-1 is interesting, but is not yet well recognized. In patients with restricting-type anorexia nervosa, obestatin, as well as acyl ghrelin, des-acyl ghrelin and nesfatin-1, are important regulators responsive to starvation and re-nutrition (38). Following a PubMed search, the current study is the first to explore the impact of ICV obestatin on these above-mentioned peptides. The results from our study show that obestatin at the doses tested, does not affect the plasma levels of acyl ghrelin and des-acyl ghrelin, the acyl ghrelin/des-acyl ghrelin ratio and nesfatin-1 2 h after ICV injection.

Previous studies have only focused on the effects of peptide administration on the gastric emptying of a solid nutrient meal in mice (27) and rats (39). Little attention has been paid to the influence of the weight of food ingested and that of food emptied into the duodenum on gastric emptying. The results from our study reveal that the weight of food ingested negatively, but significantly correlates with the gastric emptying of a solid nutrient meal, whereas the weight of food emptied into the duodenum has a tendency for negative correlation. Thus, the weight of food ingested should be cautiously taken into consideration before data interpretation using models of gastric emptying of a solid nutrient meal in the future.

The first preproghrelin gene product, acyl ghrelin, induces prokinetic activity along the entire gastrointestinal tract (40-43). When administered through ICV or intravenous injection, acyl ghrelin has been shown to accelerate the gastric emptying of a solid nutrient meal in fasted mice (44) and the gastric emptying of a non-nutrient, semi-liquid meal in fasted rats (25). It also elicits phase III-like contractions in the duodenum in acutely fed rats (45). The second ghrelin gene product, des-acyl ghrelin, via central or peripheral administration, has been shown to delay the gastric emptying of a solid nutrient meal in fasted mice (46) and to disrupt phase III-like contractions in the antrum in fasted rats (23).

In the present study, we examined the influence of the third preproghrelin gene product, obestatin, on the gastric emptying of a solid nutrient meal in rats. By contrast, unlike acyl ghrelin and/or des-acyl ghrelin, the ICV injection of obestatin (0.1-1.0 nmol), did not modify the gastric emptying of a solid nutrient meal in this study. Although GPR39 mRNA is abundant in the amygdala, hippocampus and auditory cortex (2,3), and obestatin is capable of binding to GRP39 to regulate

the functions of diverse gastrointestinal and adipose tissues (8), the current results suggest that obestatin does not act in the forebrain to affect the gastric emptying of a solid nutrient meal in conscious, fasted rats.

The findings from our study are comparable to those from a previous report measuring intra-gastric pressure after an intracisternal injection (hindbrain) of obestatin at higher doses (7.5 and 30 μg/rat) via an acutely implanted catheter in anesthetized rats (47). However, an intra-arcuate nucleus injection of obestatin has been shown to inhibit dopamine release and block the ghrelin-induced inhibition of serotonin release in the rat hypothalamus (48). Recent studies have revealed that manometric recording is a more sensitive way than gastric emptying measurement in detecting gastro-inhibitory effects of presumed anorectic peptides (28,49). Furthermore, the intravenous infusion of obestatin has been clearly shown to inhibit the percentage motor index and phase III-like contractions in the antrum and duodenum in conscious, fed rats (50). Studies using manometric methods to investigate the central effects of obestatin on gastric motility are warranted in the future.

The peripheral administration of obestatin reportedly alters gastroduodenal motility via CRF receptors (20,50). As such, h/rCRF has been used as the inhibitory control for comparison in this study. From the data obtained, it is evident that an ICV injection of h/rCRF (1 nmol) significantly delays gastric emptying of a solid nutrient meal, which is consistent with a previous report that used gastric emptying of a non-nutrient, semi-liquid meal through intracisternal administration (51). Although the half-life of h/rCRF is reportedly less than 10 min in human cerebrospinal fluid (52), the results from our current study show that the ICV h/rCRF injection (1 nmol) effectively inhibits the 2-h rate of gastric emptying of a solid nutrient meal in conscious, fasted rats.

In summary, obestatin at the doses tested does not change the plasma acyl ghrelin and des-acyl ghrelin levels, the acyl ghrelin/des-acyl ghrelin ratio and nesfatin-1 levels 2 h after ICV injection. The acute ICV administration of obestatin has no influence on the 2-h rate of gastric emptying of a solid nutrient meal in conscious, fasted rats, whereas ICV h/rCRF injection delays it. The weight of food ingested negatively affects the gastric emptying of a solid nutrient meal.

Acknowledgements

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