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Metabolic effects of chronic obestatin infusion in rats

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ABSTRACT

Obestatin is purported to be a peptide hormone encoded in preproghrelin. We studied the metabolic effects of continuous infusion of obestatin via subcutaneously implanted osmotic mini-pumps. Administration of up to 500 nmol/kg body weight/day obestatin did not change 24 h cumulative food intake or body weight in rats. Similarly, no effects were observed when obestatin was infused at 1000 nmol/kg body weight/day for seven days. This dose of obestatin infused during a 24 h fast did not alter weight loss, suggesting that obestatin has no effect on energy expenditure, and this dose did not alter glucose or insulin responses during an IPGTT. Obestatin was originally proposed to interact with GPR39 and subsequently the receptor for GLP-1. While both receptors are expressed in pancreatic islets, incubation with obestatin did not alter insulin release from islets *in vitro*. Moreover, obestatin did not bind to INS-1 β-cells or HEK cells overexpressing GLP-1 receptors or displace GLP-1 binding to these cells. Our findings do not support the concept that obestatin is a hormone with metabolic actions.

1. Introduction

Obestatin is an alleged 23 amino acid peptide hormone [33] that is predicted to be an excised product of some, but not all (e.g. monkeys) mammalian preproghrelins [5]. The proposed obestatin sequence is also not present in preproghrelins of non-mammalian vertebrates. The actions of obestatin are said to directly oppose those of ghrelin [33,19], a potent orexigenic hormone mainly produced from the stomach [15,27,32]. This notion that a cell secretes products from the same precursor, yet with opposing actions is counter-intuitive. Processing of proglucagon yields hormones with opposing actions on glucose homeostasis (glucagon and GLP-1), but they are generated by differential processing in distinct endocrine cell populations such that their release is triggered by different cues (fasting and feeding) [14]. There is no evidence that preproghrelin undergoes such cell-specific post-translational processing to avoid co-secretion of ghrelin and obestatin.

Intraperitoneal injections of obestatin at 125 and 1000 nmol/kg body weight and intracerebroventricular injections at 8 nmol/kg body weight inhibited food intake of mice for 5 h post-injection [33]. Moreover, three injections of 1000 nmol/kg body weight of obestatin daily for seven days resulted in a significant decrease in body weight of rats [33]. From a therapeutic perspective, it is important to identify whether a sustained reduction in food intake and body weight can be achieved by administering obestatin. Therefore, we studied the metabolic effects of chronic administration of pharmacological doses of synthetic obestatin in rats. We also investigated the distribution of GPR39, a proposed obestatin

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receptor [33,18] in rodent islets and tested the direct effects of obestatin on insulin release *in vitro* and its effects on glucose homeostasis and insulin levels *in vivo*. In addition, in view of a recent report [9] that obestatin acts directly on β-cells via receptors for glucagon-like peptide-1 (GLP-1), we studied obestatin binding to INS-1 β-cells and HEK cells overexpressing GLP-1 receptors. Our studies do not support the notion that obestatin interacts with GLP-1 receptors. Moreover, our results with chronic infusion of obestatin at pharmacological doses suggest that obestatin has no effects on glucose homeostasis, food intake, water intake and body weight of male Sprague-Dawley rats. Despite the presence of GPR39 immunoreactivity in islets, obestatin at the doses tested did not alter insulin release from mouse islets in culture.

2. Materials and methods

Male Sprague-Dawley rats weighing ~200–225 g and male C57BL6 mice (6–8 weeks old) were obtained from the Rodent Breeding Facility, Animal Care Centre, University of British Columbia (UBC). All animal studies adhered to the guidelines of the Canadian Council of Animal Care and were approved by the UBC Animal Care Committee. Rats were kept at 12 h dark:12 h light cycles and lights were turned on at 6 a.m. and turned off at 6 p.m. All rats ($n = 6$/group in all studies) used were weight matched, *ad libitum* fed with rat chow (Labdiet 5012; 22% protein, 4% fat, 5% fibre, 8% ash and 3% minerals) unless otherwise specified and acclimatized as described previously [28] and in all studies osmotic pumps were implanted subcutaneously. Food intake was measured as described previously [28]. Amidated rat/mouse obestatin (23 amino acids; FNAPFDVGIKLSGAQYQQHGRAL-NH_2) was synthesized and HPLC purified to ≥95% purity at the Peptide Synthesis Laboratory, Biomedical Research Center, UBC. Obestatin was freshly prepared in 0.9% saline for each study. GLP-1-[7-36] amide was purchased from American Peptide Company (Sunnyvale, California). One day (Model 2001D) and 7 day (Model 2ML1) Alzet™ osmotic mini-pumps were purchased from Durect Corporation (Cupertino, California). In all studies, pumps filled with peptides or saline were primed for a minimum of 4 h following the instructions of the manufacturer before implantation into rats. Rats were acclimatized for 4 days prior to the implantation of osmotic mini-pumps. Animals were kept in grid cages. During acclimatization, animals were transferred to the procedure room, anaesthetized using 3% isoflurane, shaved in the area where the incision was to be made and weighed. Rats quickly recovered from anaesthesia and they were returned to the animal facility. Food intake was measured by deducting the quantity of food recovered after 24-hour feeding from the initial amount of food given.

2.1. Effects of obestatin on food intake, water intake, body weight and energy expenditure

For investigating the short-term (1 day) effects of obestatin, four studies were conducted and rats were implanted with 1-day osmotic mini-pumps containing saline or 100, 200, 400 (first study) or 500 or 1000 nmol/kg/body weight (second study) obestatin or rat PYY(3-36) (Phoenix pharmaceuticals, Belmont, California; catalogue number 059-04; second study-positive control). Twenty-four hour cumulative food intake, weight gain and blood glucose levels at 24th hour (second study) were measured. The aim of the third study was to investigate the effects of obestatin on energy expenditure and to achieve this, rats were infused with saline or 1000 nmol/kg body weight/day obestatin, fasted for 24 h and the 24 h cumulative weight loss was measured. For the long-term study (fourth study), 7-day osmotic mini-pumps were used and daily food intake, water intake and body weight were measured. On day 7, plasma glucose was monitored, blood samples collected and plasma separated by centrifugation at 7 000 rpm for 9 min and stored at −20 °C until assayed using the rat/mouse leptin ELISA (ALPCO Diagnostics, Windham, NH) or rat insulin ELISA (ALPCO).

2.2. GPR39 immunoreactivity in pancreatic islets

For immunohistochemistry, paraffin-embedded male Wistar rat and C57BL6 mouse pancreas sections were deparaffinized with xylene (3 × 10 min, 25 °C) and rehydrated in a graded ethanol series. The sections were then blocked with DAKO® serum-free protein block reagent (DAKO Corporation, Carpinteria, California) for 10 min and incubated with guinea pig anti-insulin (Linco; catalogue number 4011-01F; 1:900 dilution) and rabbit anti-human GPR39 (Affinity Bioreagents, Golden, Colorado; catalogue number OPA1-15100; 1:70 dilution) antibodies for 24 h at 4 °C. The slides were then washed four times with 1X PBS and incubated with the secondary antibodies, goat anti-guinea pig Alexa Fluor 488 IgG (Green-Insulin; Chemicon International, Temecula, California; 1:500 dilution) and donkey anti rabbit Alexa Fluor 594 donkey IgG (Red-GPR39; Chemicon International; 1:1000 dilution), for 1 h at room temperature. All secondary antibodies were diluted in DakoCytomation® antibody diluent reagent (DakoCytomation, Mississauga, Ontario). Finally, the slides were washed three times in 1X PBS and mounted with Vectashield® mounting medium containing the nuclear dye DAPI (Blue; Vector Laboratories, Burlingame, California). Sections were viewed under an Axiovert 200 microscope (Carl Zeiss Canada, Port Moody, British Columbia, Canada), images were captured using a Retiga 2 000R digital CCD camera (QImaging, Burnaby, British Columbia, Canada) connected to a Power Mac G5 computer and Openlab imaging software (Improvision, Lexington, Massachusetts). As a negative control, slides containing pancreas sections were stained only with secondary antibodies.

2.3. Effects of obestatin on insulin release from mouse pancreatic islets in vitro

Islets were isolated from C57BL6 mice as described previously [30]. Two separate studies, one-hour (0.001–1000 pM obestatin) and two-hour (0.001–1000 nM obestatin) were conducted to test the effects of obestatin on insulin release *in vitro*. Briefly, 20 islets/well were plated in 24 well plates in 6.1 mM glucose containing Krebs Ringers Bicarbonate Buffer (KRBB). After overnight incubation at 37 °C and 5% CO_2, islets were pre-incubated in 10 mM glucose for 1 h. After 1 h, 10 mM glucose containing KRBB was replaced with plain 10 mM glucose-KRBB or KRBB (10 mM glucose) containing the specified