0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(1):21–26

doi: 10.1210/en.2006-0915

Effects of Obestatin on Energy Balance and Growth Hormone Secretion in Rodents

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Ghrelin stimulates food intake and adiposity and thereby increases body weight (BW) in rodents after central as well as peripheral administration. Recently, it was discovered that the gene precursor of ghrelin encoded another secreted and bioactive peptide named obestatin. First reports appeared to demonstrate that this peptide requires an amidation for its biological activity and acts through the orphan receptor, GPR-39. Obestatin was shown to have actions opposite to ghrelin on food intake, BW, and gastric emptying. In the present study, we failed to observe any effect of obestatin on food intake, BW, body composition, energy expenditure, locomotor activity, respiratory quotient, or hypothalamic neuropeptides involved in energy balance regulation. In agreement with the first report, we were unable to find any effect of obestatin on GH secretion *in vivo*. Moreover, we were unable to find mRNA expression of GPR-39, the putative obestatin receptor, in the hypothalamus of rats. Therefore, the results presented here do not support a role of the obestatin/GPR-39 system in the regulation of energy balance. (*Endocrinology* 148: 21–26, 2007)

IN 1999, THE ENDOGENOUS ligand of the GH secretagogue receptor was cloned from the stomach and designated ghrelin (1). Ghrelin is a 28-amino acid peptide derived from a precursor termed preproghrelin, which consists of 117 amino-residues. Independently, Tomasetto *et al.* (2) identified this peptide and called it motilin-related peptide, which was shown to have an identical sequence to ghrelin. Ghrelin has potent stimulatory effects on GH secretion (1) and food intake (3, 4). In addition, it was shown that ghrelin promotes gastric emptying and a positive energy balance in both humans and rodents, thus increasing adiposity and body weight (BW) (5). Circulating levels of ghrelin increase before meals, suggesting a possible role in meal initiation (6), and obese patients have lower ghrelin levels than lean subjects (7).

Recently, it was demonstrated that preproghrelin also encodes another secreted peptide, termed obestatin due to its reported anorexigenic and BW-reducing effects (8). Obestatin is a 23-amino acid peptide that has been reported to require amidation to be biologically active. It was also reported to be the endogenous ligand of the G protein-coupled receptor, GPR-39, which belongs to the GH secretagogue receptor family (8). This previous orphan receptor is localized in multiple regions of the brain as well as in peripheral tissues (9).

Obestatin has been reported to have actions opposite to ghrelin, such as decreasing food intake, BW, and delaying gastric emptying (8), and to antagonize the actions of ghrelin when both peptides are coadministered. However, obestatin did not alter GH secretion. More surprisingly, obestatin did not modify leptin serum levels, and circulating obestatin levels were not increased after fasting (8). Moreover, it has been suggested that obestatin readily crosses the blood-brain barrier but is rapidly degraded (10). Until now, all available data have suggested that obestatin is a new and relevant player in energy balance regulation, which could open up the possibility of targeting the GRP-39 receptor for the development of antiobesity drugs. In this study, we aimed to investigate, firstly, the pathways involved in the action of obestatin; secondly, whether obestatin affects other parameters important in energy homeostasis, such as energy expenditure, locomotor activity, respiratory quotient, or body composition; thirdly, the putative expression of the obestatin receptor, GPR-39, in the hypothalamus; and fourthly, any effects of obestatin on GH secretion *in vivo*.

First Published Online September 28, 2006

Abbreviations: AgRP, Agouti-related peptide; BW, body weight; CART, cocaine and amphetamine-related transcript; CCK, cholecystokinin; icv, intracerebroventricular(ly); NPY, neuropeptide Y; POMC, proopiomelanocortin; SHAM, sham vagotomy; TVX, total subdiaphragmatic vagotomy.

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TABLE 1. Primers

Oligo	Sequence (5′–3′)	GenBank accession no.
Rat cyclophilin A forward	AGC ACT GGG GAG AAA GGA TT	M 19533
Rat cyclophilin B reverse	CAT GCC TTC TTT CAC CTT CC	
Rat NPY forward	GAC CCT TCC ATG TGG TGA TG	M 15880
Rat NPY reverse	AGG CAG ACT GGT TTC ACA GG	
Rat AgRP forward	GCA GAC CGA GCA GAA GAT GT	AF206017
Rat AgRP reverse	CTT GAA GAA GCG GCA GTA GC	
Rat POMC forward	GAA GGT GTA CCC CAA TGT CG	AF510391
Rat POMC reverse	CTT CTC GGA GGT CAT GAA GC	
Rat CART forward	ACT GTC CCC GAG GAA CTT CT	NM017110
Rat CART reverse	ATT TTG AAG CAG CAG GGA AA	
Rat GPR-39 forward	CTT TGG GGC CTT CGC TGT TTAC	XM222578
Rat GPR-39 reverse	ACG CTG CTG TTT CTC CTG GTTG	

Materials and Methods

Animals

Adult male rats (10–12 wk old) or mice (8–10 wk old) were housed at 23 C under a 12-h light-dark cycle with free access to food (except where the feeding regime was altered) and water. Obestatin was diluted in saline or PBS and injected just before the dark phase or at the beginning of the light phase (depending of the experimental protocol). Animal experiments were conducted in accordance with the standards approved by the University of Cincinnati, the Faculty Animal Committee at the University of Santiago de Compostela, and by the Veterinary Office of the Canton of Zurich Health Directorate.

Effects of ip obestatin injection on food intake, BW, and body composition in mice

Obestatin (P&E GmbH, Cincinnati, IN; R.D., Indiana University, Bloomington, IN) (1 μmol/kg) was acutely injected ip in mice. Also, obestatin (150 nmol/kg) was injected ip every day for 1 wk in mice.

Effects of sc obestatin injection on food intake, BW, and body composition in rats

Obestatin (P&E GmbH, R.D.) (150 nmol/kg) or an equal volume of vehicle (saline) was administered by daily sc injection in rats for 1 wk.

Effects of ip obestatin injection on food intake in rats after total subdiaphragmatic vagotomy (TVX) or sham vagotomy (SHAM)

Ghrelin has been proposed to stimulate feeding by activating vagal afferents (11), suggesting the vagus might mediate the inhibition of feeding by obestatin as well. We therefore also examined the effect of obestatin on food intake in rats after TVX or SHAM, performed according to standard procedures. To account for the typical reductions in food intake and BW after TVX and to minimize BW differences at testing, we used heavier rats for TVX (n = 16, 392–421 g) than for SHAM (n = 9, 296–348 g). Starting 10 d before surgery, rats were given access to three palatable liquid diets (25% condensed milk in water, Migros, Zurich, Switzerland; plus Oranol multivitamin mix, Bayer AG Leverkusen, Germany; vanilla Ensure Plus, Abbott AG, Baar, Switzerland; and vanilla Clinutren, Nestlé, Vevey, Switzerland), one or two at a time for 2–3 d each. At the same time, chow intake was restricted and discontinued 2 d before surgery. After surgery, rats were offered only the liquid diets and were nursed intensively. TVX rats lost about 50 g after surgery but stabilized and returned to normal daily intakes and BW gain by about 3 wk after surgery. Seven rats that failed to do so were killed. Seven weeks after surgery, the remaining 18 rats (9 TVX and 9 SHAM) received ip obestatin (36 nmol = 91 μg/kg) or vehicle (PBS) injections (0.75 ml/kg) in a crossover trial on 2 subsequent days at dark onset after 4 h of food deprivation, and milk intake was recorded for the subsequent 20 h. Completeness of TVX was assessed functionally by lack of cholecystokinin (CCK)-8 satiation, which depends on vagal afferents (12), and histologically with retrograde labeling of vagal motor neurons in the dorsal motor nucleus by fluorogold (13). One TVX rat was excluded because CCK reduced food intake more than 30%.

Effects of intracerebroventricular (icv) obestatin injection on food intake, BW, body composition, energy balance, respiratory quotient, and locomotor activity

Brain infusion cannulas were stereotaxically placed into the lateral ventricle as previously described (14). A catheter tube was connected from the brain infusion cannula to an osmotic minipump flow moderator (model 2001D or 2ML2, Alza Corp., Palo Alto, CA). A sc pocket on the dorsal surface was created using blunt dissection, and the osmotic minipump was inserted. The incision was closed with sutures, and rats were kept warm until fully recovered. Rats were then infused with either vehicle or obestatin (8 nmol/kg·d) for 7 d.

Rats were infused icv, as described above, with either obestatin (8 nmol/kg·d) (P&E GmbH) or vehicle for 7 d into the lateral ventricle. During this time, rats were kept in metabolic cages to facilitate food intake, energy expenditure, locomotor activity, and respiratory quotient measurements and were weighed daily by the TSE system (TSE Systems Midland, MI and Bad Homburg, Germany). Lean and fat mass were measured by nuclear magnetic resonance.

Effect of ip obestatin in prefasted mice

We then examined the effects of ip obestatin (R.D.) injection (administered at the beginning of the light phase) on food intake and BW in hungry (16–18-h prefasted) mice. Total food intake, measured from time zero, was assessed at 1, 2, 3, 4, and 5 h postinjection. Doses of 125 nmol/kg and 1 μg/kg were administered.

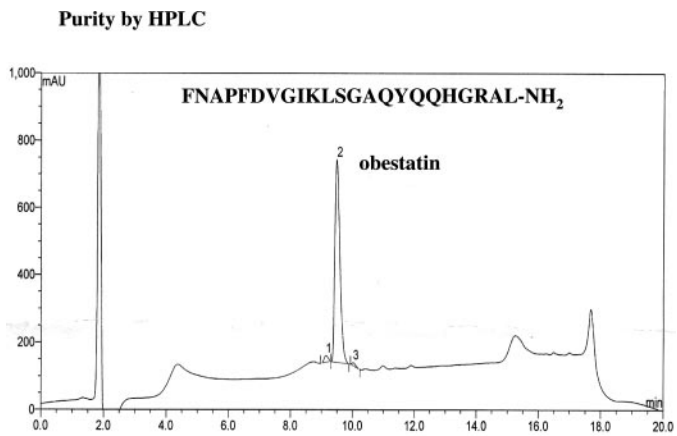

FIG. 1. Synthesis and purification by HPLC of obestatin. The sequence showed 100% homology with the sequence for obestatin previously reported (8).

TABLE 2. Effects of obestatin on food intake and body weight in rodents

Species (male)	Route of administration	Injection protocol	Obestatin dose	No. animals/ group	Cumulative FI (saline *vs.* obestatin, g)	Body weight at the end of the experiment (saline *vs.* obestatin, g)
Sprague Dawley rat	icv (pump)	Chronic (7 d)	8 nmol/kg·d (25 μg/rat)	12	62.5 ± 0.92 *vs.* 64.2 ± 0.42	357.1 ± 5.7 *vs.* 361.3 ± 2.9
Sprague Dawley rat	icv (daily injection)	Chronic (9 d)	75 nmol/kg·d (100 μg/rat)	8	30.3 ± 1.3 *vs.* 28.8 ± 1.83	319.5 ± 4.1 *vs.* 317.1 ± 7.3
Sprague Dawley rat	icv	Acute (1, 2, 4, 8, 16, 24 h)	8 nmol/kg·d (25 μg/rat)	6	29.23 ± 1.27 *vs.* 26.7 ± 2.21	ND
Sprague Dawley rat	sc	Chronic (7 d)	150 nmol/kg·d (200 μg/rat)	8	197.1 ± 5.8 *vs.* 199.8 ± 2.5	338.9 ± 4.6 *vs.* 332.1 ± 4.2
Sprague Dawley rat	ip	Acute (during 20 h)	36 nmol/kg (91 μg/rat)	9	49.6 ± 2.7 *vs.* 52.6 ± 1.9	ND
C57BL/6 mouse	ip	Acute (1, 2, 4, 6 h)	1 nmol/kg (75 μg/mouse)	8	1.77 ± 0.18 *vs.* 1.71 ± 0.11	ND
C57BL/6 mouse	ip	Chronic (7 d)	150 nmol/kg (11.25 μg/mouse)	8	26.8 ± 0.9 *vs.* 26.99 ± 0.7	28.74 ± 0.5 *vs.* 28.84 ± 0.6
C57BL/6 mouse (prefasted)	ip	Acute (1, 2, 3, 4, 5 h)	500 nmol/kg (37.5 μg/mouse)	8	0.91 ± 0.15 *vs.* 0.92 ± 0.18	ND
C57BL/6 mouse (prefasted)	ip	Acute (1, 2, 3, 4, 5 h)	500 nmol/kg (37.5 μg/mouse) (in PBS)	8	1.06 ± 0.2 *vs.* 1.12 ± 0.14	ND

ND, Not determined.

RNA extraction and real-time RT-PCR

Total RNA was extracted by Trizol Reagent (Invitrogen, Carlsbad, CA). Rat hypothalamic expression of the mRNA encoding neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine and amphetamine-related transcript (CART), and GPR-39 was assessed by real-time RT-PCR as described in detail elsewhere (15). Accordingly, cycle threshold values from each experimental sample were then used to calculate the amount of each gene and cyclophilin A mRNAs relative to the standard. For each sample, results in terms of gene expression levels were normalized to those of the internal control cyclophilin A. The oligonucleotide-specific primers for rat NPY, AgRP, POMC, CART, GPR-39, and cyclophilin A are described in Table 1.

Effect of obestatin on GH secretion

The animals (n = 8 rats/group) received ghrelin (12 nmol/kg) or ghrelin (12 nmol/kg) and obestatin (100 μg/rat). Ghrelin and obestatin were administered iv as previously described (16). Plasma GH concentrations were determined by double-antibody RIA using materials supplied by the NHPP as described previously (16). The intra- and inter-assay coefficients of variation were 2.4 and 4.8%, respectively.

Statistical analysis

Quantitative data are presented as mean ± SEM. Results were analyzed for statistically significant differences using ANOVA, followed by Mann-Whitney *U* test or a modified Student's *t* test (Bonferroni-Holm) where appropriate. $P < 0.05$ was considered significant.

Results and Discussion

Obestatin/GPR-39 and energy balance

In a recent study, Zhang *et al.* (8) reported that the peptide encoded by preproghrelin, obestatin, decreased food intake, BW, and gastric emptying. Moreover, it was suggested that obestatin acts as an endogenous opponent of ghrelin, antagonizing ghrelin's biological actions. Despite substantial efforts to detect such effects, we have been unable to replicate these results. In the present study, no significant effect of peripherally or centrally administered obestatin was seen on food intake or BW with either acute or chronic treatment. The synthesized peptides had 100% homology accordingly with the reported sequence for obestatin (8), and the purity was examined by HPLC (Fig. 1). We used the same doses as the original studies in both rats and mice (methods and results are summarized in Table 2, see also Fig. 2). In all our experiments, rats or mice treated with obestatin had daily and cumulative food intakes similar to controls and showed no difference in BW gain. Also, body composition of both rats and mice was not modified by obestatin treatment, despite a subtle, but not statistically significant, tendency toward a decrease in lean mass. Known anorexigenic drugs (MTII, exendin, CCK-8), which we used as positive controls, consistently decreased food intake in the same experiments, indicating that animals and conditions used were appropriate to detect anorectic effects. In addition, all animals were carefully adapted to injections and handling for several days as well as being given a minimum adaptation period of 1 wk to light cycle and environment. Moreover, when animals were challenged with ghrelin, both food intake and BW increased. Coadministration of ghrelin and obestatin resulted in an identical increase of food intake and BW, indicating that obestatin was unable to antagonize the effect of ghrelin (Fig. 2, B and C). Similar results were obtained in rats and mice with obestatin. Obestatin from three different suppliers was used, and no effect on food intake, BW, or other physiological parameters involved in energy homeostasis regulation such as locomotor activity, respiratory quotient, energy expenditure, or body composition was detectable (Table 2).

The reason for the discrepancies between these results and those of the original study is unclear. The authors of the original studies recommended specific conditions for optimal results. Obestatin should be frozen immediately to avoid slow formation of ice matrix and the exclusion of the peptide. It also was recommended to dilute obestatin in acetic acid and PBS-BSA to avoid loss of peptide by binding to the tube walls. Moreover, it was suggested that animals should be fasted for 16–18 h before obestatin challenge. After challenge, animals should be denied access to food for a further 15 min before measurement of food intake. In our laboratories, obestatin failed to reduce food intake even after prefasting for 18 h, regardless of the time of administration (dark or light phase), using either fresh peptide diluted in saline or in acetic acid plus PBS. The fact that the numerous conditions described above appear to be crucial for the actions of obestatin may indicate that any effects of this peptide on energy balance and metabolism are subtle. Moreover, our results, together with the reported rapid degradation of obestatin (10), suggest that any biological impact may be due to locally

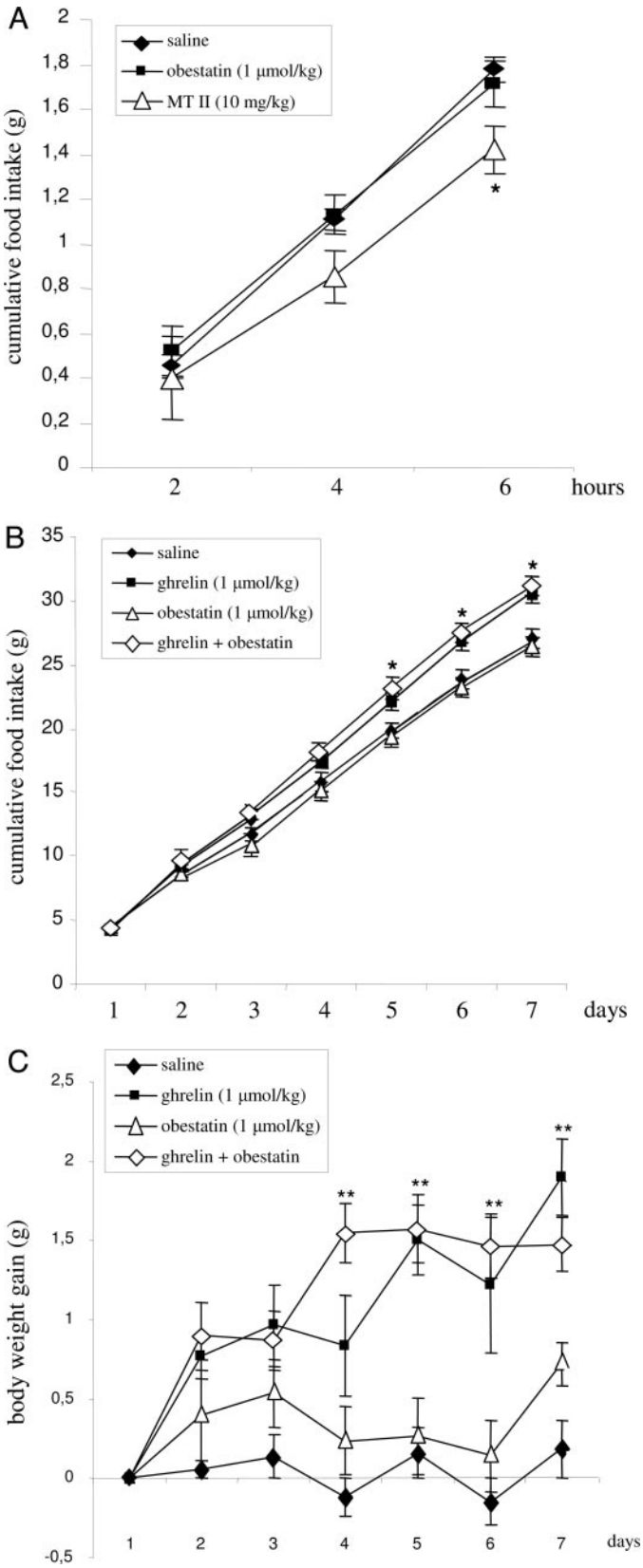

FIG. 2. A–C, Testing of obestatin. A, Melanocortin-receptor agonist MT-II (10 mg/kg), but not obestatin [ip dose of 1 μmol/kg (n = 8)] decreased 6-h food intake in mice. B, Obestatin (1 μmol/kg ip) did not modify food intake after 1 wk of administration in mice, and ghrelin (1 μmol/kg ip) increased cumulative food intake (n = 8). Obestatin was not able to antagonize ghrelin action on cumulative food intake. C, Obestatin (1 μmol/kg ip) did not decrease BW gain compared with saline control over a 7-d treatment period in mice (n = 8). Ghrelin administered at the same dose increased BW gain in mice, but obestatin failed to reduce the effect of ghrelin on BW gain. *Error bars*, SE of the mean. *, $P < 0.05$; **, $P < 0.01$.

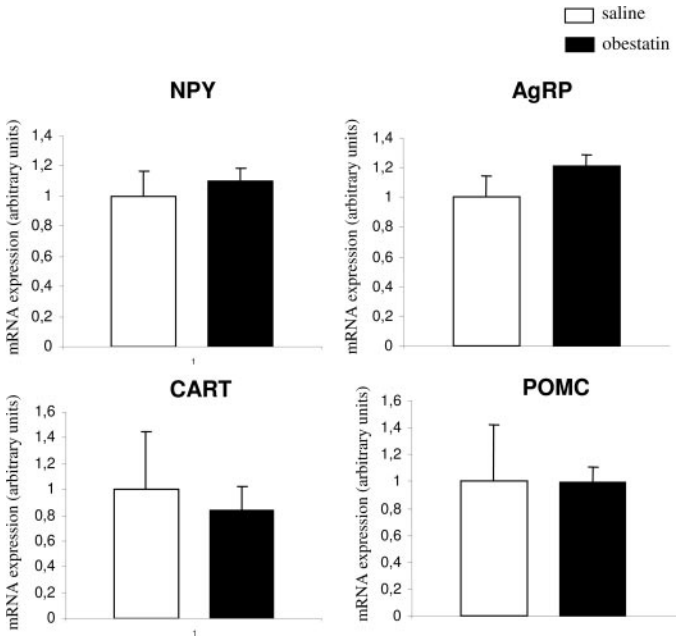

FIG. 3. Neuropeptide mRNA expression measured by real-time PCR in hypothalamus of rats treated icv with obestatin (8 nmol/kg·d) during 1 wk.

produced peptide and that functions as hormone are unlikely.

To examine whether obestatin exerted any effect at the level of hypothalamic energy balance control circuits, we assessed the mRNA expression of several neuropeptides involved in the regulation of food intake. No differences were detected in hypothalamic mRNA expression levels of NPY, AgRP, POMC, or CART after chronic icv obestatin treatment compared with saline-infused controls (Fig. 3). Obestatin is reported to act via the G protein-coupled receptor, GPR-39, which has previously been reported to be widely expressed in the brain and in peripheral tissues (9). These studies used Northern blotting to detect and quantify GPR-39 expression. In the present study, GPR-39 gene expression has been assessed by PCR, we did not detect any expression of this receptor in the rat hypothalamus, but strong expression in the ileum was detected using the same conditions (Fig. 4). These results support previous findings where GPR-39 was located in different regions of the brain, but not in the hypothalamus of mice (17). Although we were unable to find GPR-39 mRNA in the hypothalamus, it might be possible that this receptor mediates some actions in other regions of the brain. For instance, it is well known that the brain stem and the forebrain mediate the actions of several peptides involved in energy balance regulation (18). Moreover, it has been demonstrated that ghrelin also exerts several functions through these sites of the brain (19–23). Therefore, the pos-

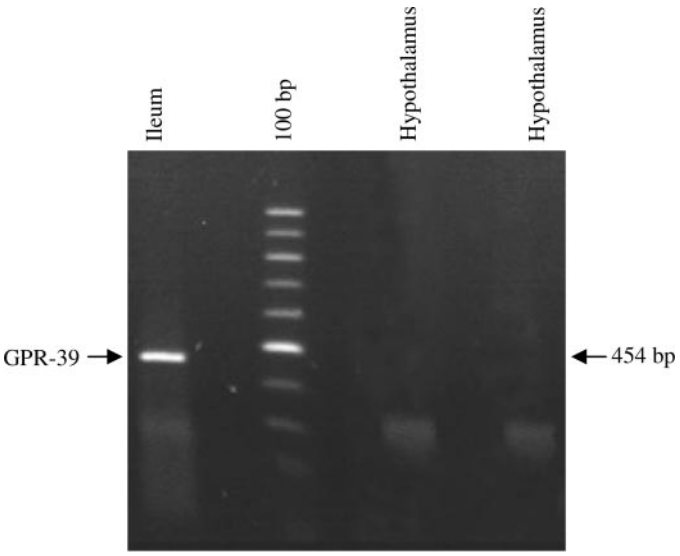

FIG. 4. GPR-39 mRNA expression measured by RT-PCR in different rat tissues.

sible roles of GPR-39 in those sites should be studied in the future.

Obestatin and GH secretion

In addition to its orexigenic effect, it is well established that ghrelin is a potent GH secretagogue. Because obestatin has been reported to act in opposition to the effects of ghrelin on food intake, it was decided to assess any potential antagonism of the effects of ghrelin on GH secretion *in vivo*. No significant effect of obestatin on spontaneous GH secretion (data not shown) was found in freely moving nonanesthetized rats. Furthermore, administration of a substantial dose of obestatin failed to influence ghrelin-induced GH secretion (Fig. 5). In summary, our data do not support a role of obestatin/GPR-39 system in energy balance regulation or in GH secretion.

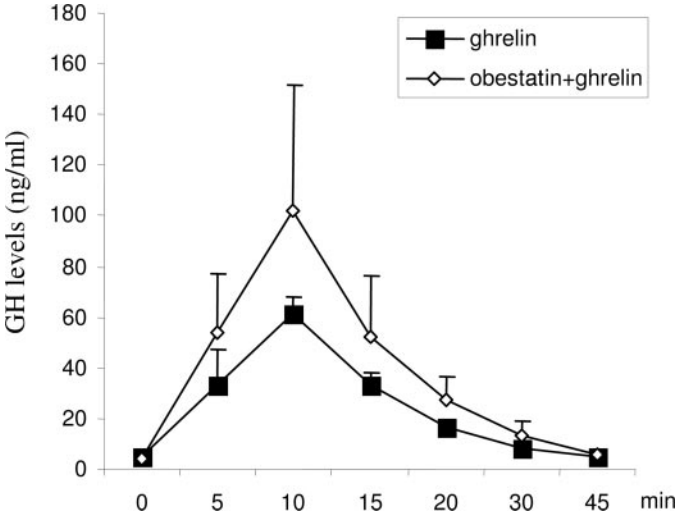

FIG. 5. Administration of obestatin (100 μg/rat) failed to influence ghrelin (12 nmol/kg)-induced GH secretion. Ghrelin and obestatin were administered iv after blood sampling at time 0 min. Plasma GH concentrations (X ± SEM) were determined by double-antibody RIA.

Acknowledgments

We thank Prof. Hsueh for continuous advice during the realization of this work.

Received July 10, 2006. Accepted September 14, 2006.

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This work was supported by Direccion General de Investigacion Cientifica y Tecnica (Grant BFU 2005-06287) and by the European Union (Grant LSHM-CT-2003-503041) (to C.D.). R.N. is a recipient of a fellowship from the Alexander von Humboldt Foundation.

P.P. followed an invitation to visit the Division of Reproductive Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine (Stanford, CA) to observe and learn the protocol used in Prof. Hsueh's lab for optimal obestatin treatment conditions. Obestatin, including compound from our laboratory, was efficient in decreasing food intake in mice in the laboratory of Prof. Hsueh. The identical protocol was repeated in our laboratory but repeatedly failed to produce those results.

The authors have nothing to disclose.

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