Obestatin Plays an Opposite Role in the Regulation of Pituitary Somatotrope and Corticotrope Function in Female Primates and Male/Female Mice

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Obestatin is a 23-amino-acid amidated peptide that is encoded by the ghrelin gene. Previous studies have shown obestatin can modulate the hypothalamic neuronal circuitry that regulates pituitary function, perhaps by modulating the actions of ghrelin. However, the direct actions of obestatin on pituitary function remain controversial. Here, primary pituitary cell cultures from a nonhuman primate (baboon) and mice were used to test the effects of obestatin on pituitary hormone expression and secretion. In pituitary cultures from both species, obestatin had no effect on prolactin, LH, FSH, or TSH expression/release. Conversely, obestatin stimulated proopiomelanocortin expression and ACTH release and inhibited GH expression/release in vitro, actions that were also observed in vivo in mice treated with obestatin. In vitro, obestatin inhibited the stimulatory actions of ghrelin on GH but not ACTH release. The inhibitory effect of obestatin on somatotrope function was associated with an overall reduction in pituitary transcription factor-1 and GHRH receptor mRNA levels in vitro and in vivo as well as a reduction in hypothalamic GHRH and ghrelin expression in vivo. The stimulatory effect of obestatin on ACTH was associated with an increase in pituitary CRF receptors. Obestatin also reduced the expression of pituitary somatostatin receptors (sst1/sst2), which could serve to modify its impact on hormone secretion. The in vitro actions of obestatin on both GH and ACTH release required the adenylyl cyclase and MAPK routes. Taken together, our results provide evidence that obestatin can act directly at the pituitary to control somatotrope and corticotrope function, and these effects are conserved across species. (*Endocrinology* 155: 1407–1417, 2014)

Obestatin is a 23-amino-acid, amidated peptide hormone that is mainly produced in the gastrointestinal tract and is derived from the posttranslational cleavage of the same peptide precursor as ghrelin (1). Obestatin and ghrelin have been shown to be stored in the same secretory vesicles (2). Obestatin is released in a pulsatile fashion, and its diurnal pattern of release is similar to that of ghrelin and GH. However, the number of obestatin pulses is less than that observed for ghrelin and GH (1, 3). It is clear that many of the actions of ghrelin are mediated through activation of the GH-secretagogue receptor (GHSR)-1a (4). However, there are conflicting opinions regarding the en-

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Abbreviations: AC, adenylyl cyclase; GHSR, GH-secretagogue receptor; GLP1R, glucagon-like peptide-1 receptor; GPR39, G protein-coupled receptor 39; Pit-1, pituitary transcription factor-1; PLC, phospholipase C; POMC, proopiomelanocortin; PRL, prolactin; qrtRT-PCR, quantitative real-time RT-PCR.

dogenous receptor for obestatin. Evidence is available indicating that both the glucagon-like peptide-1 receptor (GLP1R) (5), as well as the orphan G protein-coupled receptor 39 (GPR39), which belongs to the GHSR family (1), can be activated by obestatin, although there are other results contradicting this (6, 7). However, irrespective of the receptors involved, obestatin has been shown to regulate metabolic function at the central and the peripheral levels, which includes regulation of food intake and pancreatic, adipocyte, and cardiac function as well as cell proliferation (1, 5, 8, 9). Of note, many of these actions of obestatin are opposite to those observed for ghrelin; however, some of these actions are still controversial (10), and importantly, the potential role of obestatin in some key endocrine tissues is still unknown or yet to be fully elucidated. That is the case of one of the primary tissues for the actions of the ghrelin system, the pituitary gland, a tissue that was originally found to have the second highest obestatin-binding activity after the jejunum (8).

Specifically, the data published so far focused on the potential role that obestatin exerts at the pituitary level is quite limited, fragmentary, and unclear, and to the best of our knowledge, it has been mainly focused on the effect of obestatin on GH release in rat pituitary and in a rat tumor cell line (1, 3, 11–15), whereas the exact role that obestatin plays in regulating the function of all the pituitary cell types remains to be fully elucidated, particularly in human, primate, and mouse primary pituitary cells. Therefore, in the present study, we aimed at determining, for the first time, what the direct effect of obestatin is on the pituitary hormonal expression and release in normal primates (baboons, *Papio anubis*), a species that more closely models human physiology (16, 17). In addition, primary pituitary cell cultures from mice, as well as obestatin-treated mice and their vehicle-injected controls in vivo, were used as a model system to confirm and compare the effects of obestatin across species.

Materials and Methods

Reagents

All reagents and inhibitors of intracellular signaling pathways used in this study were purchased from Sigma-Aldrich unless otherwise specified. Human/monkey and rat/mouse obestatin, acylated ghrelin, and somatostatin were purchased from Phoenix Pharmaceuticals and NeoMPS. α-MEM, HEPES, horse serum, and penicillin-streptomycin were obtained from Invitrogen, and U73122 was purchased from Cayman Chemical.

Animals and tissue collection

The studies included in this manuscript were approved by the ethics committees of the 3 institutions participating in this research: University of Córdoba/Instituto Maimónides de Investigación Biomédica de Córdoba, University of Illinois at Chicago, and University of Turin Institutional Animal Care and Use Committees. Primate (olive baboon; *Papio anubis*; n = 5, 8–15 years of age; 15.3–21.8 kg) pituitaries were obtained from randomly cyclic control females from a breeding colony within 15 minutes after sodium pentobarbital overdose as previously reported (18, 19). Anterior pituitaries were cut into small pieces (~20–40 mg), and 2 pieces were rapidly frozen in liquid nitrogen and stored at −80°C until extraction for total RNA, and the remaining pieces were placed in sterile cold media and dispersed into single cells for culture.

Female C57BL/6J mice were purchased from Charles River at 8 weeks of age and housed under standard conditions of light (12-hour light, 12-hour dark cycle) and temperature (22°C–24°C), with free access to tap water and food (standard rodent chow; SAFE-diets). Mice were handled daily at least 1 week before euthanasia to acclimate them to personnel and handling procedures and were euthanized by decapitation, without anesthesia, under fed conditions. Plasma was collected and stored at −80°C for further analysis. Some pituitaries were processed for RNA expression analysis of GLP1R and GPR39 (n = 7) and others were dispersed into single cells (10–15 pituitaries per dispersion) for culture.

Primary pituitary cell cultures

Anterior pituitaries from female baboons and mice were dispersed into single cells by enzymatic and mechanical disruption, and cells were cultured following the methods and reagents previously reported (19, 20). Briefly, cells (100 000 cells per well) were plated onto 48-well plates in media containing 10% fetal bovine serum. After 36 hours incubation (37°C), medium was removed and cells preincubated for 1 hour with fresh serum-free medium alone, and then 1) cells were incubated with medium containing obestatin alone or in combination with acylated ghrelin (10nM) or somatostatin (100nM) for 4 or 24 hours or 2) in experiments using specific inhibitors of intracellular pathways, cells were incubated for 90 minutes with medium containing the inhibitors MDL-12330A (10μM; adenylyl cyclase [AC]), U73122 (50μM; phospholipase C), and PD-98059 (10μM; MAPK). Media were then exchanged with media containing the inhibitor combined with obestatin alone for an additional 24 hours. The doses used for all the compounds were selected based on previous reports (11, 19, 21). Controls consisted of medium alone or medium with inhibitor (experiments of intracellular pathways). At the end of the incubation with the different treatments, media were recovered for hormone analysis and cells recovered for RNA analysis.

In vivo effect of obestatin on the hypothalamic-pituitary, somatotrope, and corticotrope axes

To evaluate the in vivo effect of obestatin on the expression and release of key regulatory components of the hypothalamic-pituitary (somatotrope and corticotrope) axes, tissue samples were obtained from a group of male mice treated with obestatin and their respective controls (5–10 mice per group) previously generated in our laboratory. Details regarding the experimental procedure to generate this in vivo model have been previously described (22). Briefly, 7-week-old male C57BL/6J mice were purchased from Charles River and maintained with a standard

diet and tap water ad libitum for 1 week. Mice were fed a low-fat diet and separated into 2 groups that were ip injected for 1 week with saline or with obestatin (1 μmol/kg/d at 11:00 AM). Injections were repeated for 3 days at day 25 and at day 39 (total of 13 injections). After 8 weeks (day 56; 16 weeks of age), animals were anesthetized with tribromoethyl alcohol (Avertin; 375 mg/kg ip). The hypothalamus/pituitary tissues and plasma samples were collected and rapidly stored for the expression level and circulating hormone analyses, respectively.

Hormone release and protein analysis

GH, ACTH, prolactin (PRL), LH, FSH, and TSH concentrations in the culture media (baboons and mice) and/or plasma (mice) were measured using human or mouse commercial ELISAs (human GH, LH, FSH, PRL, ACTH, and TSH [reference numbers EIA-1787, EIA-1289, EIA-1288, EIA-1291, EIA-3647, and EIA-1790, respectively; DRG[; mouse GH [Millipore; reference EZRMGH-45K], ACTH [Phoenix Pharmaceuticals; reference EK-001-21] and PRL [GenWay; reference 40-101-325032]) following the manufacturer's instructions. To measure mouse LH levels, a specific RIA kit using a double-antibody method was used as previously reported (21, 23). Plasma IGF-I (IDS; reference AC-42F1) was also measured in mice treated with obestatin in vivo using a commercial ELISA kit. Obestatin protein was measured in the stomach and pituitary of mice fed a standard diet using the obestatin (rat/mouse) enzyme immunoassay kit (Phoenix Pharmaceuticals; reference: EK-031-90). All the assays were performed following the manufacturer's instructions where the information regarding specificity, detectability, and reproducibility for each of the assays can be accessed at the websites of the indicated companies.

RNA isolation, reverse transcription, and quantitative real-time RT-PCR of baboon and mouse transcripts

Tissues and pituitary cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene) with deoxyribonuclease treatment. The amount of RNA recovered was determined using the Ribogreen RNA Quantification Kit (Molecular Probes). Total RNA (0.15 μg for pituitary cell cultures and 1 μg for whole tissues) was reversed transcribed using random-hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNAs were amplified by quantitative real-time RT-PCR (qrtRT-PCR), where samples were run against synthetic standards to estimate mRNA copy number. Details regarding the development, validation, and application of a qrtRT-PCR to measure expression levels of baboon and mouse transcripts have been reported previously (21, 23, 24). Briefly, thermocycling and fluorescence detection was performed using a Stratagene Mx3000p real-time PCR machine and the brilliant SYBR Green QPCR Master Mix (Stratagene). The thermal cycling profile consisted of a preincubation step at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C for 30 seconds), annealing (61°C for 1 minute), and extension (72°C for 30 seconds). Final PCR products were subjected to graded temperature-dependent dissociation to verify that only 1 product was amplified. To determine the starting copy number of cDNA, reverse transcription samples were PCR amplified and the signal compared with that of standard curve (1, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies of synthetic cDNA template for each transcript of interest) run on the same plate. Specific sets of primers used in this study are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online website at http://endo.endojournals. org). New baboon sequences were obtained in the present study to amplify GLP1R and GPR39 (accession numbers KF478936 and KF478937s, respectively). To control for variations in the amount of RNA used in and the efficiency of the reverse transcription reaction, mRNA copy numbers of the baboon/mouse transcripts analyzed in pituitary cultures were adjusted by cyclophilin-A expression, whereas expression of all mouse transcripts measured in the in vivo studies were adjusted by a normalization factor calculated from the mRNA copy numbers of 3 separate housekeeping genes (hypoxanthine ribosyltransferase, β-actin, and cyclophilin-A) using the GeNorm version 3.3 application (25), where baboon/mouse cyclophilin-A or mouse normalization factor mRNA levels did not significantly vary between experimental groups within the tissue type (data not shown). Absolute mRNA levels (copy numbers per 0.05 μg total RNA) of all the gene transcripts measured in this study from the control groups of baboon and mouse primary pituitary cell cultures (24 hours after incubation in serum-free medim) are presented in Table 1.

Measurement of cell viability in response to obestatin

To determine whether obestatin altered cell viability, trypan blue (Sigma) and alamarBlue (Biosource International) assays were used as previously reported (26), following the manufacturer's instructions. Specifically, mouse primary pituitary cell cultures from male and female mice (8–10 weeks-old; 100,000 cells/well: 4 wells/treatment) were examined after a 24-hour treatment with obestatin and compared with vehicle-treated controls. For the alamarBlue assay, cells were incubated with the reagent for 4 hours, and cell viability was measured using the FlexStation 3 system (Molecular Devices). Cells were then washed 3 times with serum-free medium, and 24 hours later, cell viability was measured using the trypan blue reagent (counting a minimum of 300 cells per well).

Statistical analysis

Samples from all groups within an experiment were processed at the same time. To normalize values within each treatment and minimize intragroup variations in the different experiments (ie, different age of the tissue donor, stage of the estrous cycle or metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%). Specifically, to generate these values, individual values (adjusted by the corresponding level of housekeeping genes in the case of qrtRT-PCR) within each individual experiment were divided by the mean value of the control group and multiplied by 100, and the means of these adjusted values are presented with their associated SE. It should be emphasized that this style of data presentation does not alter the relative differences between obestatin-treated and vehicle-treated groups. Results from in vitro studies were obtained from at least 3 separate independent experiments carried out on different days and with different cell preparations (3–4 replicated per treatment per experiment). The in vivo effects of obestatin were obtained from 5 to 10 mice per group. Raw data were evaluated for heterogeneity of variance, and where found, values

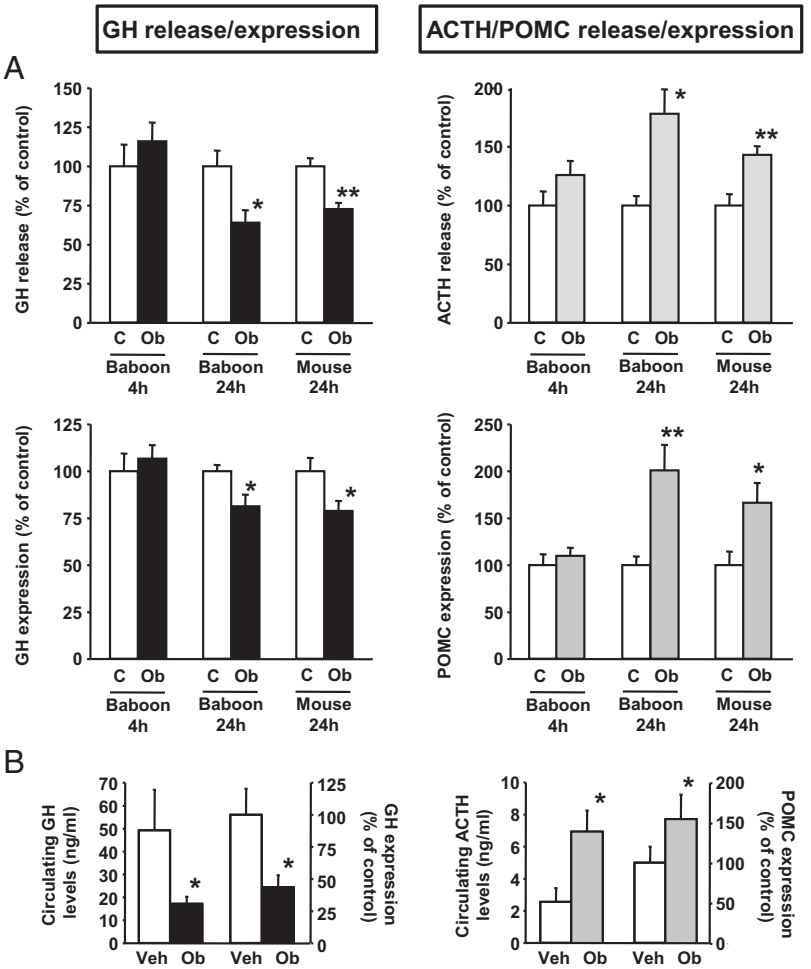

Figure 1. Effects of obestatin (Ob; 10nM) on GH and POMC/ACTH release and expression in primary pituitary cell cultures from baboons (4 and 24 hours) and mice (24 hours) (panel A) as well as in mice treated with obestatin in vivo (panel B). Data of hormonal release in vitro (primary cell cultures) and of GH/POMC expression in vitro and in vivo are expressed as percentage of vehicle-treated mice (control [C] or vehicle [Veh]; set at 100% within each experiment), whereas circulating GH and ACTH levels in mice treated with obestatin in vivo and their vehicle-treated controls are expressed as raw values. Values represent the mean ± SE (n = 3–5 individual in vitro experiments (3–4 wells per treatment per experiment [panel A] or 5–10 mice per group [panel B]). Asterisks indicate values that significantly differ from their respective control values: *, $P \leq .05$; **, $P \leq .01$. Control values for GH release are 38 ± 21 and 186 ± 94 ng/mL for baboon cultures at 4 and 24 hours, respectively, and 356 ± 147 ng/mL for mouse cultures at 24 hours. Control values for ACTH release are 4.6 ± 2.8 and 7.2 ± 4.5 ng/mL ACTH for baboon cultures at 4 and 24 hours, respectively, and 11.6 ± 7.8 ng/mL for mouse cultures at 24 hours.

were log-transformed. The in vivo and in vitro effects of obestatin vs control was assessed by one-way ANOVA followed by a Newman-Keuls test for multiple comparisons or by Student's *t* test, as appropriate ($P \leq .05$ was considered significant). All data are expressed as means ± SE. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems).

Results and Discussion

Our current understanding of the direct effects of obestatin on pituitary hormone secretions is quite scarce and unclear and is mainly derived from studies conducted in rat pituitaries and mostly focused on GH release (1, 3, 11–15). These limited studies show obestatin can have stimulatory (11), inhibitory (3, 13), or no effect (1, 3, 12, 14, 15) on basal or stimulated GH release from rat pituitaries. However, to date, no studies have shown the direct effect of obestatin on GH secretion or in other pituitary secretions in normal adult human, primate, or other nonprimate species. In this study, we provide the first evidence that obestatin directly and oppositely regulates the function of 2 pituitary cell types, somatotropes and corticotropes, in both primates and in mice. This differential effect of obestatin on GH and proopiomelanocortin (POMC)/ACTH synthesis and release was confirmed in mice in vivo.

Direct effect of obestatin on the function of pituitary cells

As shown in Figure 1A, a 4-hour incubation with obestatin did not significantly alter GH or POMC/ACTH expression or release in baboon primary pituitary cell cultures. In contrast, a 24-hour incubation with obestatin inhibited GH and stimulated POMC/ACTH expression and secretion from baboon and mouse pituitary cell cultures. Interestingly, similar to that observed at 24 hours of incubation with obestatin in vitro, mice treated with obestatin in vivo showed an inhibition in GH and an upregulation in POMC/ACTH expression and secretion (Figure 1B). A limitation of this study is the fact the in vivo assessment of mean GH levels was measured from a small sample set collected from random samples, which, due to the pulsatile nature of GH secretion, may not accurately reflect the impact of obestatin on GH output. Nonetheless, the in vitro and in vivo results strongly suggest that, at least in baboons and mice, obestatin can act as a somatotropin synthesis/release-inhibiting factor and, as a corticotropin synthesis/release-stimulating factor. Although the impact of obestatin on GH and POMC/ACTH synthesis and release was similar between mice and baboons, similar effects have not been clearly observed in rats in vitro and in

vivo. Specifically, in previous studies, obestatin did not alter (1, 3, 12, 14, 15) or stimulated (11) rat GH secretion in vivo/in vitro or did not modify rat ACTH release in vivo (13). However, it should be mentioned that discrepancies in the regulation of pituitary hormone secretion between mice and rats are not rare. For instance, although fasting enhances GH secretion in most of the mammalian species studied to date, including humans and mice, fasting suppresses GH release in rats (27). Additionally, the differences observed might also be related to the experimental approach used in this and previous studies (acute iv or intracerebroventricular administration vs prolonged ip injection), time of treatment in vivo (acute vs prolonged treatment), time of incubation in vitro (1 hour or less time used in the previous reports vs 24 hours used in the present study), cell preparation and culture conditions, and/or age studied. In line with this idea, it should be noted that, in support of our study, the single report published to date focused on the effect of obestatin on the secretion of all pituitary hormones indicates that an iv administration of obestatin tended to decrease GH levels (19% decrease) and stimulate ACTH secretion (22% increase) in blood samples collected from the jugular vein of rats; however, these differences did not reach statistical significance (13). Overall, our data strongly suggest that obestatin has a differential role in the control of GH and ACTH secretion in baboon pituitary cell cultures, and these actions were reproduced in vitro and in vivo in mouse pituitaries.

Obestatin treatment did not significantly alter the expression and/or release of PRL, LH, FSH, and/or TSH at the tested dose and time points used in primary pituitary cell cultures from primates/mice or in mice in vivo (Supplemental Figure 1), which is consistent with the only report published to date indicating that neither iv nor intracerebroventricular administration of obestatin affects the secretion of PRL or TSH in rats (13). Therefore, the present report demonstrates for the first time that obestatin exclusively exerts direct and opposite effects on the function of certain pituitary cell types and not others and that it does so similarly on primates and mice. Specifically, the effects of obestatin are restricted to inhibition of GH and stimulation of POMC/ACTH expression and release, suggesting the possibility that obestatin, together with ghrelin and somatostatin (4, 18, 19, 28), might act as a common primary regulator of GH and ACTH secretion in these species. Importantly, our results indicate that these actions of obestatin on somatotrope or corticotrope cells cannot be attributed to an effect of obestatin on cell viability because 24-hour incubation with obestatin did not alter cell viability in pituitary cell cultures from male or female mice (Figure 2).

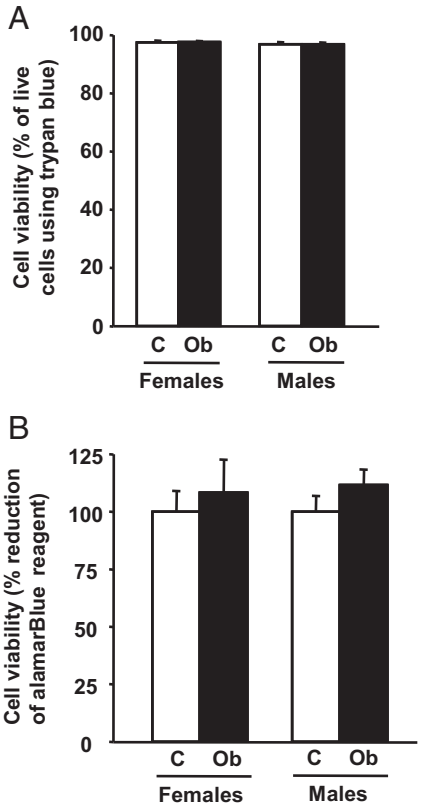

Figure 2. Effect of obestatin treatment (Ob; 10nM) on cell viability (24 hours) of mouse primary pituitary cell cultures from males and females (n = 3 from each gender; 8–10 weeks-old; 100,000 cells/well: 4 wells/treatment), assessed by trypan-blue (panel A) and alamarBlue (panel B) assays. Results are expressed as percent of vehicle-treated controls (C) and are the mean ± SE (set at 100%).

Interaction of obestatin with ghrelin and somatostatin

We next tested the direct effects of 24 hours of incubation with obestatin alone or in combination with ghrelin or somatostatin on GH and ACTH release in primary pituitary cell cultures from baboons and mice. As previously observed, obestatin alone inhibited basal GH secretion in primate/mouse primary cell cultures, whereas ghrelin alone increased GH release in both species (Figure 3, A and B, respectively). The ghrelin-stimulated GH secretion effect is consistent with previous reports in other species, including baboons (4, 19). However, to the best of our knowledge, this is the first study demonstrating that ghrelin can increase GH release from mouse primary pituitary cell cultures (Figure 3B). Interestingly, when both hormones were coincubated, obestatin blunted, but did not completely eliminate, ghrelin-stimulated GH release in primate/mouse cell cultures (Figure 3, A and B), suggesting that obestatin and ghrelin might share some common intracellular signaling pathways to regulate GH secretion in both species. These results are partially in agreement with those reported previously using different approaches,

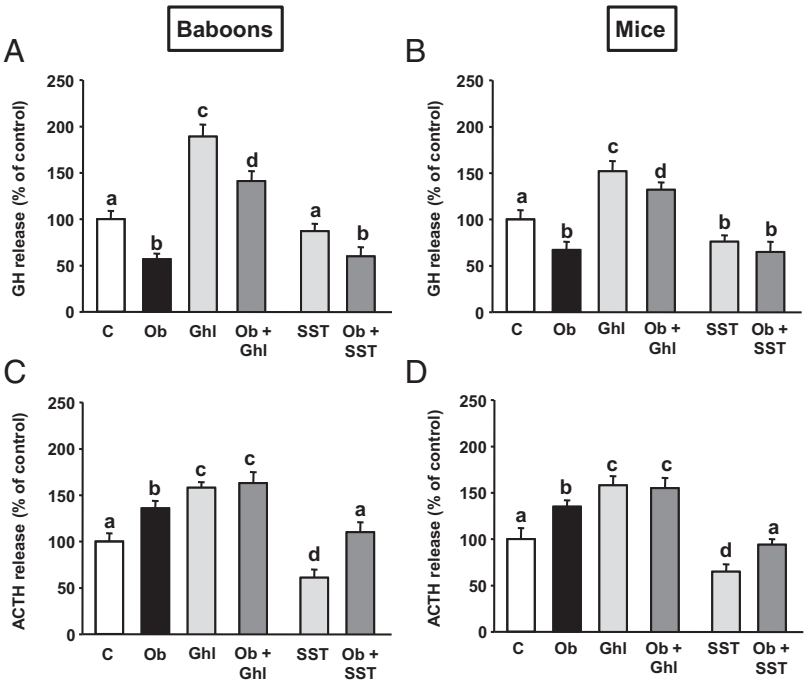

Figure 3. Interaction of Obestatin (Ob; 10nM) with common regulators of somatotrope and corticotrope function in primary pituitary cell cultures from baboons and mice. Panels A–D, Effect of 24 hours treatment of obestatin alone or in combination with ghrelin (Ghl; 10nM) or somatostatin (SST; 100nM) on the secretion of GH (baboons [panel A] and mice [panel B]) and ACTH (baboons [panel C] and mice [panel D]). Values are expressed as percentage of controls (C; set at 100% within each experiment) and represent the mean ± SE of 3 to 5 independent experiments (3–4 wells per experiment). Values that do not share a common letter are statistically different ($P \leq .05$).

which indicated that the stimulatory effect of ghrelin or its synthetic analog GHRP-2 on GH secretion in rats is not affected by an iv administration of obestatin in vivo (10 minutes after injection) (13) or by obestatin treatment in pituitary explants in vitro (3). However, in this latter report, it should be noted that it was also observed that an iv administration of obestatin in vivo was able to antagonize the ghrelin-induced increase of GH secretion between 5 and 60 minutes after injection. It has been suggested that the effect of obestatin on ghrelin-induced GH secretion is not mediated at the pituitary level (1, 3) and that both hormones likely interact at the hypothalamic level to modulate GH secretion. In support of this hypothesis, a recent report indicated that obestatin can reduce ghrelin-induced increase of GHRH release at the hypothalamic level, which probably influences the control of GH secretion (29). However, the results of the present study clearly suggest that the antagonistic effect of obestatin on ghrelin-induced GH secretion might also be exerted, at least in part, directly at the pituitary level.

Unlike the opposite actions of obestatin and ghrelin on GH release, both peptides increased basal ACTH secretion in cell cultures of primates and mice (Figure 3, C and D, respectively). Interestingly, comparison of the effects of equimolar doses of obestatin and ghrelin revealed that obestatin was less efficacious than ghrelin in inducing ACTH release in vitro in both species. Interestingly, a small but significant stimulatory effect of ghrelin on in vitro ACTH secretion is consistent with previous reports in humans and primates (4, 18). However, we and others have previously shown that ghrelin or its synthetic analogs might not be able to alter ACTH secretion in rodent pituitary cell cultures (18, 30). The discrepancies between this and previous studies might be explained by the differences on the time of incubation used (24 hours incubation in the present study [Figure 3D] vs 15–30 minutes [30] or 18 hours [18] in previous studies). Interestingly, the coadministration of obestatin and ghrelin did not evoke any further increase in the stimulatory effect on ACTH release as compared with the effects of either peptide alone, suggesting that obestatin and ghrelin might share common intracellular signaling pathways to alter ACTH secretion.

As previously reported, nanomolar doses of somatostatin did not significantly alter basal GH release in primary pituitary cultures of baboons (Figure 3A) (19), but somatostatin inhibited basal GH secretion in mice (Figure 3B) (20). Coadministration of somatostatin with obestatin did not alter the inhibitory effect of obestatin on GH release in both baboon and mouse pituitary cell cultures. Interestingly, obestatin alone stimulated ACTH secretion; however, somatostatin was able to inhibit the obestatin-stimulated ACTH release observed in both primate and mouse pituitary cell cultures to the level of basal controls (Figure 3, C and D, respectively). Overall, these results are novel and intriguing and set the stage for further future investigations to elucidate the possible physiological role of obestatin and its interaction with the primary regulators of somatotrope and corticotrope cells.

Intracellular signaling pathways involved in obestatin-regulated GH and ACTH release

We next focused our studies on defining the specific signaling pathways required by obestatin to induce GH and ACTH release on primate primary pituitary cell cultures. Given the limited source of baboon cell prepara-

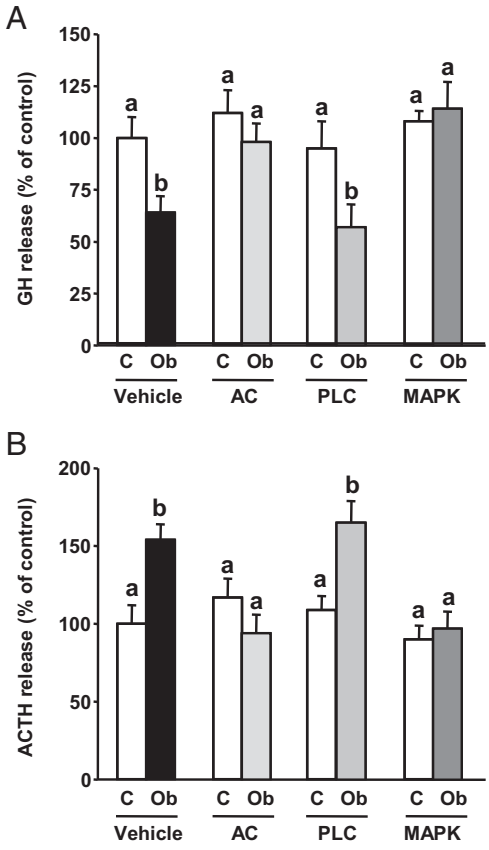

Figure 4. Intracellular signaling pathways of obestatin-regulated baboon GH and ACTH release. Effect of the inhibition of adenylyl cyclase (AC; MDL-12,330A; 10 μM), phospholipase C (PLC; U73122; 50 μM) or MAPK (PD-98,059; 10 μM) on obestatin(Ob)-inhibited GH release (panel A), and obestatin(Ob)-stimulated ACTH secretion (panel B). Values are expressed as percentage of vehicle-treated controls (C) without inhibitor (set at 100% within each experiment), and represent the mean ± SE of three independent experiments (three to four wells per experiment). Values that do not share a common letter are statistically different ($P \leq .05$).

tions, we were able to study only some selected signaling routes. Specifically, we selected inhibitors of MAPK, AC, and phospholipase C (PLC) based on previous reports indicating that these signaling routes might be activated by obestatin in other cellular systems (5, 10, 11). Administration of these inhibitors alone did not modify basal GH (Figure 4A) or ACTH (Figure 4B) release. Our results indicate that the inhibitory effect of obestatin on GH release is mediated through AC and MAPK because incubation with specific blockers of these routes, but not with PLC inhibitor, completely blocked the inhibitory effect of obestatin on GH secretion (Figure 4A). We have previously reported that ghrelin activates PLC/protein kinase C, protein kinase A, and MAPK to induce GH release in baboon pituitary cells (19), and therefore, obestatin and ghrelin require MAPK to regulate GH secretion, suggesting that the signaling pathways of both hormones may converge at the level of MAPK, which could then explain why obestatin was able to blunt, but not fully block, ghrelin-stimulated GH release in primate cell cultures (Figure 3A). Our results also demonstrate that obestatin and ghrelin activate similar signaling pathways to induce ACTH release because blockade of AC and MAPK, but not PLC activity completely abolished the stimulatory effect of obestatin (Figure 4B) and ghrelin (data not shown) on ACTH secretion. This might explain why the coadministration of both hormones did not modify the stimulatory effect on ACTH release compared with the effects of either peptide alone (Figure 3C). Thus, the present results provide primary evidence to support that the stimulatory effect of ghrelin and obestatin on ACTH release requires AC and MAPK signaling pathways in primary pituitary cell cultures.

Key regulatory components that might be directly involved in obestatin-induced in vitro and in vivo changes on somatotrope and corticotrope function

The actions of obestatin in the pituitary were not confined only to the inhibition of GH or the stimulation of POMC/ACTH synthesis and release but also included the regulation of other key regulatory components of somatotrope and corticotrope cell function in the 3 experimental models used in this study (primary pituitary cell cultures from primates and mice as well as mice treated in vivo).

Factors that might influence obestatin-induced inhibition of GH secretion

Obestatin treatment inhibited the expression of the pituitary transcription factor-1 (Pit-1), a critical factor for normal development and function of somatotrope cells (31), which might contribute to the inhibitory effect of obestatin on GH synthesis/secretion (Figure 5A). In addition, given the well-known influence of GHRH and ghrelin on GH secretion (19), our data indicate that an obestatin-induced downregulation in the expression levels of hypothalamic GHRH and ghrelin (Figure 5B), together with an overall decrease in the expression levels of pituitary GHRH receptor in the 3 experimental models used in this study (Figure 5C), might contribute to the obestatin-inhibited GH secretion observed in these models. However, it should be noted that in the case of the baboon model, this difference on GHRH receptor expression did not reach statistical significance (P value of .07). Also, we have previously reported that the mice treated with obestatin in vivo show an increase in circulating insulin levels (22), which could feed back at the level of the pituitary somatotrope and suppress GH secretion (32–34). Interestingly, the decrease in circulating GH levels in mice treated with obestatin in vivo was reflected in a 40% de-

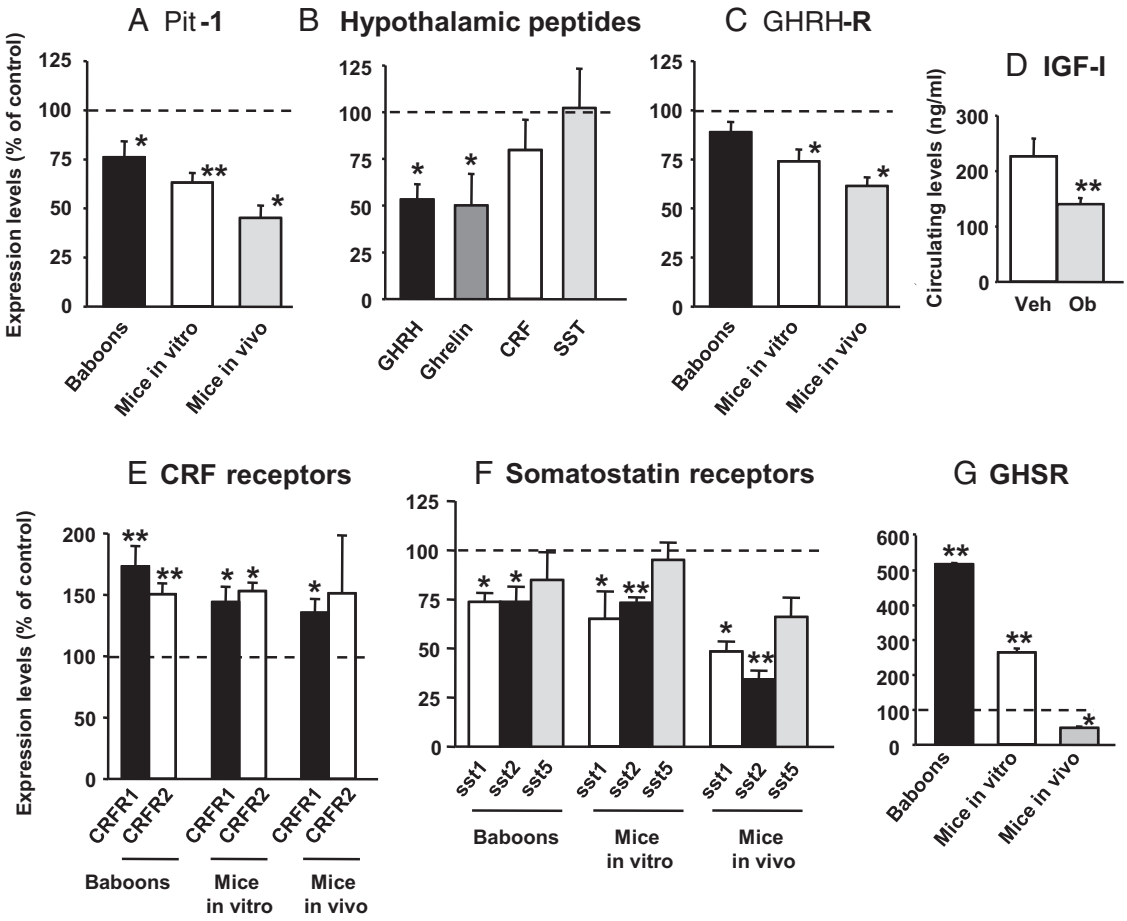

Figure 5. Key regulatory components involved in obestatin-induced alterations on somatotrope and corticotrope function in primary pituitary cell cultures from baboons and mice (24 hours incubation) and/or in mice treated with obestatin in vivo. Effects of obestatin (Ob) on Pit-1expression (panel A), hypothalamic GHRH, ghrelin, CRF, and somatostatin (SST) expression (panel B; mice treated with obestatin in vivo); pituitary GHRH-receptor expression (GHRH-R; panel C); circulating IGF-1 levels (panel D; mice treated with obestatin in vivo); pituitary CRF receptor subtypes 1 and 2 expression (CRF-R1/2; panel E); pituitary somatostatin receptors subtypes 1, 2, and 5 expression (sst1/2/5; panel F); and ghrelin receptor expression (GHSR; panel G). All the data in panels A–C and E–G are expressed as percentage of vehicle-treated controls (shown by the dotted line set at 100%), whereas circulating IGF-1 levels are expressed as raw values. Values represent the mean ± SE (n = 3–5 individual in vitro experiments [3–4 wells per treatment per experiment or 5–10 mice per group]). Asterisks indicate values that significantly differ from their respective control values: *, $P \leq .05$; **, $P \leq .01$.

crease in circulating IGF-1 levels, compared with vehicle-treated controls (Figure 5D), suggesting that these changes observed at the hypothalamic-pituitary (somatotrope) level are indeed physiologically relevant, in that they are translated into alterations in GH-regulated hepatic function leading to a decrease in circulating IGF-1 levels.

Factors that might influence obestatin-stimulated ACTH secretion

It is well-recognized that corticotropin releasing factor (CRF) and somatostatin are two key, stimulatory and inhibitory, factors in the regulation of corticotrope cell function, respectively (18, 35). Although we did not observe significant alterations in the hypothalamic CRF expression in mice treated with obestatin in vivo (Figure 5B), our results showed that obestatin treatment provoked an overall, comparable increase in the stimulatory receptors (CRF receptor subtypes 1 and 2; Figure 5E) associated with ACTH secretion in the 3 experimental models used. The increase in these receptors might contribute to the stimulatory effect of obestatin on ACTH synthesis/secretion observed in this study. In addition, obestatin treatment did not alter hypothalamic somatostatin expression (Figure 5B); however, it did evoke an overall reduction in the expression of some pituitary somatostatin receptor subtypes (sst1 and sst2 but not sst5; Figure 5F), which could also serve to modify its impact on hormone secretion, especially on ACTH secretion. Interestingly, obestatin treatment significantly stimulated GHSR expression in both cell culture systems, which might also contribute to the stimulatory effect of obestatin on ACTH synthesis/secretion (Figure 5G). However, we found that GHSR expression was significantly downregulated in mice treated with

obestatin in vivo, which suggests that other extrapituitary factors present in the environment are likely altered in response to obestatin and might predominantly contribute to convey an inhibitory effect on GHSR expression in response to obestatin treatment, and therefore, such factors could be masking the direct stimulatory effect of obestatin observed in vitro in both models. Unfortunately, the amount of plasma collected was not sufficient to measure glucocorticoid levels.

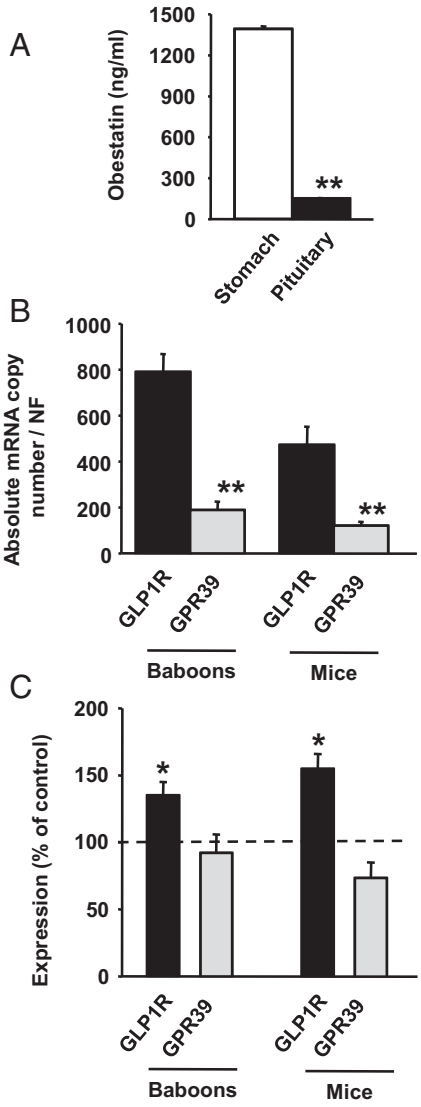

Figure 6. Presence of obestatin as well as GPR39 and GLP1R in the pituitary. Panels A and B, Obestatin protein expression levels in stomachs and pituitaries of mice (panel A; n = 3 tissues per group). Absolute mRNA copy numbers (adjusted by a normalization factor [NF]) of GLP1R and GPR39 in the pituitary of baboons and mice (panel B; n = 5). Panel C, Effects of obestatin (24 hours) on the expression of GLP1R and GPR39 in primary pituitary cell cultures from baboons and mice. Data in panel C are expressed as percentage of vehicle-treated controls (shown by the dotted line set at 100%) and represent the mean ± SE (n = 3–5 individual in vitro experiments [3–4 wells per treatment per experiment]). Asterisks indicate values that significantly differ from their respective control values: *, $P \leq .05$; **, $P \leq .01$.

Presence of obestatin as well as GPR39 and GLP1R in the pituitary

Obestatin protein was found to be expressed in mouse pituitary (Figure 6A), consistent with previous data showing obestatin protein expression in fetal and adult human pituitary (36). Interestingly, obestatin protein levels in the pituitary were much lower than those found in the stomach (Figure 6A), suggesting that circulating obestatin originating in the stomach might possibly be acting as the primary source of obestatin to the pituitary. However, we cannot discard the possibility that obestatin produced at the pituitary level may also be exerting local autocrine/paracrine effects, as previously demonstrated in other endocrine tissues (37).

We also found that both GLP1R and GPR39 are expressed in pituitary tissue (Figure 6B) and in primary pituitary cell cultures (Figure 6C and Table 1) of baboons and mice. Interestingly, our results indicate that the pituitary expression of GLP1R is greater than GPR39 in both species (Figure 6B). Moreover, similar to what was previously observed for other primary pituitary regulators of somatotrope and corticotrope function and their receptors (19, 20), long-term incubation with obestatin significantly increased the expression of GLP1R, but not of GPR39, in baboon and mouse pituitary cell cultures (Figure 6C). Although caution should be used in the interpretation of these data, our results indicate that GLP1R is significantly more expressed than GPR39 in the pituitary and, together with previous evidence, suggest the possibility that obestatin might be functionally linked with GLP1R at the pituitary level, because treatment with

Table 1. mRNA Copy Number

	Baboon Cell Cultures	Mouse Cell Cultures
GH	365 245 ± 67 391	531 866 ± 47 391
POMC	152 679 ± 26 254	346 529 ± 60 871
PRL	926 481 ± 108 912	1 239 458 ± 351 259
LH	42 581 ± 16 729	229 384 ± 66 847
FSH	46 812 ± 18 361	92 794 ± 18 732
TSH	28 537 ± 9291	113 846 ± 38 425
Pit-1	8264 ± 2481	5299 ± 1315
GHRH-R	12 945 ± 3284	7924 ± 1876
sst1	1045 ± 286	1297 ± 381
sst2	3524 ± 426	7294 ± 1684
sst5	4281 ± 596	5947 ± 1288
CRFR1	873 ± 101	2491 ± 534
CRFR2	213 ± 36	394 ± 78
GLP1R	654 ± 155	526 ± 124
GPR39	169 ± 42	79 ± 11
Cyclophilin A	175 946 ± 32 945	112 594 ± 10 438

Absolute mRNA copy number (adjusted to 0.05 μg total RNA) of gene transcripts in primary pituitary cell cultures from female baboons and mice (control groups from both species; 24 hours after incubation in serum-free media; n = 3–5 individual in vitro experiments [3–4 wells per experiment]) as determined by qrtRT-PCR. Values represent the mean ± SE.

obestatin upregulated the pituitary expression of this receptor, but not of GPR39, in a time-dependent fashion. However, future studies would be required to unequivocally ascertain the potential link between obestatin and GLP1R at the pituitary level.

Overall, the results of this study clearly indicate that obestatin oppositely regulates the hormonal expression and secretion of normal somatotrope and corticotrope function. However, given the extended duration of incubation (24 hours), it is also plausible that the effects observed on somatotrope and corticotrope cells involve both direct effects of obestatin on the target cells and secondary changes in the expression of other critical regulatory proteins conveying indirect actions. Nevertheless, it should be emphasized that we used primary pituitary cell cultures, and therefore, cell are dispersed, separated from each other, and no regulators (hypothalamic or systemic) are present in the serum-free culture media, thereby suggesting that the effects observed are likely direct and evoked in response to obestatin treatment through a putative obestatin receptor present in the pituitary. Of interest are the preliminary results obtained from a single time-course experiment (1, 4, 8, 12, 24, and 48 hours of incubation) performed in baboon primary pituitary cell cultures, where we observed that the inhibition of GH and the stimulation of POMC expression occurred in parallel to the upregulation of GLP1R expression (Supplemental Figure 2). Accordingly, it is tempting to speculate that if obestatin would exert its action through the GLP1R at the pituitary level, a sustained obestatin challenge (12–24 hours incubation) might enhance the expression and signaling of GLP1R, which might then be associated with the effects of obestatin on somatotrope and corticotrope cells. Another observation of particular interest for the somatotrope population is the parallel downregulation of the expression levels of GH and Pit-1 observed in the time-course experiment. Pit-1 is required for expression of GH, and its effect is mediated through binding to the promoter of the GH gene (31). Therefore, the parallel downregulation observed in GH and Pit-1 mRNA levels might suggest that the effect of obestatin on GH expression in baboon primary pituitary cell cultures might involve alterations in Pit-1 mRNA production. Obviously, further work will be required to complete our understanding of this complex process and to fully elucidate the molecular mechanisms underlying the effect of obestatin on GH and POMC/ACTH expression and release on baboons and mice.

Summary

The present study provides the first detailed description of the role that obestatin plays on the function of some major pituitary cell types in baboons and mice. Specifically, our results clearly demonstrate that obestatin may play a relevant, specific, and opposite role in regulating the function of 2 pituitary cell types, somatotropes and corticotropes, and that these effects are conserved across 2 distinct mammalian species (primates and mice). In addition, our results revealed that obestatin requires AC and MAPK activity to exert its inhibitory role on somatotrope cells and its stimulatory effect on corticotrope cells. Finally, this study describes some of the cellular and molecular mechanisms whereby obestatin influences somatotrope and corticotrope function in vitro and in vivo in both species. Altogether, our results provide the first comprehensive experimental evidence to support a potential role of obestatin in the direct control of these pituitary cell types in mice and, most importantly, in a primate model.

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