Obestatin inhibits vasopressin secretion: evidence for a physiological action in the control of fluid homeostasis

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Abstract

Obestatin, a product of post-translational processing of the ghrelin prohormone, has been reported to act in the brain to inhibit thirst. We extended our initial studies on water drinking by examining the effects of obestatin on hypovolemia-induced water and saline drinking and vasopressin release in male rats. Intracerebroventricular administration of obestatin significantly inhibited water, but not saline (0·3 M NaCl) drinking in response to a hypovolemic challenge. Obestatin also inhibited, in a dose-related fashion, dehydration-induced vasopressin secretion without affecting plasma oxytocin levels. Vasopressin release induced by central angiotensin II administration was attenuated significantly by prior administration of obestatin. Finally, central administration of an antiserum specific to obestatin resulted in an exaggerated basal vasopressin release and an increased vasopressin response to overnight water deprivation. Antiserum treatment also resulted in significantly increased *ad libitum* water drinking and drinking in response to dehydration. We conclude that this product of post-translational processing of the ghrelin prohormone may be an important contributor to the physiologic regulation of fluid and electrolyte homeostasis.
Journal of Endocrinology (2008) **196,** 559–564

Introduction

We have previously reported that obestatin, a 23 amino acid peptide derived from the same prohormone as ghrelin (Zhang *et al.* 2005), when injected into the lateral cerebroventricle inhibits water drinking in response to dehydration and angiotensin II administration (Samson *et al.* 2007). This antidipsogenic effect has been confirmed by Hsueh *et al.* (Zhang *et al.* 2007). While a number of studies have reported obestatin to inhibit food intake (Zhang *et al.* 2005, Bresciani *et al.* 2006, Sibilia *et al.* 2006, Green *et al.* 2007, Zizzari *et al.* 2007), other groups have not been able to reproduce such observations (Seoane *et al.* 2006, Chartrel *et al.* 2007, Nogueiras *et al.* 2007). In our hands the ability of obestatin to inhibit food intake appeared secondary to its action on water drinking (Samson *et al.* 2007). We also demonstrated direct neuronal actions of obestatin in subfornical organ (SFO), a potential site of action of peptides of both peripheral and central origin to inhibit not only water drinking, but also sodium appetite and vasopressin secretion. Here, we sought to determine if the antidipsogenic effect of obestatin could be extended to hypovolemia-induced thirst and salt appetite and if, in addition, the peptide could exert significant effects on vasopressin secretion. The physiological relevance of the pharmacological actions of obestatin was examined by passive immunoneutralization of endogenous obestatin.

Materials and Methods

Animals

All procedures have been approved by the animal care committee of Saint Louis University. Adult male rats (Sprague–Dawley, Harlan, Indianapolis, IN, USA) were maintained (12 h light:12 darkness cycle, lights-on 0600 h, 23–25 °C) with *ad libitum* access to food and water, unless otherwise indicated. Under ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA)/xylazine (TranquiVed, Vedco Inc., St Joseph, MO, USA) anesthesia (60 mg/8 mg mixture/ml, 0·1 ml/100 g body weight, i.p. injection) rats were placed in a stereotaxic device and a 23 gauge, stainless steel cannula (17 mm) implanted into the right lateral cerebroventricle as described previously (Antunes-Rodrigues *et al.* 2004, Samson *et al.* 2007). Rats were allowed to recover to presurgery weights, minimally 5 days prior to experimentation. Placement and patency of the lateral ventricular cannula were verified (Samson *et al.* 2007) by the dipsogenic response to angiotensin II (50 pm A II).

Hypovolemia-induced thirst and salt appetite were examined with a two-bottle preference test (Blackburn *et al.* 1993). Rats were acclimated to two drinking bottles, one with tap water, the other with 0·3 M NaCl, prior to experimentation. Animals were anesthetized by isoflurane gas inhalation (3% in O_2 for induction, 2% in O_2 for

DOI: 10.1677/JOE-07-0364
0022–0795/08/0196–559
Online version via http://www.endocrinology-journals.org

maintenance of anesthesia, IsoSol, Vedco, Inc.) and 5 ml polyethylene glycol solution (PEG, Carbowax PEG 20 000; Fisher Scientific, Pittsburgh, PA, USA; of 15% weight/volume in saline, 37 °C) injected subcutaneously. Animals were then denied access to food and water for 18 h to complete the hypovolemic challenge protocol (Blackburn *et al.* 1993). Ten minutes prior to returning the water and saline (0·3 M NaCl) drinking bottles to the cages, either 2 µl saline vehicle or vehicle containing 3·0 nm obestatin, a dose previously demonstrated by us to inhibit water consumption (Samson *et al.* 2007), was administered intracerebroventricularly. Cumulative intakes of water and saline were measured every 15 min for 1 h and every 30 min for the next 4 h. Food was then returned to the cages and fluid intakes monitored once more at 24 h. There were no significant differences in body weights between the rats administered obestatin or saline vehicle before or after the protocol was completed. Data were expressed in terms of ml water or saline consumed per 100 g body weight.

The effect of obestatin on physiologically driven vasopressin secretion was examined in rats deprived of water, but not food, for 18 h prior to experimentation. Animals were moved to a quiet room 2 h prior to injection of vehicle (2 µl, sterile 0·9% NaCl, i.c.v.) or vehicle containing 1·0 or 3·0 nm obestatin (0900–1000 h). Rats were killed by decapitation 15 or 30 min later and trunk blood collected into heparinized tubes. Samples were maintained on ice and then centrifuged (3000 ***g***, 4 °C, 30 min) to allow collection of plasma for subsequent determination of vasopressin (AVP) and oxytocin (OT) levels by RIA (Samson 1985, Samson *et al.* 1985).

The effect of obestatin on pharmacologically driven vasopressin secretion was examined in *ad libitum* fed and watered rats (0900–1000 h). Water bottles were removed from the cages and animals administered 2 µl saline vehicle (sterile 0·9% NaCl, i.c.v.) or vehicle containing 1·0 or 3·0 nanomole obestatin, 10 min prior to the administration of A II (50 picomole in 2 µl, i.c.v., Qadri *et al.* 1993). Five minutes following A II injection, the rats were killed by decapitation and trunk blood collected as described above.

In a final series of experiments, the effects of central administration of anti-obestatin antiserum on vasopressin secretion and thirst were determined. The effect of anti-obestatin treatment on basal vasopressin secretion was examined in *ad libitum* fed and watered rats. Two hours after being moved to a quiet room (0900–1000 h), the animals received an i.c.v. injection of 3 µl normal rabbit serum (non-immune serum, Sigma Chemical Co.) or 3 µl anti-obestatin antiserum (H&L purified, G-031-92, Phoenix Pharmaceuticals, Belmont, CA, USA). This antiserum is selective for obestatin and displays no cross-reactivity with ghrelin. Tissue staining for obestatin in the myenteric plexus is absent when this antiserum is preabsorbed with excess obestatin (Dunn *et al.* 2006). In addition, using this antiserum in western blot analysis of extracts of stomach and hypothalamus, a single band of immunoreactivity was detected that migrated similarly to synthetic obestatin (data not shown). Animals were left undisturbed with access to food and water for 1 h, at which time they were killed by decapitation and trunk blood collected as described above.

The effect of anti-obestatin treatment on dehydration-induced vasopressin secretion was examined in overnight water-restricted animals. Two hours after being moved to a quiet room, the animals received an i.c.v. injection of 3 µl normal rabbit serum (NRS) or 3 µl anti-obestatin antiserum as described above. Thirty minutes later, the rats were killed and trunk blood collected.

The effect of anti-obestatin administration on water and food intakes in *ad libitum* fed and watered animals was examined as described previously (Samson *et al.* 2007) with the exception that instead of i.c.v. administration of peptide, animals received cerebroventricular injections of 3 µl normal rabbit serum or 3 µl anti-obestatin antiserum, at the beginning of a 30-min interval of food and water restriction (1530–1600 h). Food and water were returned to the metabolic cages at 1600 h and intakes monitored at 30-min intervals until 2000 h and again at noon and 1600 h on the following day when the animals were weighed.

The effect of anti-obestatin administration on dehydration-induced water drinking was examined in overnight water-restricted (food present) animals. Two hours after being moved to a quiet room, the animals received an i.c.v. injection of 3 µl normal rabbit serum or 3 µl anti-obestatin antiserum as described above (0900–1000 h). Water bottles were returned to the cages 30 min later and intakes monitored for the following 5 h and again at 24 h.

Determination of plasma vasopressin and OT content

AVP content in plasma was determined by RIA as described previously (Samson 1985) following extraction of 1·0 ml plasma using C-18 chromatography. The lower limit of sensitivity of our AVP RIA (defined as 95% B/B_0) is 0·125 pg per tube and the intra-assay variability determined in replicate serum pool samples was $<5\%$. Since several assays were conducted during these experiments, we included samples from the same serum pool in each assay and the inter-assay coefficient of variability was $<6\%$. Plasma OT levels were determined as described previously (Samson *et al.* 1985) following extraction using cold methanol (0·3 ml plasma/0·6 ml methanol). The lower limit of detection of the OT RIA was 0·5 pg per tube. The inter- and intra-assay coefficients of variability were $<8\%$. Recoveries for both the AVP and OT extractions were consistently $>90\%$. Values are reported as mean plasma hormone levels (pg/ml, $\pm$S.E.M).

Statistical analysis

Differences between groups or within groups across time were determined by ANOVA with Scheffe's multiple comparison testing. In experiments with only two experimental groups, the independent *t*-test was employed. An outcome with a probability of $<5\%$ was considered significant. All data are presented as means and standard errors of the mean.

Results

Central administration of 3·0 nm obestatin, a dose we have previously demonstrated to inhibit thirst (Samson *et al.* 2007), significantly reduced water, but not saline drinking, in response to PEG-induced hypovolemia (Fig. 1). The inhibition by obestatin attained significance after 2 h of drinking and remained significant for at least three more hours. There were no significant differences in water or saline intakes 24 h after the bottles were returned to the cages.

Anti-obestatin antiserum administration resulted in a significantly increased water drinking in *ad libitum* fed and watered rats (Fig. 2a). The effect was already apparent at the first 30 min of observation and remained significant ($P<0·05$) well into the lights-out period. Even at noon (antiserum-treated: 8·1±1·0, NRS-treated: 6·1±0·9 ml/100 g body weight) and 1600 h (antiserum-treated: 10·0±1·2, NRS-treated: 8·0±1·0) the following day, the animals in the antiserum treatment group continued to display increased, cumulative water intakes; however, these elevations did not attain statistical significance. Similarly, water intakes over the next 24-h interval did not differ significantly between groups (data not shown). While food intakes were elevated in antiserum-treated animals when compared with controls (Fig. 2b), these differences failed to reach a statistical significance at any time point. Body weights (data not shown) did not differ significantly between groups on the day prior to i.c.v. injections, or for the subsequent 3 days.

Antiserum administration significantly increased cumulative water drinking in overnight water-restricted rats (Fig. 3). The effect attained significance only at 4 ($P<0·5$) and 5 ($P<0·01$) h following water bottle return; however, it remained statistically significant 24 h later ($P<0·001$). Again, body weights did not differ between treatment groups.

Plasma AVP levels were significantly elevated following overnight water restriction (see Fig. 4a, dehydrated controls versus Fig. 5, normally hydrated controls). Obestatin administration i.c.v. significantly lowered the plasma AVP levels in water-restricted animals without significantly altering plasma OT levels (Fig. 4b). The inhibitory action of obestatin on AVP secretion at 15 min was dose-related (1·0 nm obestatin, $P<0·05$; 3·0 nm obestatin, $P<0·001$;

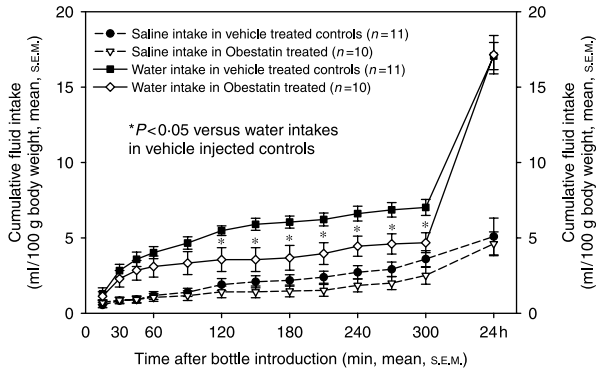

Figure 1 Intracerebroventricular administration of 3·0 nm obestatin significantly inhibits water drinking, but not saline drinking, in male rats following polyethylene glycol-induced hypovolemia. *$P<0·05$ versus water intake in vehicle-treated controls.

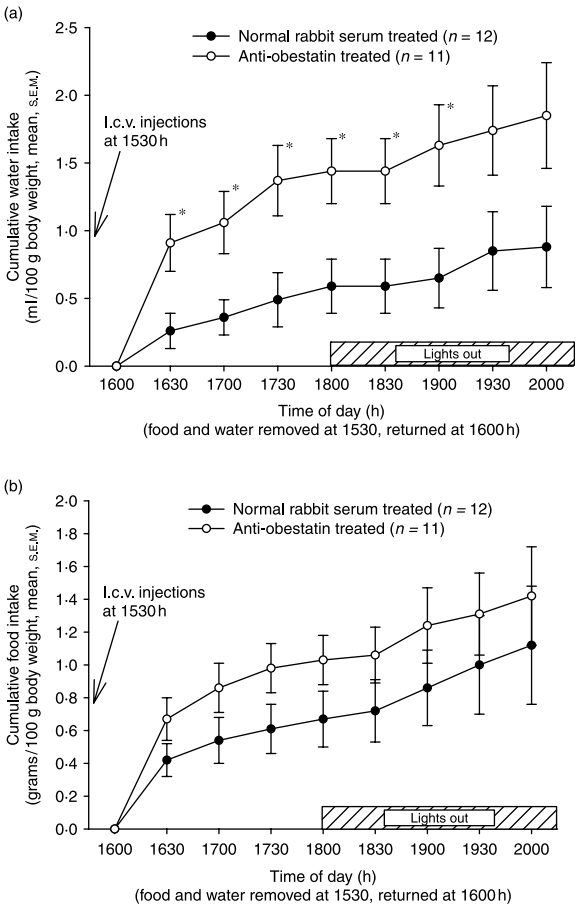

Figure 2 Effect of passive immunoneutralization of endogenous obestatin on (a) *ad libitum* water drinking and (b) food intake in male rats. *$P<0·05$ versus intakes in NRS-treated controls.

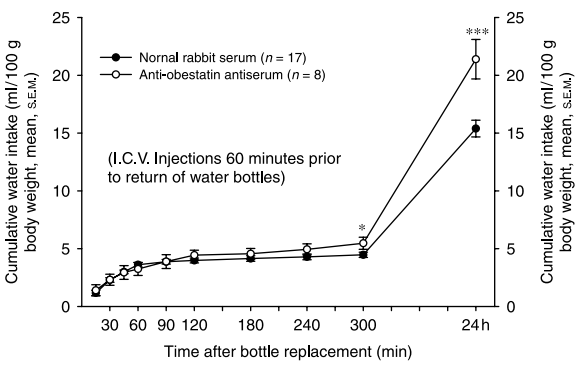

Figure 3 Effect of passive immunoneutralization of endogenous obestatin on water drinking in overnight water-restricted male rats. *$P<0·05$, ***$P<0·001$ versus intakes in NRS-treated controls.

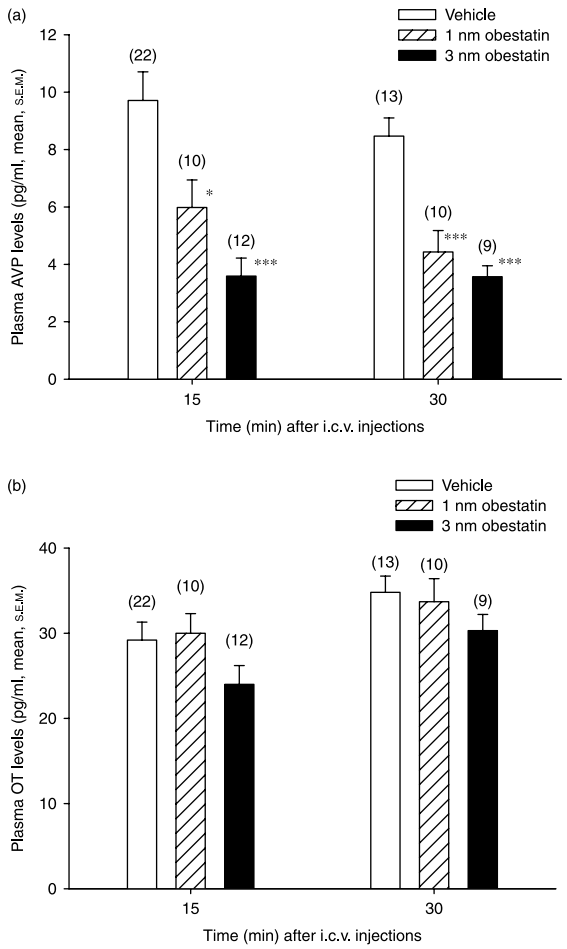

Figure 4 Intracerebroventricular administration of obestatin significantly reduces overnight dehydration-induced vasopressin (a) but not oxytocin (b) levels in male rats. *$P<0{\cdot}05$, ***$P<0{\cdot}001$ versus levels present in vehicle-treated controls.

obestatin versus vehicle) and was still evident 30 min after peptide administration ($P<0{\cdot}001$, both the doses).

Central administration of 50 pm angiotensin II resulted in a significant elevation of plasma AVP levels ($P<0{\cdot}001$), without any significant effect on plasma levels of OT (Fig. 5). Pretreatment with 1·0 or 3·0 nm obestatin 10 min prior to the angiotensin II administration resulted in a significant reversal of the stimulatory actions of angiotensin II. The AVP levels in angiotensin II-administered animals pretreated with 1·0 nm obestatin were significantly lower than those in saline-pretreated rats ($P<0{\cdot}05$), but still significantly greater than levels in control animals (saline-pretreated, saline-administered instead of angiotensin II, $P<0{\cdot}05$). On the other hand, plasma AVP levels in rats pretreated with 3·0 nm obestatin and then administered angiotensin II did not differ significantly from those present in saline-pretreated and saline-administered controls. OT levels were not affected by obestatin administration.

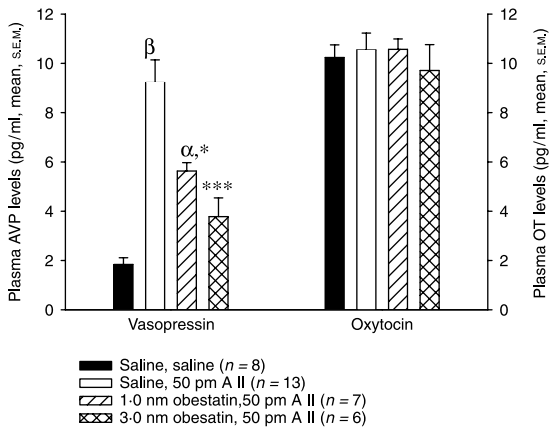

Figure 5 Intracerebroventricular administration of angiotensin II significantly elevates plasma vasopressin, but not oxytocin, levels in normally hydrated rats and this effect is significantly reduced by pretreatment with obestatin. *$P<0{\cdot}05$, ***$P<0{\cdot}001$ versus saline-pretreated, A II-injected rats. $^{\alpha}P<0{\cdot}05$ versus saline-pretreated, saline-injected controls. $^{\beta}P<0{\cdot}001$ versus saline-pretreated, saline-injected controls.

Basal AVP levels in *ad libitum* fed and watered animals were significantly elevated following i.c.v. administration of anti-obestatin antiserum ($4{\cdot}2\pm0{\cdot}5$ pg AVP/ml plasma, $n=15$) compared with levels present in NRS-treated controls ($1{\cdot}4\pm0{\cdot}2$, $n=8$). Plasma AVP levels present in NRS-treated controls did not differ significantly from untreated or saline-injected controls.

The elevated plasma AVP levels observed in water-restricted animals administered normal rabbit serum i.c.v. were not significantly different than those observed in water-restricted control animals (Fig. 6). However, plasma AVP levels were significantly elevated above control in water-restricted animals administered anti-obestatin antibodies 30 min before killing. Plasma OT levels were not significantly altered by non-immune serum or anti-obestatin administration.

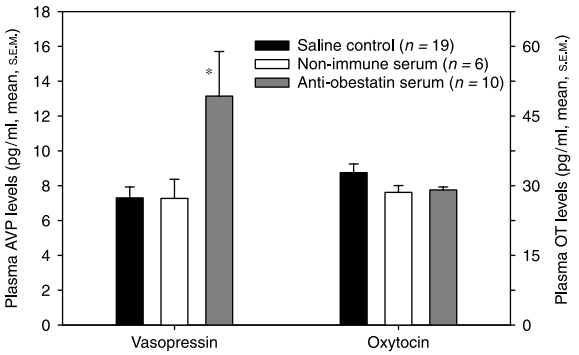

Figure 6 Intracerebroventricular administration of anti-obestatin antibodies significantly elevates dehydration-induced vasopressin, but not oxytocin, secretion in male rats. *$P<0{\cdot}05$ versus vehicle- or normal rabbit serum (non-immune serum)-injected controls.

Discussion

We have extended our earlier studies on the central actions of obestatin on water drinking behavior (Samson *et al*. 2007) by demonstrating that in response to hypovolemia, obestatin selectively inhibits water, but not 0·3 M NaCl drinking. More importantly, both physiologically and pharmacologically driven AVP secretion is inhibited by similar doses of obestatin. The effect appeared to be selective for AVP, as plasma levels of OT remained unaffected. Thus, it is not surprising that in the two-bottle preference test (PEG-induced hypovolemia) we observed no effect of obestatin on saline drinking, a behavior influenced by OT release (Blackburn *et al*. 1993). The pharmacologic action of obestatin to inhibit AVP secretion may have physiologic relevance since passive immunoneutralization of endogenous obestatin resulted in exaggerated AVP levels under basal conditions and in response to water deprivation. Furthermore, the antidipsogenic effects of synthetic obestatin appear to reflect a potentially important action of the endogenous peptide to restrain water drinking since both *ad libitum* and dehydration-induced water intakes were significantly elevated in anti-obestatin treated animals. As in our previous publication (Samson *et al*. 2007), we conclude that any effects of obestatin of exogenous or endogenous origin on feeding are likely to be secondary to its antidipsogenic action since cumulative food intakes did not differ significantly between antiserum and NRS-treated animals.

These studies do not identify the specific site of action of obestatin to inhibit thirst or vasopressin secretion; however, we have previously reported that obestatin exerts direct membrane effects on dissociated SFO neurons (Samson *et al*. 2007). Thus, it is possible that in these studies obestatin exerted its antidipsogenic and AVP inhibiting effects within the SFO. Although controversy exists (Lauwers *et al*. 2006, Moechars *et al*. 2006, Holst *et al*. 2007, Tremblay *et al*. 2007), to date the only identified receptor that may bind obestatin *in vivo* is G protein-coupled receptor 39 (GPR39). In one study (Jackson *et al*. 2006), GPR39 mRNA was not observed in the hypothalamic sites; however, in the original description of obestatin, Zhang *et al*. 2005 were able to demonstrate the presence of the message in mouse hypothalamus by reverse transcriptase-PCR methodologies. It is not clear whether or not the SFO was included in the tissues examined by either group. While the original identification of GPR39 as a possible receptor for obestatin remains to be verified, it is possible that another, yet to be identified receptor mediates the actions of the peptide. Alternatively, additional splice variants of the GPR39 gene product (Egerod *et al*. 2007) may exist in the hypothalamus, which were not detected in the initial studies (Jackson *et al*. 2006). The SFO and perhaps more directly the paraventricular or supraoptic nuclei remain attractive potential sites for the effects of the peptide described here, since we administered obestatin in our animals behind the blood–brain barrier. At least three other groups have reported the cellular effects of obestatin behind the blood–brain barrier (Dunn *et al*. 2006, Szentirmai & Krueger 2006, Carlini *et al*. 2007) and, because it has been reported that peripherally administered obestatin is cleared from the circulation very quickly and not likely to cross the barrier (Pan *et al*. 2006), we hypothesize that our passive immunoneutralization results reflect the sequestration of brain-derived obestatin, released from populations of preproghrelin expressing neurons previously reported in multiple CNS sites (Koijima & Kangawa 2005). Indeed, the antiserum we employed in that study has been demonstrated to be specific for obestatin as preabsorption with synthetic obestatin eliminated the immunohistochemical identification of obestatin-positive cells in myenteric plexus, cells that also stain positively for preproghrelin (Dunn *et al*. 2006), and immunoreactive obestatin extracted from stomach and hypothalamus is visualized as a single band with appropriate mobility in western blot analysis.

In summary, we have demonstrated that in addition to a pharmacologic action to inhibit water drinking, obestatin acts in the brain to reduce the secretion of AVP in response to both pharmacologic and physiologic stimuli. We hypothesize that the complementary actions of obestatin to inhibit thirst and AVP secretion reflect a physiologically relevant action of the endogenous peptide to buffer total body fluid content. Certainly, our demonstration that the central administration of anti-obestatin antibodies results in an exaggerated AVP secretion under basal conditions, and in response to water deprivation, suggests that these pharmacologic effects of the peptide may have a physiologic relevance. Indeed, the ability of the passive immunoneutralization of endogenous obestatin to elevate basal AVP levels in *ad libitum* fed and watered rats suggests that brain-derived peptide may function to protect the animal against inappropriate secretion of that hormone. Much remains to be learned about the regulation of obestatin production and release, its sites of action, and its full biologic activity; however, our findings described here and previously (Samson *et al*. 2007) do draw attention to both behavioral and endocrine actions of the peptide that may provide further insight into the physiology of fluid homeostasis.

Acknowledgements

This work was supported by NIH Grant HL66023 to W K S and A V F. The authors have no conflict of interest to disclose.

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Received in final form 26 October 2007
Accepted 27 November 2007
Made available online as an Accepted Preprint
27 November 2007