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Comparative Analysis of the Effects of Ghrelin and Unacylated Ghrelin on Luteinizing Hormone Secretion in Male Rats

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Ghrelin, the endogenous ligand of GH secretagogue receptor type 1a, has emerged as pleiotropic modulator of diverse biological functions, including energy homeostasis and, recently, reproduction. Although inhibitory actions of ghrelin on LH secretion and puberty onset have been reported previously, the receptor mechanisms mediating these actions, and the potential gonadotropic effects of the unacylated isoform of ghrelin (UAG), remain unclear. In this work, the effects of single and repeated administration of ghrelin or UAG on LH secretion were compared in pubertal and adult male rats. In addition, the effects of ghrelin were assessed in models of transient or persistent hypergonadotropism. Daily injection of ghrelin or UAG throughout puberty similarly decreased LH levels and partially delayed balanopreputial separation. Likewise, chronic infusion of ghrelin or UAG to adult males resulted in significant decreases in circulating LH and FSH concentrations. Moreover, acute injection of ghrelin induced a transient reduction in LH levels in freely moving males, an effect that was fully mimicked by administration of UAG. Yet in contrast to ghrelin, UAG failed to modify GH secretion. Finally, injection of ghrelin moderately, but significantly, reduced the duration of LH secretory responses to the potent gonadotropin secretagogue kisspeptin-10, whereas ghrelin infusion in a model of chronic elevation of serum gonadotropin levels (the transgenic growth retarded male rat) evoked a significant reduction of LH concentrations. Altogether our present results further substantiate the inhibitory effect of ghrelin on basal and stimulated LH secretion in a wide array of experimental conditions. Moreover, our data are the first to demonstrate the ability of UAG, originally considered an inert form of the molecule, to mimic the actions of acylated ghrelin on LH release. These observations reinforce the contention that ghrelin, as putative signal for energy insufficiency, may operate as negative modifier of male puberty and LH secretion, an effect that might be, at least partially, conducted through a GH secretagogue receptor type 1a-independent mechanism. (*Endocrinology* 147: 2374–2382, 2006)

GHRELIN IS A 28-amino-acid peptide, primarily secreted by the stomach, and originally identified as the endogenous ligand of the GH secretagogue receptor (GHS-R) (1, 2). The GHS-R is a member of the large family of G protein-coupled, seven-transmembrane domain receptors, of which two major subtypes, generated by alternative splicing of a single gene, have been described so far: the full-length type 1a receptor and the truncated GHS-R type 1b (3, 4). The GHS-R1a is defined as the functionally active, signal transducing form of the receptor. In contrast, the GHS-R1b lacks the transmembrane domains 6 and 7 and is apparently devoid of high-affinity ligand binding and signal transduction capacity (3). Apart from its high degree of conservation among species, a striking feature of ghrelin molecule is its ability to incorporate, though acylation, an *n*-octanoyl group at Ser3 (1), the first posttranslational modification of this type reported in a secreted protein (5). Indeed, it is well established that octanoylation at Ser3 is absolutely mandatory for the binding of ghrelin to GHS-R1a and the stimulation of GH secretion (6). By extension, it has been assumed that, acting through GHS-R1a, acylated ghrelin is responsible for the whole set of endocrine activities of ghrelin (see below). However, a wealth of evidence has very recently emerged indicating that unacylated ghrelin (UAG), whose concentration in plasma and circulating half-life exceed those of octanoylated ghrelin, is not merely an inert form of the hormone. Indeed, UAG has been shown to exert specific peripheral actions, including cardiovascular, adipogenic, and (anti)proliferative effects (6–8). Moreover, evidence for central neuroendocrine effects of ghrelin independent of GHS-R1 has also been recently reported (9).

In the context of the interaction of ghrelin with the neuroendocrine axes, fragmentary information now indicates that ghrelin may participate in the modulation of the hypothalamic-pituitary-gonadal function, with a predominantly inhibitory effect on the reproductive system in primates, sheep, and rats (10–17). Expression of ghrelin has been demonstrated in human and rodent placenta, and ghrelin has been reported to inhibit early embryo development *in vitro*

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Abbreviations: AS, Albino Swiss; BPS, balanopreputial separation; GHS-R, GH secretagogue receptor; Tgr, transgenic growth retarded; UAG, unacylated isoform of ghrelin.

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and pregnancy outcome *in vivo* (10–12). In addition, ghrelin has been shown to suppress LH secretion *in vivo* and decrease LH responsiveness to GnRH *in vitro* (13–16). Moreover, repeated administration of ghrelin induced a partial delay in the timing of puberty in male rats (12). Finally, transcripts for ghrelin and its cognate receptor have also been identified in rat and human gonads (6, 17), and ghrelin has been reported to inhibit stimulated testicular testosterone secretion (16). Given its proposed role as peripheral signal for energy insufficiency (18), the above data suggest that ghrelin exerts a negative influence on the gonadotropic axis, contributing to the complex neuroendocrine network linking energy status and fertility. Despite these reported observations, the physiological role of ghrelin in regulating gonadotropin secretion remains unclear. Similarly, the receptor mechanisms mediating the actions of ghrelin on the reproductive axis are yet to be determined.

Most of the studies conducted in this neglected aspect of ghrelin physiology have focused on the effects of acute injection of acylated ghrelin on basal LH secretion (see Refs. 6 and 19). However, the consequences of acute or chronic administration of the unacylated form of the molecule have not been documented. In this study, we investigated the effects of UAG on gonadotropin secretion and examined whether the action of ghrelin in the gonadotropic axis is influenced by coadministration of UAG. In addition, we evaluated the effects of acylated ghrelin on transiently or persistently elevated LH secretion. Collectively, our data reinforce the contention that ghrelin is a negative regulator of gonadotropin (LH) secretion in the rat. The fact that such an action was mimicked by the unacylated form of the molecule in a number of experimental settings suggests a previously unsuspected, potential neuroendocrine role of UAG in the control of the gonadotropic axis.

Materials and Methods

Experimental designs

The procedures described for each experimental design were approved by the corresponding local ethical committees and were conformed to the institutional and national guidelines for care and use of experimental animals. Rat ghrelin, with *n*-octanoyl modification at Ser3, and rat UAG were purchased from Global Peptides (Fort Collins, CO; experiments 1, 3, and 4) and Phoenix Pharmaceuticals Ltd. (Belmont, CA; experiments 2 and 5). Mouse KiSS-1 (110–119)-NH_2, termed hereafter as kisspeptin-10, was obtained from Phoenix Pharmaceuticals Ltd.

Experiment 1: effect of chronic intermittent administration of ghrelin or UAG in pubertal male rats

Male Wistar rats (n = 10–11/group) received twice daily (at 0900 and 1900 h) sc injections of ghrelin (1.0 nmol per 100 μl), UAG (1 nmol/μl) or vehicle (100 μl physiological saline) between postnatal d 34 and d 43. This period was selected on the basis of previous references on the normal timing of puberty in the male rat (20) and local data on the occurrence of balanopreputial separation (BPS) in our animal stock. Because puberty is particularly sensitive to changes in energy stores, special efforts were taken to minimize the potential bias of major differences in body weight on the different end points under analysis. Thus, ghrelin-treated rats were pair fed with vehicle-injected animals. To this end, daily food intake was recorded in untreated animals, and an equal fixed amount of standard chow was daily provided to control and ghrelin-treated animals. In all experimental animals, body weight and food intake were monitored daily, and the occurrence of BPS, defined as complete separation of prepuce from gland penis (balano), was recorded. The rats were killed by decapitation on d 43, at 1 h after last sc injection, when trunk blood was collected for determination of circulating LH and FSH concentrations. Additional blood samples were obtained by jugular venipuncture under light ether anesthesia on d 36 and d 41, at 1 h after sc injection of vehicle, ghrelin, or UAG.

Experiment 2: effect of chronic continuous administration of ghrelin and UAG in adult male rats

Adult (~12 wk old) male Sprague Dawley rats (n = 6 per group) were prepared with a single-bore jugular vein cannula connected via a regulator to an osmotic minipump (Alzet model 2001; Alza Corp., Palo Alto, CA) implanted sc under halothane anesthesia. Minipumps delivered vehicle alone [sterile saline containing BSA (1 mg/ml) and heparin (10 U/ml)] or vehicle containing rat ghrelin (1.0 nmol/h) or des-octanoyl ghrelin (1.0 nmol/h) for 7 d. An additional group of animals were infused for 7 d with the GHS-R1a-specific agonist, L163,255 (160 μg/d) (21). Body weight and food intake were monitored daily throughout the 7-d infusion period. At the end of the infusion period, the rats were killed by decapitation, when samples of trunk blood were collected for LH and FSH determinations.

Experiment 3: effect of single iv injection of ghrelin or UAG on circulating LH in adult male rats

Adult (~12 wk old) male Sprague Dawley rats (n = 8 per group) were prepared with intracardiac (jugular vein) cannulae, as described in experiment 2. After 48 h recovery, the animals were subjected to serial blood sampling, after iv administration of ghrelin or UAG, under freely moving conditions. In detail, blood samples (250 μl) were taken every 15 min over a 150-min period. To obtain basal levels, the animals were sampled three times before iv injection of a single bolus of ghrelin (3.0 nmol/rat), UAG (3.0 nmol/rat), or vehicle (physiological saline). A fourth group received coadministration of ghrelin and UAG (3.0 nmol each). Separated serum was stored at −20 C before determination of LH concentrations.

Experiment 4: effect of ghrelin on kisspeptin-stimulated LH secretion in adult male rats

Adult (~12 wk old) male Sprague Dawley rats (n = 8 per group) were prepared with intracardiac (jugular vein) cannulae, as described in experiment 2. After 48 h recovery, the animals were subjected to serial blood sampling, after coadministration of ghrelin and kisspeptin, under freely moving conditions. In detail, blood samples (250 μl) were taken every 15 min over a 150-min period. To obtain basal levels, the animals were sampled three times before iv injection of a single bolus of vehicle, kisspeptin-10 (7.5 nmol/rat), ghrelin (3.0 nmol/rat), or ghrelin and kisspeptin-10. Of note, kisspeptin has been recently identified as the most potent stimulator of the GnRH/LH axis (22, 23), and our previous data demonstrated that the dose selected (7.5 nmol) was able to evoke a transient approximately 10-fold increase in circulating LH levels (22).

Experiment 5: effect of patterned iv infusions of ghrelin in hypergonadotropic transgenic growth-retarded (Tgr) male rats

To determine whether ghrelin can suppress chronically elevated gonadotropin secretion, patterned infusions of ghrelin were administered to a model of persistent hypergonadotropism, the Tgr rat. In this transgenic model, expression of human GH in the arcuate GH-releasing factor neurons leads to a reduction in the amplitude of the spontaneous episodes of GH secretion and an accompanying dwarfism (24). An intriguing observation recently made by our group is that, in the course of adulthood, male Tgr rats also become hypergonadotropic, with persistently elevated serum levels and pituitary contents of both gonadotropins (Davies, J. S., N. M. Thompson, M. C. Christian, L. Pinilla, F. J. P. Ebling, M. Tena-Sempere, and T. Wells, manuscript submitted). Hemizygous Tgr rats and their wild-type albino Swiss (AS) littermates were derived from the original colonies at the National Institute for Medical Research (London, UK) and bred in the Transgenic Unit (School of Biosciences, Cardiff University, Cardiff, UK). Groups of adult male Tgr

rats (14 wk old; weighing 216–242 g; n = 6/group) were acclimatized in metabolic cages for 4 d before the implantation of a single-bore jugular vein cannula under halothane anesthesia. After 48 h recovery, an automated infusion system was used to deliver an iv infusion of either vehicle [sterile saline containing BSA (1 mg/ml) and heparin (10 U/ml)], given either continuously (at 100 μl/h) or intermittently (300 μl, 2-min pulses every 3 h), or vehicle containing ghrelin given either continuously (24 nmol/d) or in 10-μg (300 μl) pulses every 3 h, for 7 d. Body weight and food intake were monitored daily throughout the 7-d infusion period. At the end of the infusion period, the rats were killed by cervical dislocation. A terminal blood sample from each experimental animal was removed by cardiac puncture for determination of circulating LH and FSH levels. Terminal blood samples were also withdrawn from a cohort of uncannulated, age-paired AS littermates, taken for reference purposes.

Hormone measurements by specific RIAs

Circulating levels of LH and FSH were measured in a volume of 25–50 μl using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (NIH; Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 and FSH-I-9 were labeled with ^{125}I by the chloramine-T method, and the hormone concentrations were expressed using the reference preparation LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation were 8 and 10%, respectively, for LH and 6 and 9%, respectively, for FSH. The sensitivity of the assays was 5 pg/tube for LH and 20 pg/tube for FSH. In addition, in selected experimental samples (experiment 3), serum GH levels were measured by a double-antibody RIA, using kits provided by the NIH. Rat GH-I-7 was labeled as described above, and hormone concentrations were expressed using reference preparation GH-RP-2. Intra- and interassay coefficients of variation were 6 and 9%, respectively, and the sensitivity of the assay was 5 pg/tube. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

Presentation of data and statistics

LH and FSH determinations were conducted in duplicate, with a total number of six to 12 samples/determinations per group. Hormonal data are presented as mean ± SEM. In addition, when relevant (see experiment 3), integrated LH secretory responses were expressed as the area under the curve, calculated following the trapezoidal rule, over a 105-min period after iv administration of ghrelin peptides. Hormonal data were analyzed for statistically significant differences using one-way ANOVA followed by Student-Newman-Keuls multiple range test or, in case of serial blood sampling, repeated-measures ANOVA followed by Student-Newman-Keuls test (SigmaStat 2.0; Jandel Corp., San Rafael, CA). χ^2 evaluation was used for statistical comparison of data on percentages of BPS (SigmaStat 2.0). Unless otherwise stated, $P \leq 0.05$ was considered statistically significant.

Results

Gonadotropin secretion after chronic administration of ghrelin or UAG to pubertal male rats

The effects of chronic intermittent administration of ghrelin or UAG on serum LH and FSH levels were first evaluated in pubertal male rats; a developmental period during which inhibitory effects of ghrelin on different reproductive parameters have been described (11). Twice-daily injection of ghrelin or UAG to pair-fed animals had no significant effect in total body weight (Fig. 1A) or cumulative weight gain (data not shown). In contrast, mean LH concentrations were significantly reduced in ghrelin-treated males at d 36 and d 41, an effect that was fully mimicked by repeated administration of UAG (Fig. 1B). Intriguingly, at the end of the treatment period (d 43), mean serum LH levels in ghrelin- and UAG-injected groups were similar to those of

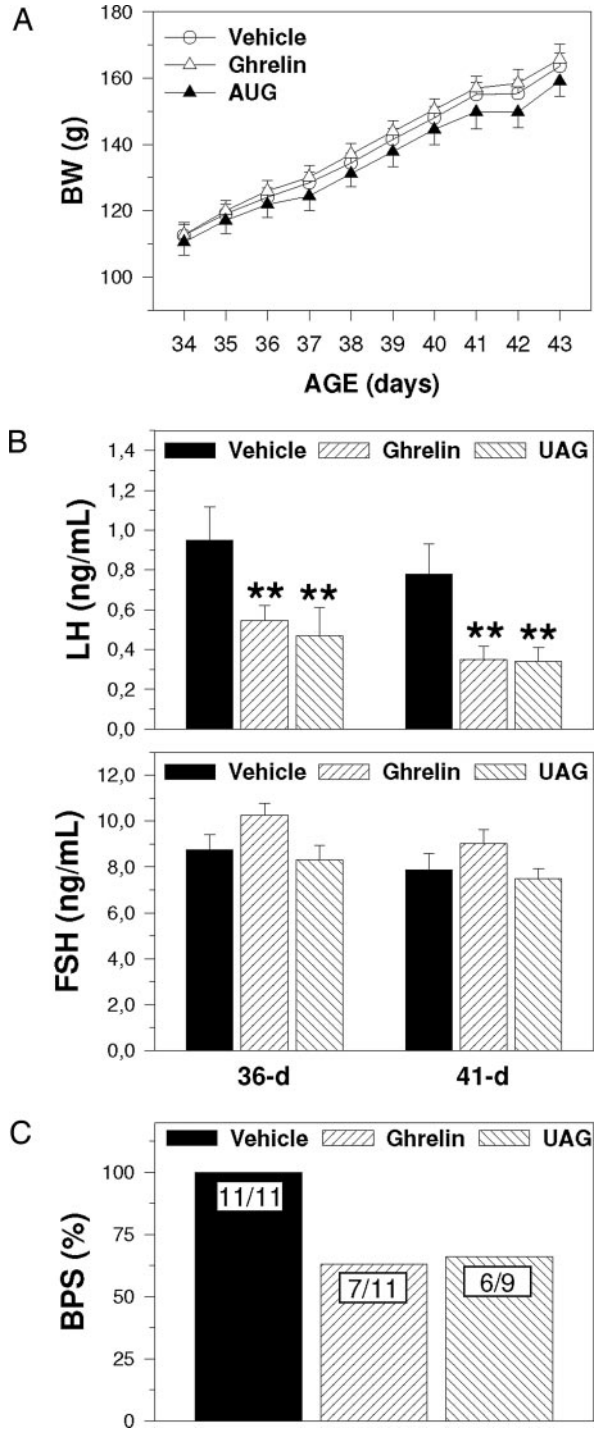

FIG. 1. Effects of chronic intermittent administration of ghrelin or UAG on serum gonadotropin levels and occurrence of preputial separation in pubertal male rats. Ghrelin or UAG was repeatedly injected at a dose of 1.0 nmol per 12 h between postnatal d 34 and d 43. A, Total body weight (BW) records in the experimental groups are presented. B, Serum LH and FSH levels in vehicle-, ghrelin-, and UAG-treated animals are shown. Samples were obtained at d 36 and d 41 1 h after last injection of the peptide. C, The cumulative percentage of males with complete BPS at d 43 is presented. The experimental groups were composed of nine to 11 animals. When applicable, values are given as the mean ± SEM. **, $P < 0.01$ *vs.* corresponding vehicle-injected animals (ANOVA followed by Student-Newman-Keuls multiple range test).

vehicle-treated controls (0.86 ± 0.20 ng/ml in ghrelin-treated and 0.82 ± 0.09 ng/ml in UAG-treated *vs.* 0.85 ± 0.18 ng/ml in controls). In contrast, chronic treatment with ghrelin or UAG failed to modify serum FSH levels at any age point studied (Fig. 1C). Finally, the impact of chronic ghrelin and UAG treatments on BPS, as external biomarker of puberty, was comparatively evaluated. The age of occurrence of BPS in controls rats was 41.6 ± 0.7 d, and 100% of animals (11 of 11) presented complete preputial separation at d 43. In this context, chronic administration of ghrelin partially prevented normal timing of occurrence of BPS because complete preputial separation was detected in only 63.5% (seven of 11) of ghrelin-treated animals at d 43 (Fig. 1D). Likewise, repeated administration of UAG resulted in a similar reduction in the percentage of pubertal males showing complete BPS at the end of the treatment period (66.6%; six of nine animals). Statistical analysis of these differences (using χ^2 tests) yielded $P < 0.069$, *i.e.* close to the limit of statistical significance.

Gonadotropin secretion after chronic administration of ghrelin or UAG to adult male rats

In addition, the effects of chronic administration of ghrelin or UAG on gonadotropin secretion were monitored in adult male rats. Continuous infusion of ghrelin or UAG had no significant effect in total body weight (Fig. 2A), yet cumulative weight gain was significantly reduced after 7 d of UAG infusion ($P < 0.05$; data not shown). In keeping with data from pubertal males, circulating LH levels decreased significantly at the end of the treatment periods of chronic infusion of ghrelin or UAG (Fig. 2B). Likewise, terminal FSH levels were significantly lower in ghrelin- and UAG-infused animals than in their corresponding controls (Fig. 2C). Interestingly, iv infusion of the GHS-R1a-specific agonist, L163,255, also reduced significantly plasma LH concentrations (0.248 ± 0.06 *vs.* 0.678 ± 0.11 ng/ml in vehicle-infused rats; $P < 0.01$) but had no effect on circulating FSH levels (5.66 ± 0.43 *vs.* 5.66 ± 0.60 ng/ml in vehicle-infused rats).

Effect of a single iv injection of ghrelin or UAG on circulating LH levels in adult male rats

Because results from experiments involving chronic administration indicated that ghrelin and UAG equally decreased serum LH levels in pubertal and adult male rats, the phenomenon was further explored in adult male rats after iv injection of a single bolus of ghrelin, UAG, or a combination of both. Injection of ghrelin induced a transient decrease in serum LH levels (compared with corresponding preinjection levels), LH concentrations being halved 15 min after injection (Fig. 3A). Likewise, injection of UAG induced a similar lowering in serum LH concentrations in conscious animals at 15 min, a phenomenon that was sustained up to the end of the sampling period (Fig. 3B). Finally, coadministration of ghrelin and UAG induced a consistent suppression of circulating LH levels (Fig. 3C). Accordingly, when integrated LH secretory responses to ghrelin, UAG, or their combination were calculated over the period of the study, it became apparent that both ghrelin and UAG, or their equimolar mixture, equally decreased net LH secretion *vs.* their corresponding control values (Fig. 3D). Nevertheless, discrete differences were detected in the time course of the suppression of LH secretion by ghrelin, UAG, or the coadministration of both peptides. Thus, serum LH levels were not significantly decreased 60 min after injection of ghrelin, but LH concentrations remained lower than preinjection values throughout the study period after administration of UAG or ghrelin plus UAG, yet the decrease of LH levels 15 min after coadministration of ghrelin and UAG was in the limit of statistical significance. Finally, in the same animals, UAG was completely ineffective in inducing GH secretion, despite the ability of ghrelin, either alone or in combination with AUG, to evoke a robust, time-dependent elevation in circulating GH (Fig. 4).

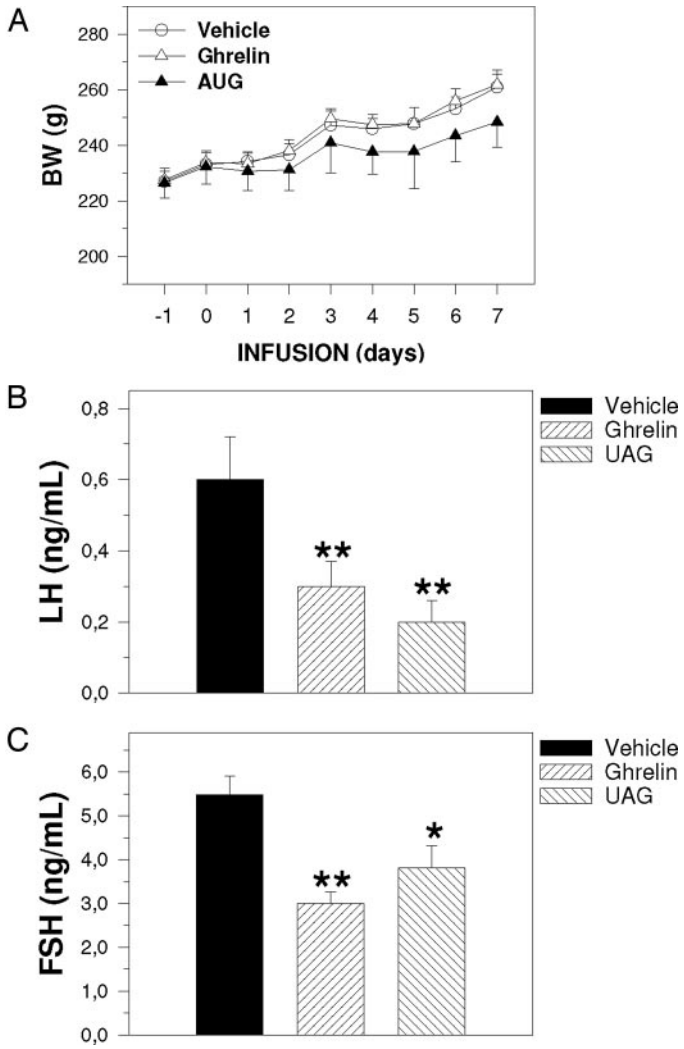

FIG. 2. Effects of chronic iv administration of ghrelin or UAG on serum gonadotropin levels in adult male rats. Ghrelin or UAG was chronically infused at a dose of 1.0 nmol per 1 h for 7 d. A, Total body weight (BW) records in the experimental groups are presented. B and C, In addition, serum LH and FSH levels at terminal samples from vehicle-, ghrelin-, and UAG-treated animals are shown. The experimental groups were composed of six animals. Values are given as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ *vs.* corresponding vehicle-injected animals (ANOVA followed by Student-Newman-Keuls multiple range test).

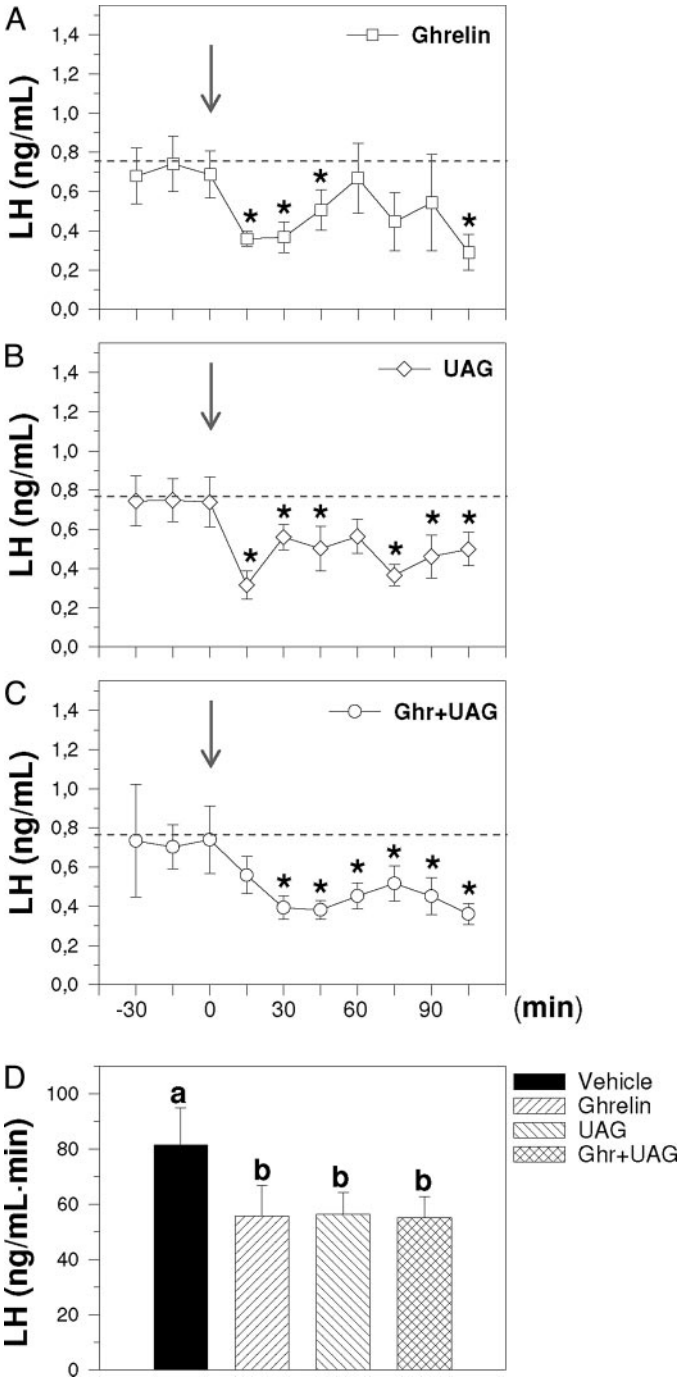

FIG. 3. Effects of iv injection of a bolus of ghrelin, UAG, or their equimolar combination on serum LH levels in freely moving adult male rats. A–C, Mean serum LH profiles in males (n = 8 per group) injected with vehicle, 3.0 nmol ghrelin, 3.0 nmol UAG, or ghrelin+UAG are presented. Administration of the peptides took place after stabilization of the animals following the third sampling point (indicated by the *arrow*). D, Mean integrated LH secretory responses, calculated as the area under the curve over the study period (105 min after iv injections), are shown for vehicle- and ghrelin-injected groups. Values are given as the mean ± SEM. *, $P < 0.05$ *vs.* integrated preinjection values from all the experimental groups (denoted by the *scattered line*; repeated-measures ANOVA followed by Student-Newman-Keuls test); in the *lower panel*, *bars with different superscript letters* are significantly different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

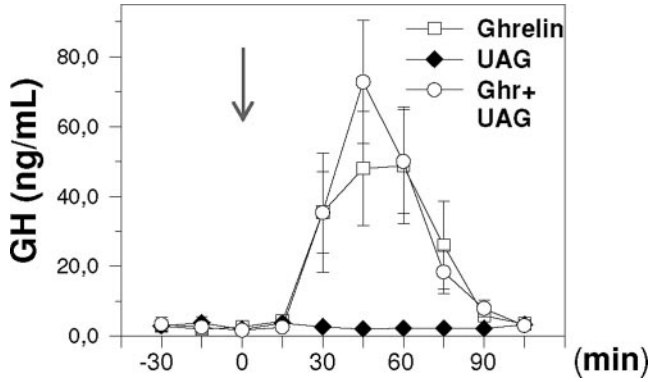

FIG. 4. Effects of iv injection of a bolus of ghrelin, UAG, or their equimolar combination on serum GH levels in freely moving adult male rats. Mean serum LH profiles in males (n = 8/group) injected with vehicle, 3.0 nmol ghrelin, 3.0 nmol UAG, or ghrelin + UAG are presented. Administration of the peptides took place after stabilization of the animals following the third sampling point (indicated by the *arrow*). Values are given as the mean ± SEM. **, $P < 0.01$ *vs.* integrated preinjection values (repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).

Effects of ghrelin on kisspeptin-induced LH secretion in adult male rats

The effect of ghrelin on (transiently) stimulated gonadotropin secretion was determined by assessing the magnitude of kisspeptin-induced LH responses with or without coadministration of acylated ghrelin. Of note, kisspeptins have recently emerged as major gatekeepers of the reproductive axis, playing a key role in puberty onset and fertility (25). Indeed, apart from GnRH itself, kisspeptin-10 is likely the most powerful stimulator of LH secretion known so far (22). Intravenous administration of kisspeptin-10 elicited a robust and sustained increase in circulating LH levels, with peak values at 15–30 min after injection, and elevated LH levels persisting for at least 105 min after injection (Fig. 5A). Coadministration of ghrelin failed to significantly modify the magnitude of the acute phase of response to kisspeptin-10 because LH levels were similar to those evoked by kisspeptin alone up to 60 min after iv injection (Fig. 5B). Thereafter, however, serum LH levels in ghrelin + kisspeptin-treated animals became lower than those of animals injected with kisspeptin alone (Fig. 5, A and B). Indeed, when kisspeptin responses were represented as net increased over their corresponding controls (injected with vehicle or ghrelin alone), it was evident that coadministration of ghrelin significantly shortened the duration and net magnitude of LH secretory responses to kisspeptin (Fig. 5C).

Effect of patterned iv infusions of ghrelin in hypergonadotropic Tgr male rats

The effects of chronic patterned infusions of ghrelin on serum LH and FSH levels were monitored in the Tgr model of persistent hypergonadotropism. Of note, adult male Tgr rats become overtly hypergonadotropic, with persistently elevated serum LH and FSH levels, and increased pituitary contents of both gonadotropins (Davies, J. S., N. M. Thompson, M. C. Christian, L. Pinilla, F. J. P. Ebling, M. Tena-Sempere, and T. Wells, manuscript submitted). Ghrelin in-

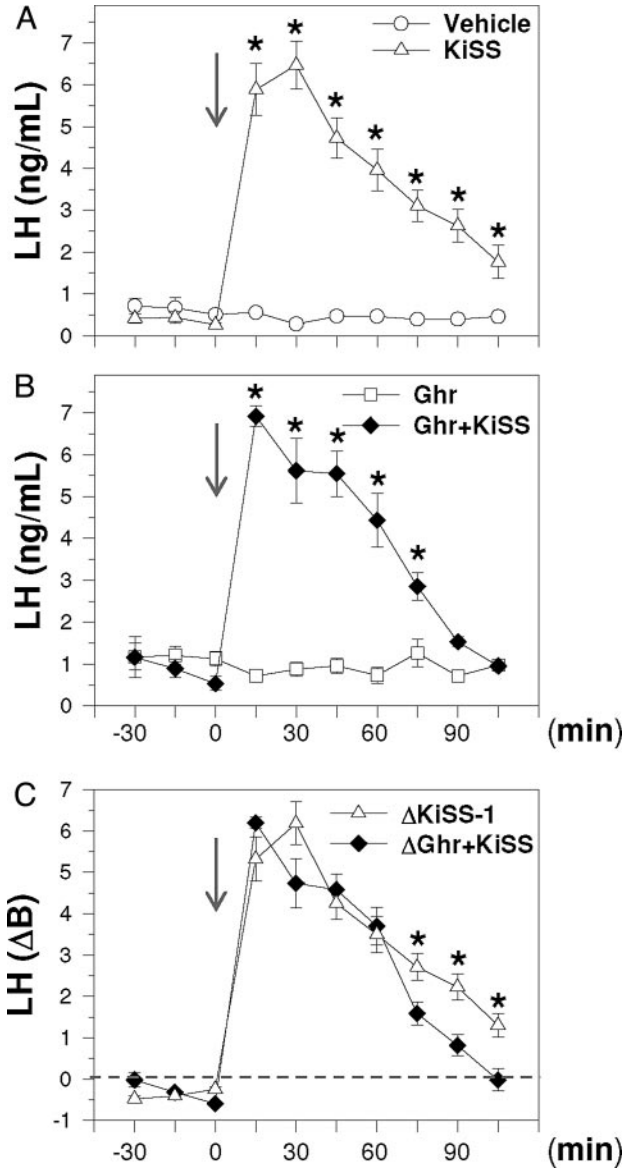

FIG. 5. Effects of combined acute administration of ghrelin and the potent LH secretagogue kisspeptin-10 in freely moving adult male rats. A, Mean serum LH profiles in males (n = 8/group) iv injected with vehicle or kisspeptin-10 are presented. B, Mean serum LH profiles in males iv injected with ghrelin alone or ghrelin plus kisspeptin-10 are shown. Administration of the peptides took place after stabilization of the animals following the third sampling point (indicated by the *arrow*). C, In addition to LH profiles, net LH secretory responses (ΔB) to kisspeptin-10 in the presence or absence of exogenous ghrelin are presented, calculated for each time point as the difference of stimulated levels *vs.* corresponding reference values (ΔB: vehicle-injected *vs.* kisspeptin alone; ghrelin-injected *vs.* ghrelin+kisspeptin). Values are given as the mean ± SEM. *, $P < 0.05$ *vs.* corresponding preinjection values and reference (vehicle or ghrelin injected) group (repeated-measures ANOVA followed by Student-Newman-Keuls multiple range test).

fusion to hemizygous Tgr adult male rats for 7 d induced an increase in total body weight, which was statistically significant for the pulsatile pattern of administration (Fig. 6A). Adult Tgr males showed the expected elevation in circulating LH and FSH levels over corresponding gonadotropin concentrations in wild-type AS littermates, presented for reference purposes (Fig. 6, B and C). The elevated LH concentrations in Tgr males were partially suppressed by both continuous and pulsatile patterns of infusion of acylated ghrelin at the end of the treatment periods (Fig. 6B). Of note, mean LH levels in animals with pulsatile infusion of ghrelin appeared to be slightly lower than in those of continuous administration, yet such a difference was not statistically significant. In contrast, mean FSH levels at the end of the infusion periods were not affected by either continuous or pulsatile infusion of ghrelin (Fig. 6C).

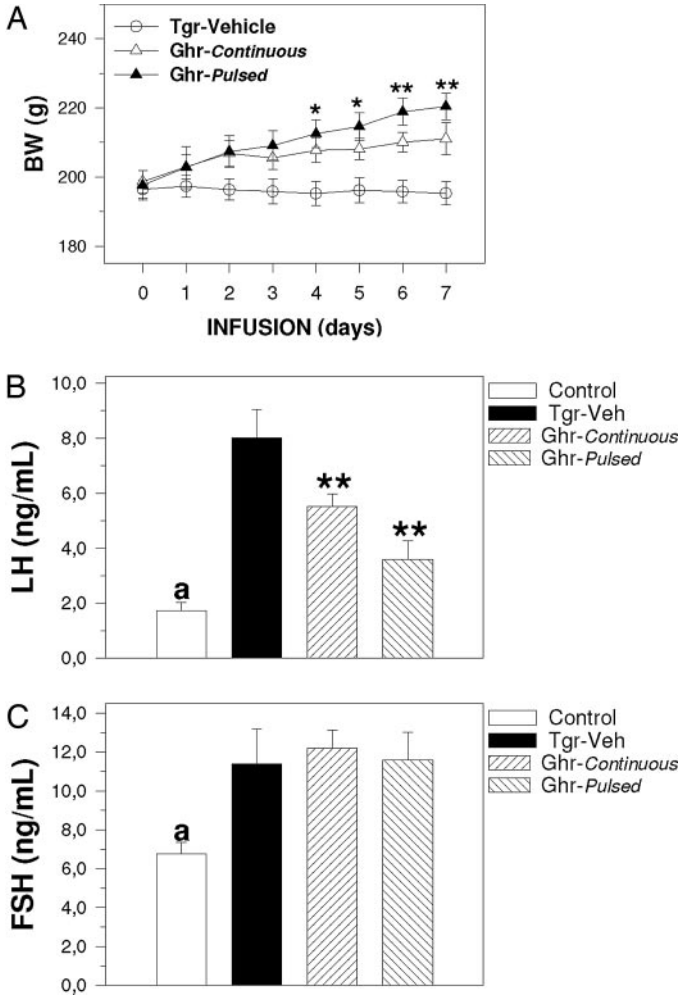

FIG. 6. Effects of chronic patterned infusion of acylated ghrelin on serum gonadotropin levels in a model of persistent hypergonadotropism, the Tgr male rat. Patterned ghrelin infusions were conducted in Tgr rats for 7 at two different schemes: continuous administration or pulsatile injection. A, Total body weight (BW) records in the experimental groups are presented. B and C, In addition, LH and FSH levels in terminal blood samples are shown. For reference purposes, mean LH and FSH levels in age-paired, control wild-type AS littermates are also presented. The experimental groups were composed of six animals. Values are given as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ *vs.* corresponding vehicle-injected Tgr animals; a, $P < 0.01$ *vs.* Tgr groups (ANOVA followed by Student-Newman-Keuls multiple-range test).

Discussion

A wealth of evidence indicates that ghrelin likely operates as a pleiotropic signal for energy insufficiency (18). Among

its wide range of actions, fragmentary evidence suggests that ghrelin might regulate the networks controlling the gonadotropic axis and gonadal function, thereby contributing to the physiological systems linking the energy status and reproduction (9). This contention, however, remains to be fully explored. In the present study, we addressed two critical aspects of the potential effects of ghrelin on the gonadotropic axis. First, we investigated whether the unacylated form of ghrelin was able to mimic, or modulate, the effects of ghrelin on LH (and FSH) secretion. Second, because most of the studies of the potential actions of ghrelin on the gonadotropic axis had been restricted to the assessment of the effects of single administration of the acylated peptide on basal LH levels (13, 15, 16), we examined whether ghrelin could regulate gonadotropin secretion in models of transient or persistent hypergonadotropism.

Our data demonstrate that chronic administration (repeated injection or continuous infusion) of ghrelin consistently decreased mean LH secretion in pubertal and adult male rats. Our observations in pubertal animals corroborate previous data from our group showing that administration of ghrelin (at half of the dose of the present study) was able to suppress serum LH levels and to partially disrupt the normal timing of the onset of puberty (12). Indeed, in our current experiment, twice-daily sc administration of ghrelin prevented the occurrence of complete preputial separation in more than 35% of treated males at d 43, *i.e.* an age point when all control males presented BPS. Preputial separation has been conventionally accepted as suitable external index of pubertal development in male rats (26). Thus, the observations that chronic treatment with ghrelin induces a decrease in serum LH levels and an apparent delay in BPS concurrently suggest that at high levels, as observed in conditions of negative energy balance (see Ref. 6 and cites therein), ghrelin might (partially) suppress the activation of male reproductive axis at puberty. Of note, the observed differences in the occurrence of BPS appeared shortly below the limit of statistical significance using conventional χ^2 tests. This was due, at least partially, to the limited size of the experimental groups and the qualitative nature of the variable (presence or absence of BPS) under analysis. However, in the context of the inhibitory hormonal effects reported herein, we find this effect biologically relevant. Nonetheless, we believe that the most salient finding of this set of data was that repeated administration of UAG, initially regarded as an inert form of the molecule, was able to fully reproduce the effects of acylated ghrelin in terms of inhibition of LH secretion and apparent delay in timing of preputial separation. In this sense, although it has been recently suggested that ghrelin may regulate body weight by a central mechanism independent of GHS-R1a (9), our observation is, to our knowledge, the first evidence for a neuroendocrine effect of des-octanoyl ghrelin, and prompted us to explore the phenomenon in greater detail.

The ability of UAG to reproduce the inhibitory effects of ghrelin on serum LH levels was also observed in adult male rats. Chronic continuous infusions of ghrelin or UAG equally decreased circulating LH concentrations at the end of 1 wk administration. In addition, as in our infusion experiments, both forms of ghrelin were able to lower serum LH levels in adult males after acute injection of a bolus of each peptide. In contrast, in the very same samples, only ghrelin (but not UAG) was able to elicit clear-cut GH secretory responses, in keeping with previous references (see Refs. 5–7 and references therein). Overall, these observations reinforce the validity of our current findings and support the contention that UAG is able to elicit specific regulatory actions (*e.g.* suppression of gonadotropin secretion) but not others (*e.g.* stimulation of GH release).

Interestingly, transgenic mice overexpressing UAG were recently demonstrated to have unaltered levels of circulating LH and FSH in single time-point measurements (27), a finding that is in apparent contrast with our present data. Several possibilities may account for such a divergence, including differences in the genetic background and species as well as the occurrence of potential compensatory mechanisms in the transgenic model. In addition, desensitization to the effects of persistently elevated UAG levels might have occurred in constitutive ghrelin overexpressors. In this sense, desensitization events have been previously reported for the GH secretagogue effect of ghrelin and synthetic GHS (28). This possibility might explain also our present finding that mean LH levels in pubertal males were not significantly different from those of control animals at the end of period of ghrelin administration (d 43).

Finally, the apparent lack of gonadotropin alterations in this transgenic model may derive from the fact that the reproductive phenotype of these animals has been only partially or superficially evaluated. This also appears to be the case for ghrelin null mice that, although devoid of overt reproductive defects, when cross-bred with ob/ob mice (which exhibit severe reproductive deficits and infertility) rescued the gonadal ob/ob phenotype (Smith, R., personal communication; viewed at NIH Conferences in Neuroscience: http://videocast.nih.gov/PastEvents.asp?c=16&s=11). This observation is in line with the findings reported herein because it evidences that genetic removal of the inhibitory influence of ghrelin/UAG might be sufficient to compensate for the lack of a stimulatory/permissive signal in reproduction, such as leptin.

The fact that similar inhibitory effects were observed in our initial experiments with repeated injections of ghrelin or UAG prevented us from testing the effects of their combined administration in the context of chronic treatment. In contrast, the potential interaction between octanoyl and des-octanoyl ghrelin was explored after acute coadministration of the peptides to conscious animals. Our results demonstrated that UAG did not significantly modify the inhibitory effect of ghrelin on LH secretion. This observation suggests the convergence of ghrelin and UAG signaling in the inhibitory control of LH secretion, implying that UAG is not provided with potential antagonistic effects on this specific function, in contrast with the proposed role of des-octanoyl ghrelin as counterbalance for several metabolic responses to acylated ghrelin (29, 30).

The receptor mechanism(s) mediating the observed common effects of ghrelin and UAG on LH secretion is a key issue that remains to be experimentally elucidated, but several possibilities can be considered. The most plausible option is that the observed inhibitory action is mediated through a

pathway independent of GHS-R1a. In this sense, despite the initial contention that octanoylation at Ser3 is mandatory for its biological activity (1, 2), strong evidence is now available demonstrating that UAG (which does not bind GHS-R1a) is able to mimic at least some of the metabolic, cardiovascular, (anti)proliferative, and adipogenic actions of ghrelin (5–8, 29–31). An alternative explanation is that exogenously administered UAG is octanoylated *in vivo*, thereby solely acting thought GHS-R1a. Although this phenomenon was not analytically tested, such a possibility appears highly unlikely as, after its acute administration, UAG was able to inhibit LH secretion with a similar time course to ghrelin but was unable to elicit GH release. Moreover, transgenic models in which ghrelin gene is persistently overexpressed do show an approximately 50-fold increase in total ghrelin levels without a significant increase acylated ghrelin (27). As a whole, these observations support the contention that the inhibition of LH secretion after UAG administration is genuinely mediated via a GHS-R1a-independent mechanism. Nonetheless, the possibility that, at least partially, the inhibitory effect of acylated ghrelin on LH secretion may be conducted via GHS-R1a cannot be totally excluded on the basis of our current data. Indeed, chronic infusion of the agonist of GHS-R1a, L-163,255, was able to lower mean LH levels in adult male rats, without altering FSH concentrations. Further pharmacological studies, including dose-response analyses for ghrelin and UAG, and testing of different types GHS would be of help to unmask whether common or different receptors are involved in this phenomenon.

Besides comparative analysis of the effects of ghrelin and UAG on gonadotropin secretion, the impact of acylated ghrelin in conditions of transient or persistent hypergonadotropism was explored. For the former, the ability of ghrelin to modulate kisspeptin-induced LH release was evaluated. It is worth noting that KiSS-1 peptides have recently emerged as potent stimulators of gonadotropin secretion, playing a pivotal role in puberty onset and dynamic regulation of the gonadotropic axis (22, 25). Indeed, hypothalamic KiSS-1 neurons appear to function as gatekeeper of the GnRH system. Moreover, our recent data suggest that the central KiSS-1 system plays a major role in relaying metabolic cues (*e.g.* in conditions of negative energy balance) to the reproductive axis (32). On this basis, we explored the potential cross-talk between ghrelin and kisspeptin in the control of LH secretion. Although ghrelin was not able to diminish the peak amplitude of LH secretory bursts in response to a maximal dose of kisspeptin-10, it clearly reduced the total duration of such responses. Furthermore, it is to be noted that somewhat maximal doses of kisspeptin were used in the present study, thus leaving open the possibility that ghrelin might have decreased more dramatically the LH responses to lower doses of kisspeptin. Overall, it is tempting to propose that conditions of hyperghrelinemia, as those observed in situations of low body mass index (6), might negatively impact LH secretion, at least partially, by inhibiting the releasing ability of endogenous kisspeptin.

Likewise, chronic ghrelin infusion in Tgr rats evoked a significant reduction of mean serum LH levels. It should be noted that the Tgr rat constitutes a model of persistently elevated LH and FSH levels in which, in contrast to orchidectomy, the integrity of peripheral feedback loops controlling gonadotropin secretion is preserved (Davies, J. S., N. M. Thompson, M. C. Christian, L. Pinilla, F. J. P. Ebling, M. Tena-Sempere, and T. Wells, manuscript submitted). Although the mechanism(s) responsible for the elevation of serum and pituitary gonadotropin levels in this model remains to be elucidated, the reported decrease in circulating LH concentrations after ghrelin administration to Tgr rats is coincident with our previous data from gonadectomized animals (14) and strongly suggests that, regardless of the duration and mechanisms responsible for the state of hypergonadotropism, ghrelin is able to inhibit chronically elevated basal LH secretion. From a mechanistic standpoint, such an inhibitory effect may stem from the ability of ghrelin to decrease GnRH-induced LH secretion at the pituitary level (14) and/or its direct actions at the hypothalamus because we have recently obtained evidence for the capacity of ghrelin to reduce GnRH secretion by hypothalamic fragments from ovariectomized female rats *ex vivo* (Pinilla, L., M. Tena-Sempere, and E. Aguilar; manuscript submitted).

Finally, an intriguing observation is that, whereas ghrelin consistently decreased circulating levels of LH in a great variety of experimental contexts, a concomitant reduction in serum FSH levels was observed only in basal conditions after chronic infusion of high doses of ghrelin to adult male rats. Different factors, such as the developmental stage, strain, doses, and patterns of ghrelin administration as well as the prevailing FSH levels, may account for this apparent discrepancy. Nonetheless, when considered collectively, these observations suggest that, whereas FSH secretion might also be susceptible of modulation by ghrelin (*e.g.* in conditions of severe hyperghrelinemia), LH secretion is more sensitive to the putative modulatory action of this gut-derived hormone.

In summary, since its discovery as the endogenous counterpart of the synthetic GHSs, an enormous body of data has enabled definition of ghrelin as a ubiquitous, pleiotropic modulator of a wide array of endocrine and nonendocrine functions (5–8). Our current data strengthen the contention that, among other neuroendocrine actions, ghrelin may participate, as a predominantly inhibitory signal, in the control of the gonadotropic axis, thereby contributing (in conjunction with other hormonal signals and metabolic cues) to the dynamic regulation of reproduction in the context of energy homeostasis. Moreover, our current results add complexity to our current knowledge of the influences of ghrelin on the reproductive axis, demonstrating its ability to inhibit basal and stimulated LH levels in large diversity of experimental conditions. More importantly, our data provide the first evidence for the capacity of des-octanoyl ghrelin to mimic the actions of the acylated molecule in terms of inhibition of LH secretion and puberty onset in the male. Because we have previously shown that continuous infusions of ghrelin also suppress spontaneous GH secretion (33), these observations raise the possibility that persistently elevated ghrelin may serve as a metabolic cue for the widespread attenuation of hypothalamic neuroendocrine activity in conditions of negative energy balance. In addition, our results suggest that these actions of ghrelin may be mediated, at least partially, by GHS-R1a-independent pathways. A more precise defini-

tion of these receptor mechanisms, and their physiological significance, warrants further investigation.

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