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Effects of Single or Repeated Intravenous Administration of Kisspeptin upon Dynamic LH Secretion in Conscious Male Rats

S. Tovar, M. J. Vázquez, V. M. Navarro, R. Fernández-Fernández, J. M. Castellano, E. Vigo, J. Roa, F. F. Casanueva, E. Aguilar, L. Pinilla, C. Dieguez, and M. Tena-Sempere

Department of Cell Biology, Physiology and Immunology (V.M.N., R.F.-F., J.M.C., E.V., J.R., E.A., L.P., M.T.-S.), University of Córdoba, 14004 Córdoba, Spain; and Departments of Physiology (S.T., M.J.V., C.D.) and Medicine (F.F.C.), University of Santiago de Compostela, 15705 Santiago de Compostela, Spain

The ability of kisspeptins, ligands of the G protein-coupled receptor 54, to potently elicit LH secretion is now undisputed. Yet, most of the pharmacological characterization of their gonadotropin-releasing effects has been conducted after intracerebral administration. In contrast, the effects of peripheral injection of kisspeptin remains less well defined. In this study, dynamic LH secretory responses to iv administration of kisspeptin-10 in different experimental settings are presented, and compared with those evoked by kisspeptin-52, using a protocol of serial blood sampling in conscious, freely moving male rats. LH responsiveness to peripheral administration of kisspeptin appeared extremely sensitive, as doses as low as 0.3 nmol/kg (0.1 μg/rat) evoked robust LH bursts, the magnitude of which was dose-dependent and apparently maximal in response to 3.0 and 30 nmol/kg kisspeptin-10. The ability of kisspeptin-10 to stimulate LH release was fully preserved, and even doubled in terms of relative increases, after short-term fasting despite suppression of prevailing LH levels. Repeated injections of kisspeptin-10 (four boluses, at 75-min intervals) evoked associated LH secretory pulses, the magnitude of which remained constant along the study period. Moreover, in this setting, *in vivo* LH responses to a terminal injection of GnRH were preserved, whereas basal and depolarization-induced GnRH release *ex vivo* was significantly enhanced. Finally, iv administration of kisspeptin-52 elicited dynamic LH responses analogous to that of kisspeptin-10; yet, their net magnitude and duration was slightly greater. In summary, we present in this study a series of experiments on the effects of systemic (iv) injection of single or repeated doses of kisspeptin upon dynamic LH secretion in conscious male rats. Aside from potential physiologic relevance, our present data might contribute to setting the basis for the rational therapeutic use of kisspeptin analogs in the pharmacological manipulation of the gonadotropic axis. (*Endocrinology* 147: 2696–2704, 2006)

SECRETION OF PITUITARY gonadotropins, LH and FSH, is primarily driven by the pulsatile release of the hypophysiotropic hypothalamic decapeptide GnRH (1, 2). The master position of GnRH neurons in the hierarchy of signals controlling the gonadotropic axis makes it the final target of a large number of regulators of central (*e.g.* glutamate, γ-amino-butyric acid, neuropeptide Y, noradrenaline) and peripheral (*e.g.* gonadal steroids, metabolic hormones) origin (1–6). However, although our knowledge on the mechanisms and signals involved in GnRH regulation has expanded dramatically in the last decades, characterization of the networks controlling the synchronous discharge of the disperse population of hypothalamic GnRH neurons, at different functional situations and development stages of the reproductive axis, remains partially incomplete.

The ligand-receptor kisspeptin (KiSS-1)/G protein-coupled receptor 54 (GPR54) pair was originally identified in the context of tumor biology, as a metastasis-suppressor signaling system (7–9). The KiSS-1 gene encodes a number of structurally related peptides, globally termed kisspeptins, which include metastin-54 (kisspeptin-52 in the rat) and kisspeptin-10 (9). All kisspeptins share their C-terminal region, where they show a distinctive Arg-Phe-NH_2 end-motif, hallmark of the RFamide peptide superfamily (9). The biological actions of kisspeptins are conducted via interaction with the G protein-coupled receptor GPR54 (7–10). Notably, the different forms of kisspeptin display similar high-affinity binding for GPR54 in heterologous cell systems (9).

By late 2003, the known biological functions of the KiSS-1/GPR54 system appeared to be (mostly) restricted to its inhibitory action upon tumor cell migration. At that time, however, a new reproductive "dimension" of this system was unraveled by the seminal observations of de Roux *et al.* (11) and Seminara *et al.* (12), who demonstrated that inactivating mutations and deletions in the gene encoding GPR54 were associated to lack of puberty onset and hypogonadotropic hypogonadism, both in humans and rodents. Thereafter, different groups, including ours, reported molecular, physiological, and pharmacological studies on the potential reproductive functions of kisspeptins. Indeed, in the last 2 yr, a number of studies have been published proving the extraordinary potency of kisspeptins in inducing gonadotropin release in different species, such as the rat, mouse, sheep, monkey, and, very recently, the human (13–22). Such a gonadotropin-releasing action of kisspeptins is thought to de-

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Abbreviations: AUC, Area under the curve; BW, body weight; GPR54, G protein-coupled receptor 54; KiSS-1, kisspeptin.

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rive primarily from a direct stimulatory action upon the hypothalamic GnRH system, as activation of GnRH neurons and GnRH release by kisspeptins has been very recently demonstrated (17, 20, 23, 24). Yet, the possibility of additional sites of action of kisspeptins (*e.g.* at the pituitary) cannot be ruled out (18, 19). Altogether, the available evidence clearly point out that the KiSS-1 system is a major gatekeeper of the GnRH/gonadotropin axis, and hence of reproductive function, in mammals (25–27).

The ability of kisspeptins to potently elicit LH and FSH secretion makes them a suitable target for the pharmacological manipulation of the gonadotropic axis. Indeed, kisspeptin-10 and metastin have been proven to evoke very robust LH secretory bursts, after delivery through different routes (intracerebral, iv, ip, and sc). However, most of these studies aimed at providing the physiological basis for the central actions of KiSS-1 in the control of gonadotropin secretion, thus using intracerebral injection as experimental setting (14–21, 23, 24). In contrast, although the ability of peripheral injection of kisspeptin to elicit LH secretion is undisputed (13, 17, 18, 21), the profiles of gonadotropin responses to systemic kisspeptin administration remain ill defined. For instance, in the rat, exhaustive dose-response and time-course analyses of the LH-releasing effects of kisspeptin have been only conducted after central injection (14, 18, 19), whereas the effects of peripheral administration of the peptide have been explored only at single time-point measurements or very high doses (13, 17). In the aforementioned scenario, we found it relevant to provide a comprehensive analysis of the pattern of dynamic LH secretory responses to systemic injection of kisspeptin, at different doses, protocols of administration (*i.e.* single and repeated injections), and functional states of the gonadotropic axis. As experimental setting, iv injection of kisspeptin-10 (and, in selected settings, kisspeptin-52), followed by serial blood sampling in conscious, freely moving male rats, was applied. Of note, during the final stage of preparation of this manuscript, an analogous study on dynamic LH responses to repeated kisspeptin-10 administration in juvenile monkeys was reported (28).

Materials and Methods

Animals and drugs

Adult Sprague Dawley male rats bred in the vivarium of the University of Santiago de Compostela were used. The animals were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C), with free access to pelleted food and tap water unless otherwise stated. Upon initiation of the experiments, the animals were individually caged. All the experimental procedures were approved by the Ethical Committees for animal experimentation of the Universities of Cordoba and Santiago de Compostela, and were conducted in accordance with the European Union normative for care and use of experimental animals. The animals were humanely killed by decapitation at the end of the experimental settings. Mouse/rat KiSS-1 (110–119)-NH_2, the rodent analog of the human C-terminal KiSS-1 decapeptide KiSS-1 (112–121)-NH_2, was obtained from Phoenix Pharmaceuticals Ltd. (Belmont, CA). This peptide fragment, which has been previously shown to maximally bind and activate GPR54 in transfected CHO cells (8, 9), will be referred hereafter as kisspeptin-10. In addition, for experiment 5, rat metastin, termed hereafter kisspeptin-52, was purchased from Phoenix Pharmaceuticals Ltd. The decapeptide GnRH was obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental designs

In experiment 1, the effects of a range of doses of kisspeptin-10 upon the dynamic profiles of LH secretion were evaluated after systemic injection of the peptide. A protocol of iv injection of kisspeptin and serial blood sampling in conscious, freely moving rats was applied as described in detail elsewhere (18, 19). To this end, adult male rats (258.0 ± 10.5 g BW; n = 8 animals per group) were implanted with intracardiac cannulae, following standard procedures (29), and blood samples (250 μl) were taken every 15 min over a 360-min period. The animals were sampled three times before iv injection of KiSS-1 or vehicle (physiological saline). Three different doses of kisspeptin-10 were tested for iv injection: 0.3, 3.0, and 30 nmol/kg BW; equivalent to 0.1, 1.0, and 10 μg per animal, respectively. During the sampling period, the volume of blood withdrawn was replaced hourly by a warmed suspension of blood cells in sterile saline.

In experiment 2, dynamic LH secretory responses to systemic (iv) injection kisspeptin were studied in a model of suppressed function of the gonadotropic axis. To this end, adult male rats (n = 8) were implanted with intracardiac cannulae and, after a 48-h recovery, underwent a 48-h period of food deprivation, with free access to tap water; age-paired animals fed *ad libitum* served as controls. Fed and fasted animals were subjected to iv injection of an effective dose of kisspeptin-10 (30 nmol/kg BW) or vehicle. Procedures for acclimatization of the animals, serial blood sampling, and replacement were similar to those described for experiment 1, except for the total time of sampling that was limited to 135 min (preinjection period, 30 min; postinjection period, 105 min; total of 10 blood samples taken per animal).

In an additional set of experiments, the effects of repeated iv injection of effective doses of kisspeptin-10 upon dynamic LH responses *in vivo*, and hypothalamic GnRH release *ex vivo*, were explored. Thus, in experiment 3, adult male rats (n = 8) were implanted with intracardiac cannulae, and subjected to a protocol of repeated injections of a dose of 30 nmol kisspeptin-10 per kilogram of BW (four boluses, at 75-min intervals) or vehicle. Serial blood sampling was applied at 15-min intervals, as described for experiments 1 and 2. The animals were sampled two times before iv injection of the first bolus of kisspeptin-10 or vehicle. At 120 min after the last injection of vehicle or kisspeptin, all the animals were injected with an effective dose of 10 μg GnRH (equivalent to 32.5 nmol/kg BW), and serial blood sampling was continued at 15-min intervals for an additional 90-min period. Volume of blood withdrawn was replaced hourly by a warmed suspension of blood cells in sterile saline.

In addition, in experiment 4, a similar protocol of repeated systemic injections of 30 nmol kisspeptin-10 per kilogram of BW or vehicle (four boluses, at 75-min intervals) was performed in adult animals (n = 10–12 per group), without serial blood sampling. Two hours after the last injection of kisspeptin or vehicle, the animals were decapitated and whole hypothalamic fragments were excised by a horizontal cut of about 2 mm depth with the following tissue limits: 1 mm anteriorly from the optic chiasm, the posterior border of mammillary bodies, and the hypothalamic fissures, as described in detail previously (24). The hypothalamic explants from animals treated *in vivo* with vehicle or kisspeptin were placed into individual incubation chambers containing 250 μl of phenol red-free DMEM for a 30-min preincubation, using a Dubnoff incubator at 37 C with constant shaking (60 cycles per minute), under an atmosphere of 95% O_2-5% CO_2. After this period, the preincubation medium was replaced by fresh DMEM to test for basal GnRH releasing capacity for an additional 30-min period, when the media were collected. Finally, the hypothalamic fragments were further challenged, for 30 min, by a depolarizing concentration of 56 mM KCl to test for stimulated secretory capacity, in keeping with our previous references (30). At the end of the incubation periods, media were boiled to inactivate endogenous protease activity, and kept at −80 C until assayed for GnRH levels.

Finally, in experiment 5, the profile of dynamic LH responses to kisspeptin-10 was compared with that of kisspeptin-52, using protocols of iv injection and serial blood sampling in freely moving conditions, as described in experiments 1 and 2. An equimolar dose of 3 nmol/kg BW was tested for kisspeptin-10 and kisspeptin-52. Groups of adult male rats (n = 7) were implanted with intracardiac cannulae, and blood samples (250 μl) were taken every 15 min over a 360-min period. The animals were sampled three times before iv injection of kisspeptin-10 or kisspep-

tin-52. Procedures for acclimatization of the animals and blood replacement were similar to those described for experiment 1.

Hormone measurement by specific RIAs

Serum LH levels were determined in a volume of 25–50 μl using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 was labeled with ^{125}I by the chloramine-T method, and the hormone concentrations were expressed using the reference preparation LH-RP-3 as standard. Intraassay and interassay coefficients of variation were less than 8 and 10%, respectively. The sensitivity of the assay was 5 pg/tube. In addition, GnRH levels in the incubation media were measured using a commercial RIA kit (Peninsula Laboratories, San Carlos, CA), following the instructions of the manufacturer. The sensitivity of the assay was 1 pg/tube. Accuracy of hormone determinations was confirmed by assessment of serum and medium samples of known LH and GnRH concentrations, respectively, used as external controls.

Presentation of data and statistics

Hormonal determinations (LH in serum samples, GnRH in medium samples) were conducted in duplicate, with a minimal total number of seven to eight samples per time point for *in vivo* experiments (experiments 1–3 and 5), and 10–12 determinations per group for *ex vivo* experiments (experiment 4). When appropriate, aside from individual time-point determinations, integrated LH secretory responses were estimated as the area under the curve (AUC), calculated following the trapezoidal rule, over the 120-min period after administration of kisspeptin-10 in experiments 1 and 2, during the 75-min interpulse periods in experiment 3, and over the 120-min period after administration of kisspeptin-10 or kisspeptin-52 in experiment 5. In addition, in experiment 3, integrated LH responses to terminal GnRH stimulation were calculated over the 90-min period after injection, using a similar procedure. Calculation of integrated secretory responses as AUC using the trapezoidal rule is described in detail elsewhere (31). When appropriate (experiment 1), the ED_{50}, defined as the dose of kisspeptin-10 able to induce 50% of the maximal LH response, was determined by nonlinear regression (SigmaStat 2.0; Jandel Corp., San Rafael, CA). Hormonal data are presented as mean ± SEM. Results were analyzed for statistically significant differences using repeated measures or one-way ANOVA followed by Student-Newman-Keuls multiple range test. $P \leq 0.05$ was considered significant.

Results

Effects of iv injection of kisspeptin upon dynamic LH secretion in conscious rats

In our first approach, the effects of single iv injection of a range of doses of kisspeptin-10 upon the dynamic secretory patterns of LH were monitored in conscious animals, under freely moving conditions. In detail, three doses of kisspeptin-10 were comparatively evaluated (0.3, 3.0, and 30 nmol/kg; equivalent to 0.1, 1.0, and 10 μg/rat), and hormonal responses were estimated as LH secretory profiles, at 15-min intervals over a total 360-min sampling period, as well as net (integrated) LH secretion during 120 min after kisspeptin injection. To note, the highest dose of kisspeptin used was previously proven by our group to significantly elicit LH secretion after its iv administration to male rats (18). Our analysis showed that all doses of kisspeptin tested effectively stimulated LH release within 15 min of injection, with maximal levels ranging between 4- to 8-fold increase over preinjection values and vehicle-injected controls. Nonetheless, the peak amplitude of LH pulses, as well as the magnitude of integrated secretory responses to kisspeptin, clearly appeared dose-dependent. Thus, the mean amplitude of LH pulses elicited by 0.3 nmol/kg kisspeptin-10 was approximately half of that evoked by the doses of 3.0 and 30 nmol/kg, and the duration of the pulses was considerably shorter, as LH levels had returned to preinjection values at 45 min after injection of 0.3 nmol/kg kisspeptin-10, but not until 90–105 min after administration of higher doses. Accordingly, the magnitude of the integrated LH secretory responses to kisspeptin was significantly greater for 3.0 and 30 nmol/kg kisspeptin-10 than for the lowest dose. To be noted, although mean integrated LH responses to 30 nmol/kg kisspeptin-10 appeared to be slightly higher than those elicited by the dose of 3.0 nmol/kg, such difference was not statistically significant, and both doses evoked LH pulses of similar (maximal) peak amplitude (Fig. 1). This allowed for calculation of the ED_{50} of kisspeptin-10, in terms of LH release, after iv administration, with a value of approximately 0.5 nmol/kg BW kisspeptin-10.

Effects of iv injection of kisspeptin upon dynamic LH secretion in fasting rats

The ability of systemic (iv) injection of kisspeptin-10 to elicit significant LH secretory responses was also tested in conditions of suppressed functionality of the gonadotropic axis. To this end, adult male rats were subjected to short-term (48-h) fasting, iv administration of an effective dose of 30 nmol/kg kisspeptin-10 was applied, and serial blood sampling was conducted in freely moving conditions. Of note, maximally effective doses were selected for this experiment as we aimed to determine whether systemic administration of kisspeptin-10 would be sufficient to overcome the inhibition of basal functioning of the gonadotropic axis in conditions of metabolic stress by short-term fasting. Hormonal responses were estimated by means of LH secretory profiles, at 15-min intervals over a total 135-min sampling period, as well as integrated LH secretion after kisspeptin injection. Food deprivation for 48-h induced a moderate, but significant, decrease in body weight in the experimental animals (259.5 ± 5.0 g in fasted animals *vs.* 288.5 ± 7.5 g body weight (BW) in controls; $P < 0.01$). In animals fed *ad libitum*, iv injection of 30 nmol/kg kisspeptin-10 elicited robust LH secretory responses, which were roughly similar in terms of peak amplitude and duration to those observed in our previous experiments (Ref. 18 and present results); such responses were defined by a mean 6.75-fold increase in terms of integrated LH secretion *vs.* corresponding vehicle-injected controls. Food deprivation for 48 h induced a significant suppression of basal LH secretion *vs.* corresponding control values in rats fed *ad libitum*, as evidenced by the decrease of individual LH levels and net (integrated) LH secretion in vehicle-injected animals during the study-period. Nonetheless, iv injection of a bolus of 30 nmol/kg kisspeptin-10 was able to potently elicit LH secretory bursts in fasted animals, which were similar in terms of absolute peak amplitude, duration, and net secretion to those evoked in fed rats. Moreover, as basal LH levels were significantly decreased, relative responses to kisspeptin (estimated as fold increase over corresponding vehicle-injected values) were 2-fold higher in fasted animals than in control-fed rats (Fig. 2).

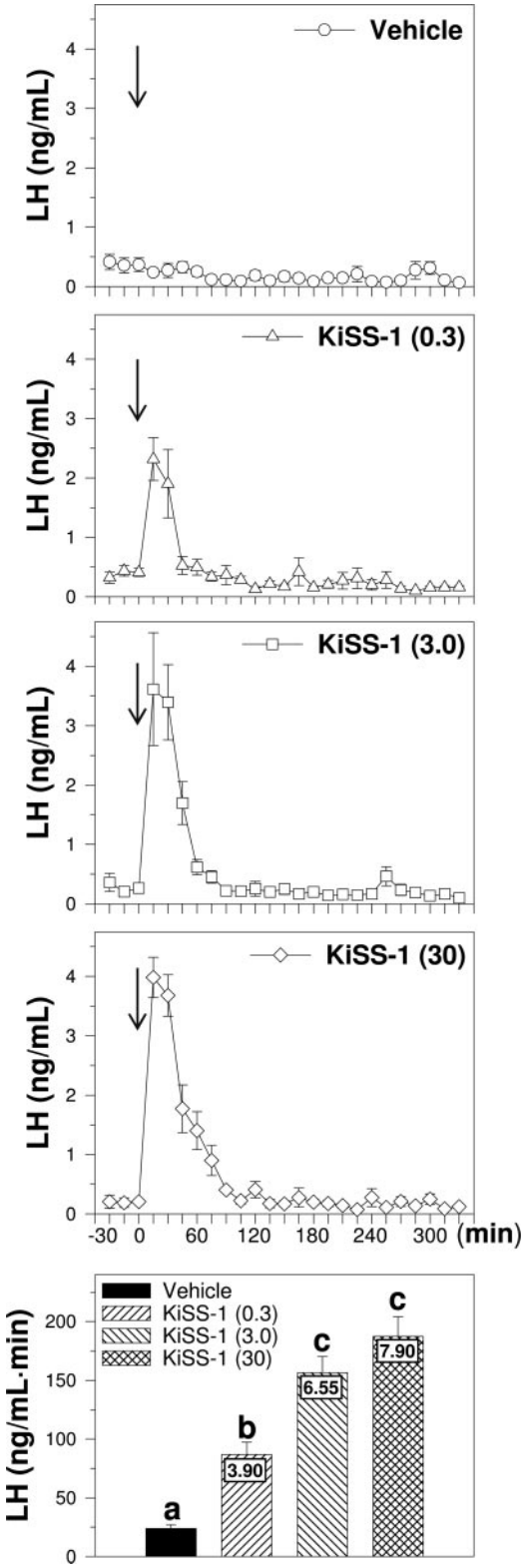

FIG. 1. Effects of iv administration of a single bolus of kisspeptin-10, at different doses, upon the pattern of LH secretion in conscious adult male rats. Three different doses of kisspeptin-10 (0.3, 3.0, and 30 nmol/kg BW) were tested for iv injection, and serial blood sampling, at 15-min intervals, in freely moving conditions was conducted. Vehicle-injected animals served as controls. For each group, the time-point when iv injection was applied is indicated by *arrows*. In addition to dynamic LH profiles, net LH secretion evoked by the different doses of kisspeptin is presented in the *lower panel*, as integrated hormone responses (AUC) over the 120-min period after injection of kisspeptin-10 or vehicle. In addition to absolute responses, relative increases (in terms of fold increase) over respective mean control values injected with vehicle are indicated in the *insets*. Groups with *different superscript letters* are statistically different ($P < 0.01$; ANOVA followed by Student-Newman-Keuls multiple range test).

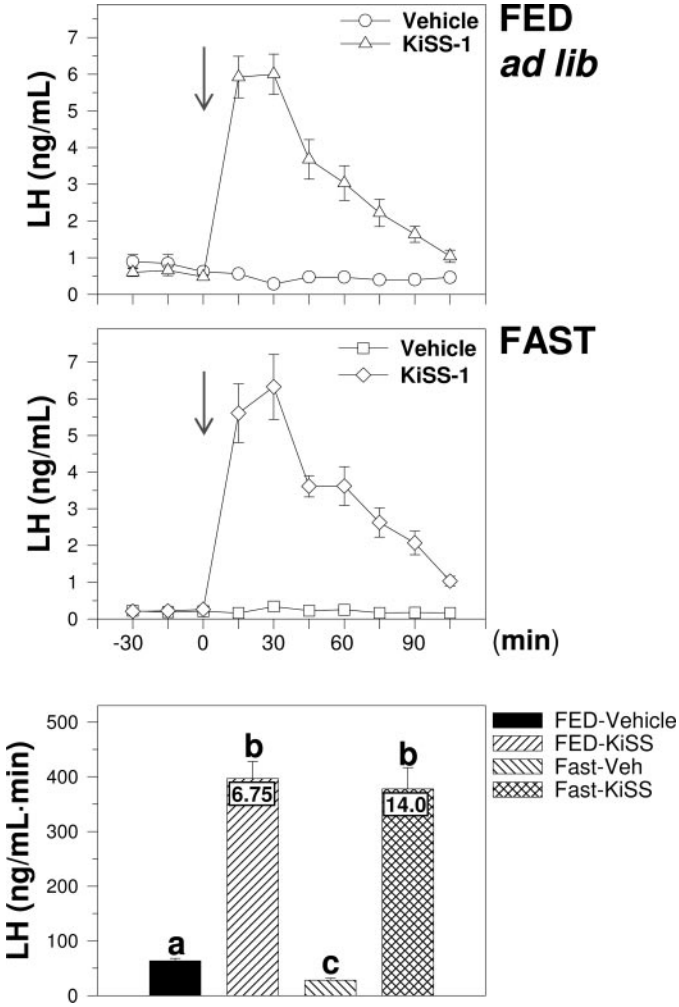

FIG. 2. Effects of iv administration of a single bolus of kisspeptin-10, at an effective dose of 30 nmol/kg BW, upon the pattern of LH secretion in conscious adult male rats either fed *ad libitum* or after short-term food restriction. Fasting was extended for 48 h, and serial blood sampling, at 15-min intervals, in freely moving conditions, was conducted for 105 min after iv injection of kisspeptin-10 or vehicle (indicated by *arrows*). In addition to dynamic LH profiles, net LH secretion evoked by kisspeptin-10 in fed and fasted animals is presented in the *lower panel*, as integrated hormone responses (AUC) over the 105-min period after injection of kisspeptin-10 or vehicle. In addition to absolute responses, relative increases (in terms of fold-increase) over respective pair-fed control values injected with vehicle are indicated in the *insets*. Groups with *different superscript letters* are statistically different ($P < 0.01$; ANOVA followed by Student-Newman-Keuls multiple range test).

Effects of repeated kisspeptin injection on dynamic LH secretion and GnRH response in vivo

To ascertain the consequences of repetitive administration of kisspeptin in terms of induction (and eventual desensiti-

zation) of LH secretion, dynamic LH responses to repeated iv injection of maximally effective doses of kisspeptin-10 were explored in conscious male rats, at freely moving conditions. As experimental protocol, administration of four boluses of 30 nmol/kg kisspeptin-10 or vehicle at 75-min intervals, and serial blood sampling every 15 min, was selected. The time-interval for kisspeptin administration was fixed on the basis of the mean duration of LH secretory pulses obtained in experiment 1, and was roughly coincident with that of previously published protocols of systemic administration of elicitors of LH secretion in the rat (32). In addition, to monitor pituitary responsiveness to GnRH at the end of the treatment protocols, terminal LH responses to a maximally effective dose of 10 μg GnRH were assayed in vehicle- and kisspeptin-injected animals. Analysis of hormonal profiles over a 450-min period evidenced that repeated administration of kisspeptin-10 was able to evoke a series of associated LH secretory bursts, which were grossly similar to each other in terms of peak amplitude and duration (Fig. 3). In addition, such repeated LH pulses were analogous to those induced by the single injection of kisspeptin-10 (Figs. 1 and 2). Interestingly, the magnitude of kisspeptin-induced LH bursts (expressed as integrated LH secretion during the interpulse period) appeared to progressively increase from the first to the third pulse; the mean net LH secretion being approximately 35% higher at the third pulse than at the first one. Yet, such difference did not reach statistical significance (Fig. 4A).

In addition, terminal iv injection of a dose of 10 μg GnRH similarly induced robust LH bursts in animals preinjected with four boluses of either vehicle or kisspeptin-10 (Fig. 3). Although the slope and peak amplitude of the LH surge appeared to be slightly higher in animals pretreated with vehicle, the net magnitude of such integrated LH responses to GnRH was not statistically different from that of kisspeptin preinjected animals (Fig. 4B). Of note, regardless of the pretreatment regimen, net LH responses to GnRH were significantly greater ($P < 0.01$) than each individual LH pulse elicited by iv injection of kisspeptin.

Effects of repeated kisspeptin injection in vivo upon GnRH secretion ex vivo

A similar protocol of repetitive injection of kisspeptin or vehicle *in vivo* was used to determine the effects of persistently elevated kisspeptin tone upon the capacity of the hypothalamic tissue to secrete GnRH *ex vivo*. An approach involving static incubation of rat hypothalamic fragments in the presence of medium alone (basal release), or after challenge with a depolarizing dose of KCl (stimulated release), was used. In animals pretreated with four boluses of either vehicle or kisspeptin-10 *in vivo*, KCl-induced depolarization similarly evoked a approximately 2.5-fold increase in GnRH release to the incubation media over corresponding basal values in the presence of medium alone, in keeping with

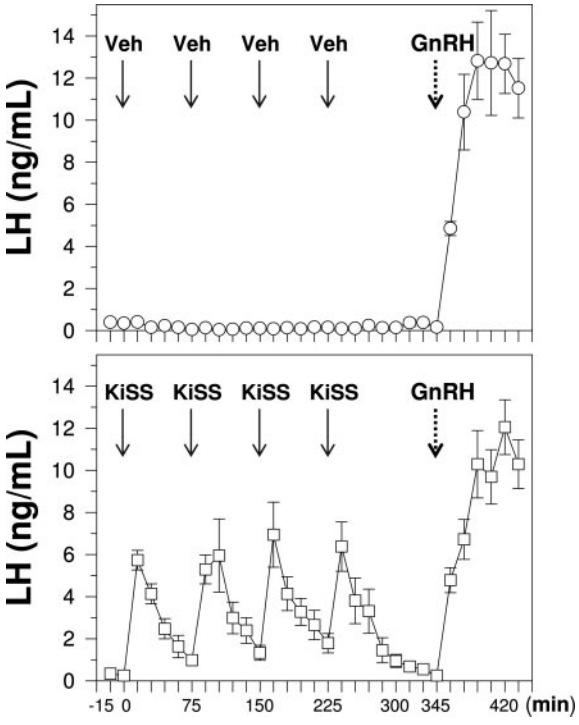

FIG. 3. Effects of repeated iv administration of an effective dose of kisspeptin-10 upon the pattern of LH secretion in conscious adult male rats. A protocol of repetitive iv injection of four boluses of 30 nmol/kg BW kisspeptin-10 at 75-min intervals was applied, and serial blood sampling, every 15 min, in freely moving conditions, was conducted for a 450-min period. Vehicle-injected animals served as controls. For each group, the time-points when iv injections of vehicle or kisspeptin were applied are indicated by *solid arrows*. In addition, terminal provocative tests were carried out in animals pretreated with either vehicle or kisspeptin, by means of iv administration of an effective dose of 10 μg/rat GnRH, 120 min after the last pulse (denoted by *dotted arrows*).

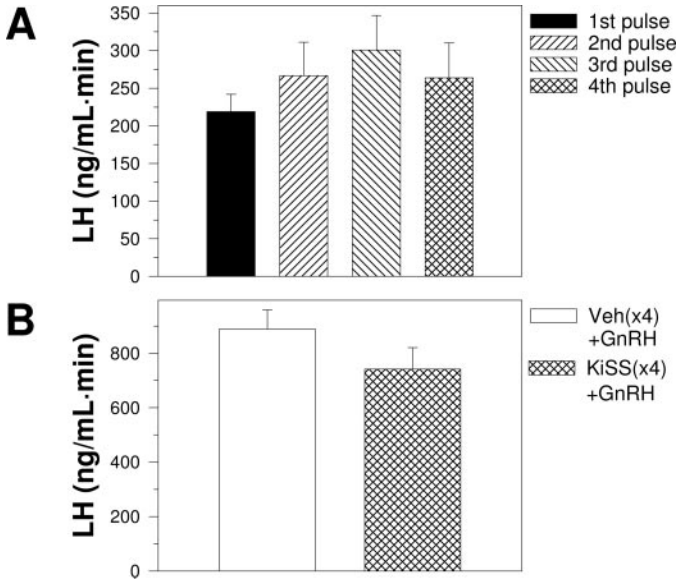

FIG. 4. Summary of the effects of repeated iv administration of kisspeptin-10, as well as of terminal iv injection of GnRH, in terms of integrated LH secretory responses in conscious adult male rats. In the *upper panel* (A), the magnitudes of the LH pulses induced by each of the four boluses of 30 nmol/kg kisspeptin-10, calculated as AUC over the 75-min interpulse period, are presented. In addition, in the *lower panel* (B), net LH secretion induced by iv injection of 10 μg/rat GnRH in animals pretreated with four boluses of vehicle or kisspeptin is shown. No significant differences were detected among the different data points.

previous references (30). Interestingly, analysis of basal and stimulated secretory responses revealed that both spontaneous and depolarization-induced GnRH secretion *ex vivo* was significantly higher in explants from animals pretreated with four injections of kisspeptin-10 *in vivo* (Fig. 5).

Comparative analysis of dynamic LH responses to kisspeptin-10 and kisspeptin-52

Finally, the profiles of dynamic LH responses to systemic administration of kisspeption-10 were compared with those evoked by an equimolar dose of full-length metastin (*i.e.* rat kisspeptin-52). In line with results from experiment 1, iv administration of 3.0 nmol/kg kisspeptin-10 elicited robust LH bursts, with peak levels at 15–30 min after injection and progressive decline thereafter; serum LH levels were similar to preinjection values at 75–90 min after kisspeptin-10 administration. Likewise, iv injection of kisspeptin-52 evoked a massive LH discharge, the peak levels of which were similar to those induced by kisspeptin-10. However, the decay in serum LH concentrations after injection of kisspeptin-52 was partially delayed because LH levels remained elevated over preinjection values up to 120 min after kisspeptin-52 injection. Indeed, LH concentrations in kisspeptin-52-injected animals were significantly higher than the corresponding levels in rats treated with kisspeptin-10, between 60–120 min after administration of the peptides. Accordingly, the magnitude of the integrated LH response to kisspeptin-52, during the 120 min after iv injection, was significantly higher than that of kisspeptin-10 (Fig. 6).

Discussion

The present study provides an overview of the effects of systemic (iv) administration of kisspeptin upon dynamic LH secretory profiles in the male rat, either after a single bolus (over a range of doses or at different functional states of the reproductive axis) or after repeated injection of kisspeptin-10. In addition, selective comparison of LH secretory responses to iv injection of kisspeptin-10 and kisspeptin-52 (*i.e.* rat full-length metastin) is presented. Of note, detailed analyses on the dose dependency and time course of the gonadotropin releasing effects of kisspeptin had been mostly conducted after central injection of the peptide, whereas peripheral responses remained less well defined. Furthermore, direct comparison of the effects of different forms of kisspeptin after systemic delivery had not been presented. In this sense, the ability of systemic administration of kisspeptin to potently elicit LH secretion had been undisputedly demonstrated on the basis of previous studies in the rat and monkey (13, 17, 18, 21). Moreover, Dhillo *et al.* (22) very recently reported the gonadotropin releasing effects of peripheral administration of metastin in humans. Yet, in most cases, those previous studies were limited to the evaluation of the effects of high doses of kisspeptin, at limited timepoints after administration of a single bolus in control animals. Our present data extend those previous observations and further substantiate the extraordinary potency of kisspeptin-10 to persistently elicit LH secretion even at low

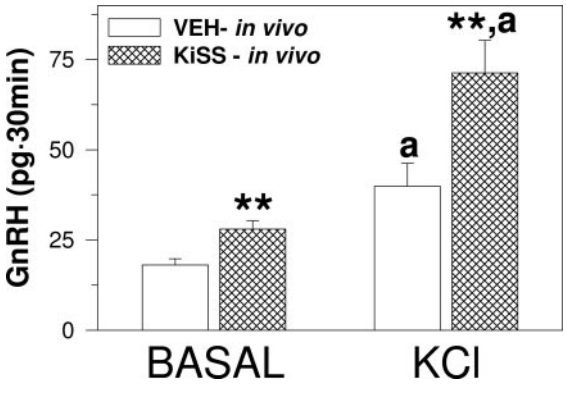

FIG. 5. Effects of repeated administration of an effective dose of kisspeptin-10 *in vivo* upon the pattern of GnRH secretion by hypothalamic explants *ex vivo*. A protocol of peripheral injection of four boluses of kisspeptin-10 or vehicle similar to that of Fig. 3 was implemented. At 120 min after the last injection, whole hypothalamic preparations from animals pretreated with vehicle or kisspeptin *in vivo* were explanted and incubated for 30 min in the presence of medium alone (DMEM; *basal secretion*). After this period, hypothalamic fragments from the same experimental groups were further challenged for an additional 30-min period with an unspecific depolarizing stimulus (56 mM KCl; *stimulated secretion*). **, $P < 0.01$ *vs.* corresponding values from animals pretreated with vehicle *in vivo*; *a*, $P < 0.01$ *vs.* corresponding basal values incubated in the presence of DMEM alone (ANOVA followed by Student-Newman-Keuls multiple range test).

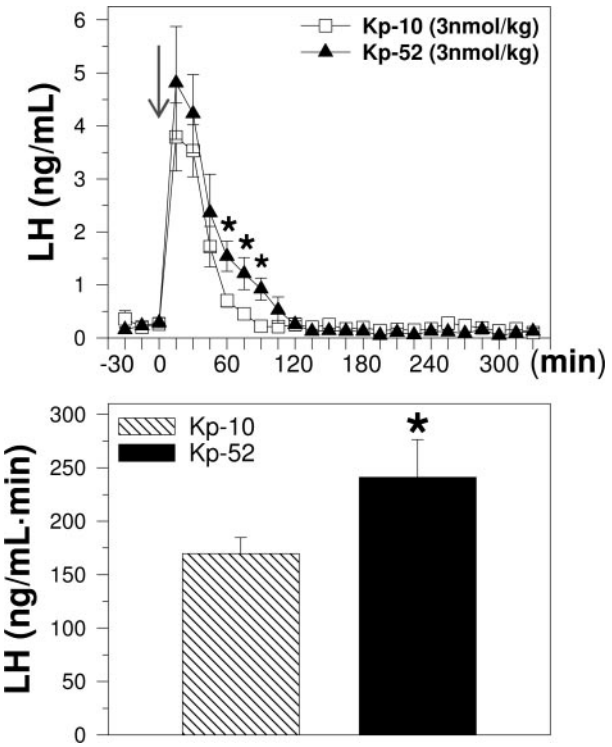

FIG. 6. Comparative analysis of the effects of iv administration of a single bolus of kisspeptin-10 or kisspeptin-52, at an equimolar dose of 3.0 nmol/kg BW, upon the pattern of LH secretion in conscious adult male rats. Serial blood sampling, at 15-min intervals, in freely moving conditions, was conducted. The time-point when iv injection was applied is indicated by the *arrow*. In addition to dynamic LH profiles, net LH secretion evoked by kisspeptin-10 and kisspeptin-52 is presented in the *lower panel*, as integrated hormone responses (AUC) over the 120-min period after kisspeptin injection. *, $P < 0.05$ *vs.* corresponding values in kisspeptin-10-injected animals (ANOVA followed by Student-Newman-Keuls multiple range test).

doses, after fasting-induced suppression of the gonadotropic axis, and after repeated administration of the peptide *in vivo*.

One the most conspicuous findings reported in this study is the ability of very low doses of kisspeptin-10 to efficiently evoke unambiguous LH responses after its systemic (iv) injection. Thus, a dose as low as 0.3 nmol/kg BW (equivalent to 0.1 μg/rat) was able to elicit a consistent LH secretory burst, the mean peak amplitude and integrated secretory mass of which were 4- to 5-fold higher than values of vehicle-injected controls. Based on previous experimental evidence (27), it is assumed that such LH-releasing effect is mediated via activation of GnRH at the hypothalamus. Yet, although the kinetics of the passage of kisspeptin-10 through the blood-brain barrier remains to be determined, the rapid pattern of response to peripheral administration suggests that systemically delivered kisspeptin-10 may regulate GnRH release directly at GnRH neuron nerve terminals located at the median eminence-arcuate nucleus complex, which is mostly placed outside the blood-brain barrier (33). Interestingly, previous studies on the effects of peripheral administration of other well-known stimulators of LH release, such as the agonist of glutamate receptors *N*-methyl-aspartic acid, reported significant LH pulses in male rats only after injection of 20 mg/kg (equivalent to 133 μmol/kg BW; see Ref. 32), a dose five orders of magnitude higher than the lowest dose of kisspeptin-10 (on equimolar basis) tested in this study. Although differences between glutamate agonists and kisspeptin-10 in terms of systemic clearance, passage through the blood-brain barrier, and sites of action may account for part of such divergence, this comparison illustrates the enormous biopotency of kisspeptin-10 in inducing LH secretion even after systemic administration. Indeed, other neuropeptidergic stimulators of GnRH/LH release (such as galanin-like peptide) do not appear to be effective after systemic administration. Another interesting aspect of our dose-response analyses is that nearly similar, maximal LH responses were achieved after iv injection of 3.0 and 30 nmol/kg kisspeptin-10 (equivalent to 1.0 and 10 μg/rat), suggesting that this is the range of doses where maximal LH responses to systemic injection of kisspeptin-10 are achieved. This allowed prediction of an ED_{50} of approximately 0.5 nmol/kg kisspeptin-10 for systemic delivery. Indeed, it is noticeable that, despite being 100-fold lower, the dose of 0.3 nmol/kg was able to induce consistent LH responses that were nearly half of those elicited by the highest doses tested. These findings might be relevant to provide a protocol for moderate, but sustained, stimulation of the gonadotropic axis by peripheral delivery of kisspeptin.

The functionality of the gonadotropic axis critically relies on the presence of sufficient energy stores that are signaled to the reproductive centers by leptin as well as other peripheral endocrine factors (6, 34). Conditions of negative energy balance are linked to variable degrees of central hypogonadotropism, which is believed to be mediated by the suppression of hypothalamic GnRH secretion through as yet unknown effector mechanisms. We have recently described that, in fasting conditions, hypothalamic expression of KiSS-1 gene is decreased, whereas intracerebral administration of exogenous kisspeptin-10 partially restored puberty onset and stimulated gonadotropin responses in peripubertal animals at undernutrition (24). Our present results demonstrate that, despite significant reduction in basal levels, LH secretory responses to kisspeptin were fully preserved in terms of absolute secretion, and even enhanced in terms of relative increases over corresponding control values, after short-term fasting in adult male rats. Taken together, these data strongly suggest that replacement of kisspeptin (either by central or systemic pulse administration) is sufficient to overcome the defective function of the gonadotropic axis in undernutrition. This observation may pose interesting implications for the design of physiologically sound protocols for reactivation of the reproductive axis in conditions of negative energy balance.

Aside from the effects of a single bolus, the consequences of repeated, intermittent administration of kisspeptin in terms of dynamic LH secretory responses were explored. This was considered relevant because the GnRH/LH axis appears to be exquisitely sensitive to the pattern of stimulation. Thus, whereas low-dose or pulsatile GnRH delivery results in efficient activation of LH secretion, continuous exposure to high doses of GnRH induces desensitization of LH responses; a phenomenon that involves down-regulation of pituitary GnRH receptors (see Refs. 35 and 36 and citations therein). Assuming that kisspeptin evokes LH secretion via induction of GnRH release (17, 20, 23, 24), this analysis might be useful to decipher the function and regulation of the KiSS-1 neuronal system in the neuroendocrine control of the gonadotropic axis. Repeated injection of maximally effective doses of kisspeptin (at 75-min intervals) elicited a sustained pattern of LH pulses, without decrement in terms of peak amplitude, duration, and secretory mass. Moreover, a trend toward increase in net LH secretion, which did not reach statistical significance, was observed after repeated kisspeptin-induced LH pulses, thus ruling out the possibility of potential desensitization events after (short-term) repeated peripheral administration of kisspeptin. Furthermore, absence of down-regulation of pituitary responsiveness to GnRH is suggested by the conserved LH responses in terminal GnRH provocative tests in animals pretreated with four boluses of kisspeptin; yet, the use of supra-physiological doses of GnRH in this setting does not allow us to exclude the possibility of subtle changes in pituitary sensitivity. In addition, *in vivo* pretreatment with multiple injections of kisspeptin appeared to moderately enhance the GnRH releasing capacity of hypothalamic tissue *ex vivo*, both at basal conditions as well as after an unspecific depolarization stimulus. Altogether, these data suggest that the protocol of repeated administration of kisspeptin reported in this study may provide an effective approach for sustained, short-term activation of the gonadotropin axis, alone or in combination with GnRH. The fact that equimolar doses of GnRH evoked supra-maximal LH pulses that largely exceeded those induced by kisspeptin-10 might indicate that peripheral administration of kisspeptin, by inducing the secretion of the endogenous releasable pool of GnRH, might constitute a physiological procedure for stimulation of gonadotropin secretion, without the risk of desensitization events linked to more continuous or pharmacological stimulation of the GnRH/LH axis.

Although the aforementioned tests involved the use of kisspeptin-10 as full agonist of GPR54 with amenable molecular characteristics (*e.g.* low molecular weight) for the design of protocols of pharmacological manipulation of the gonadotropin axis, our study also contains the first systematic comparison of the effects of kisspeptin-10 and kisspeptin-52 (the rat ortholog of human metastin) upon dynamic LH secretory profiles in conscious male rats. Of note, whereas different kisspeptin forms have been shown to be equally potent at the GPR54 level (9), no detailed analysis on potential differences of their biological activity after systemic delivery has been reported. Yet, significant structural differences exist between kisspeptin-10 and kisspeptin-52 which may account for divergent hormonal responses after their peripheral administration. Our functional tests demonstrated that the profiles of LH response to both forms of kisspeptin were roughly similar, with analogous peak values at 15–30 min after peptide injection and a progressive decline thereafter. However, the duration of secretory responses to kisspeptin-52 appeared significantly protracted, as LH levels between 60–120 min were higher than the corresponding values in kisspeptin-10-injected animals. This resulted in a moderate, but significant, increase in the net magnitude of integrated LH responses to kisspeptin-52. From a pharmacological perspective, this observation may reflect an extended half-life of circulating kisspeptin-52/metastin, in agreement with its higher molecular weight; a phenomenon which may pose interesting therapeutic implications. From a physiological standpoint, the relevance of this finding is partially obscured by the fact that, to date, it is not clear which form(s) of kisspeptin is present in the bloodstream. Likewise, the role, if any, of circulating kisspeptin in the control of the gonadotropic axis remains to be settled.

In conclusion, our present study provides an integral analysis of the effects of systemic (iv) injection of kisspeptin-10 upon dynamic LH secretion, over a range of doses, at different functional states of the gonadotropic axis, and after repeated administration of the peptide, in conscious animals. In addition, we report in this study the first comparative evaluation of the dynamic LH responses to iv administration of kisspeptin-10 and kisspeptin-52/metastin, which were moderately higher for the latter. Overall, our results demonstrate that, even at very low doses, peripheral delivery of kisspeptin is able to evoke unambiguous LH responses, and that replacement with exogenous pulses of kisspeptin is sufficient to rescue defective LH secretion in undernutrition. In addition, our data show that short-term, repetitive iv injection of kisspeptin promotes a sustained trend of LH pulses, without decrement in the amplitude and duration of the hormone bursts, neither inducing down-regulation of hypothalamic GnRH release nor desensitization of GnRH responses at the pituitary level. These observations are strikingly similar to those very recently reported by Plant *et al.* (28) after repeated iv administration kisspeptin-10 in juvenile monkeys. Aside from potential physiologic relevance, our current results may contribute to set the basis for the rational use of kisspeptin analogs in the pharmacological manipulation of the gonadotropic axis.

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Address all correspondence and requests for reprints to: Manuel Tena-Sempere, Physiology Section, Department of Cell Biology, Physiology and Immunology, Faculty of Medicine, University of Córdoba, Avenida Menéndez Pidal s/n, 14004 Córdoba, Spain. E-mail: fi1tesem@uco.es.

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