EXPERIMENTAL STUDY

Six-week treatment with hexarelin in young dogs: evaluation of the GH responsiveness to acute hexarelin or GHRH administration, and of the orexigenic effect of hexarelin

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Abstract
In this study we evaluated, in six young (5–7 year-old) beagle dogs, the effects of a 6-week administration of hexarelin (250 μg/kg s.c. twice daily) on the GH response to an acute challenge with hexarelin or GHRH (2 μg/kg i.v.), delivered before and after 3 and 6 weeks of treatment. The GH peak response to acute hexarelin or GHRH initially increased, with a maximum observed at the 3rd week, and then decreased to basal values (GHRH) or less (hexarelin) at the 6th week. These data would indicate that hexarelin initially primed the pituitary to acute administration of further hexarelin or of GHRH, followed by downregulation of the GH response to hexarelin and preservation of the response to GHRH. We then studied the rebound increase in GH secretion after withdrawal of an infusion of somatostatin (4 μg/kg per h for 1.5 h), a likely stimulus of endogenous GHRH function. The pattern obtained was similar to, though not superimposable upon, that ensuing after acute hexarelin or GHRH administration. Parallel evaluation of the acute orexigenic effect of hexarelin evinced a different time-course of the behavioural response, namely an acute feeding response to hexarelin that was abolished at the 3rd week and returned to normal at the 6th week. The differing timing of the neuroendocrine or behavioural response to hexarelin would suggest the existence of different subtypes of central nervous system GH-releasing peptide receptors.

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Introduction
In the past few years, the search for compounds as alternatives to either growth hormone (GH) or GH-releasing hormone (GHRH) has resulted in the development of a new family of oligopeptides, named GH-releasing peptides (GHRPs) (1). This family now comprises a number of different short peptidyl and non-peptidyl molecules, many of which have already proven to be effective in stimulating GH secretion in either animals (2–4) or humans (5–9).

GHRPs exert other neuroendocrine and behavioural actions: though with different potency, they slightly activate the hypothalamic–pituitary–adrenal axis (10–12) and also affect food intake (13–15) and sleep (16, 17). The intra- or extrahypothalamic mechanisms, or both, that underlie these central effects remain elusive.

In a previous study on the action of GHRP on somatotrophic function, we showed that, in aged dogs, a 16-week treatment with a high dosage of hexarelin, a hexapeptide analogue of these compounds, progressively potentiated the GH response to its own acute administration during the first month of treatment (18). However, evaluation of the GH response to acute hexarelin challenge at the 6th week of peptide administration revealed a decrease to GH values lower than those before treatment, though a washout interval of 2 weeks restored the original pattern of GH responsiveness to hexarelin (18). In that study, the potential ability of long-term treatment with hexarelin to interfere with GHRH function was not investigated, though it was likely that such an effect was occurring (19–21).

In this context, it is of note that we (22, 23) and others (24) have proposed the use of the rebound GH release that follows withdrawal of an infusion of somatostatin as a useful tool with which to evaluate endogenous GHRH function.

In our previous long-term study (18), the acute and chronic stimulation of GH release induced by hexarelin was apparently free from overt adverse effects. However, Clark and his colleagues (25) have raised the issue that the concomitant GH and cortisol release elicited by GHRPs may represent, in the long term, a diabetogenic stimulus with adverse effects on the use of GHRPs in the treatment of humans.
In the present study, we examined the effects of a long-term treatment with hexarelin in young dogs, evaluating its impact on the acute response to GHRH and, though inferentially, the potential alterations induced by hexarelin in hypothalamic GHRH function. Finally, we evaluated parallel changes in cortisol concentrations and food intake.

Materials and methods

Animals

Experiments were performed in the dog, a species that behaves like humans for many aspects of GH regulation and presents with a GH secretory profile similar to that observed in humans (2, 18). Six young (ages 5–7 years; weights 8–10 kg: two male and four female), well-trained beagle dogs were used in this study. Animals were fed normal dry food (Diete Standard, Charles River, Calco, Italy) once a day, at 1600 h, with water available ad libitum. The food not eaten was removed at 1900 h, so that dogs were fasting until 0830 h of the experimental day (time of the first blood sampling), except in the experiments on food intake, which were performed at 1400 h (see below). Animals were subject to a regimen of 12 h light: 12 h darkness, with lights on at 0700 h. Before and during the study, body weights of all dogs were stable and they had no observable disease.

All the experimental procedures were in accordance with that previously authorized by the Committee on Animal Care and Use of the University of Milan.

Treatment schedule

Hexarelin (Europeptides, Argenteuil, France) was administered twice daily at 0900 h and 1700 h, in a dose of 250 \( \mu \)g/kg s.c. The entire treatment period lasted 6 weeks.

Once in a week, 5 ml blood were drawn from the cephalic vein in all dogs, at 0830 h, for the determinations of insulin-like growth factor (IGF)-I and cortisol. The different experiments – acute hexarelin or GHRH testing, infusion of somatostatin, and food intake (see below) – were performed in a randomized sequence, with 24-h intervals, starting before hexarelin treatment or at the end of the 3rd or 6th week of hexarelin treatment.

Acute hexarelin testing

After one baseline blood sample had been taken (−30 min), dogs received hexarelin (250 \( \mu \)g/kg i.v.) at time 0 and then samples were obtained at 15, 30, 45, 60 and 90 min post-injection.

Acute GHRH testing

Dogs were challenged with the administration of GHRH (2 \( \mu \)g/kg i.v.; GRF(1−29)-NH\(_2\), Pharmacia & Upjohn, Milan, Italy). Blood samples were taken at −30, 0 (the time of GHRH injection) and at 15, 30, 45, 60 and 90 min post-injection.

Somatostatin infusion

An indwelling, non-thrombogenic i.v. catheter was positioned and maintained in the cephalic vein for the duration of each individual experiment. A solution of 0.9% saline was delivered (5 ml/h) for 30 min, after which an i.v. infusion of 4 \( \mu \)g/kg per h somatostatin (5 ml/h; Stilamin. Serono, Milan, Italy) was delivered for 1.5 h. The infusion of somatostatin was then stopped and 0.9% saline (5 ml/h) was again infused for 1.5 h.

Blood samples were drawn 30 min before the somatostatin infusion, at 0 min (the time of starting the somatostatin infusion), at 30-min intervals during the somatostatin infusion, and 15−30 min after withdrawal of the somatostatin infusion.

The extent of the GH response at the termination of the somatostatin infusion was taken to reflect the endogenous GHRH function (22–24).

Evaluation of food intake

The dogs were given, in a large cup, a premeasured quantity of their normal food (30 g/kg) at 1400 h. The test meal (time 0) was offered 5 min after administration of hexarelin (250 \( \mu \)g/kg s.c.) or saline (0.1 ml/kg s.c.) (time −5 min). Each cup, with its remaining content of food, was carefully weighed, to the nearest 0.1 g, at 0, 5, 10, 20, 30, 60, 90, 120 and 150 min after the test meal. After each measurement of the food eaten, the remaining food was quickly made available to the dog again.

Sample determinations

All blood samples were collected in chilled tubes containing 400 kallikrein inhibitor units of aprotinin (Sigma Tau, Milan, Italy) and 1.2 mg EDTA/ml blood and centrifuged. The plasma was then frozen at −80°C until required for assay.

GH radioimmunoassay

Highly purified canine GH, used for iodination and as a standard, and monkey anti-canine GH antiserum were obtained through the courtesy of Dr A F Parlow (Pituitary Hormones and Antisera Center, Torrance, CA, USA). The sensitivity of the assay was 0.4 ng/ml. The intra-assay coefficients of variation were 3.8 and 4.1% at concentrations of 12.5 and 3.1 ng/ml respectively. To avoid possible interassay variation, all samples of a given experiment were assayed in a single RIA.
IGF-I concentrations

Serum concentrations of IGF-I were determined by RIA of acid-extracted samples (26). Serum was mixed 1:1 with acetic acid 2 mol/l and incubated for 1 h at room temperature. The incubation mixture (200 μl) was applied to a Sephadex G-50 column (0.9×28 cm) and eluted with acetic acid 1 mol/l and NaCl 0.02 mol/l at a flow rate of 5 ml/h. The IGF-I fraction, which eluted at Kₐ values between 0.37 and 0.75, was lyophilized and assayed by a homologous RIA with reagents provided by Amersham, Milan, Italy. The recovery from gel filtration was 90%. The sensitivity of the assay was 0.1 ng/ml; intra- and interassay variations were less than 10%.

Cortisol concentrations

Plasma concentrations of cortisol were determined by RIA provided by ICN, Milan, Italy.

Statistical analysis

GH was evaluated either as absolute mean values (ng/ml) ± S.E.M. or as area under the GH response curve (AUC₀₋₉₀ min: ng/ml per h), calculated by the trapezoid method. IGF-I (ng/ml) and cortisol (μg/dl) were expressed as absolute mean values ± S.E.M. Food intake was expressed as a percentage of the food ingested at the test meal after hexarelin or saline administration, with subsequent cumulative values.

No differences in analytical levels between male and female dogs were observed in the different experimental conditions, therefore these data were pooled.

Statistical comparisons of the mean values were performed by Student’s t-test for unpaired or (where necessary) paired data, preceded by ANOVA; P<0.05 was taken to be statistically significant.

Results

Acute hexarelin testing

Baseline GH values before hexarelin or after the 3rd and 6th weeks of hexarelin treatment did not differ significantly (time 0 min: 0.5 ± 0.1, 0.5 ± 0.2, 0.6 ± 0.1 ng/ml, respectively; P = NS) (Fig. 1). There was, however, a striking change in the GH responsiveness to the acute hexarelin challenge: the means of individual GH peak responses were 79.9 ± 18.0 ng/ml before hexarelin treatment, 168.3 ± 12.8 ng/ml after the 3rd week and 37.0 ± 26.8 ng/ml after the 6th week of treatment (Fig. 1). In all dogs, plasma GH concentrations peaked between 15 and 30 min post-injection, and remained increased at 60 min.

Determinations of the AUC₀₋₉₀ values showed that the GH response to the peptide challenge increased significantly after the 3rd week of treatment (from 2574.8 ± 649.5 to 6211.2 ± 599.0 ng/ml per h, P<0.01), but, after the 6th week, decreased to values less than those present before treatment (1308.4 ± 903.9 ng/ml per h, P<0.01) (Fig. 2).

Acute GHRH testing

Baseline GH values before hexarelin or after the 3rd and 6th weeks of hexarelin treatment did not differ significantly (time 0 min: 0.5 ± 0.1, 0.5 ± 0.0, 0.6 ± 0.1 ng/ml, respectively; P = not significant) (Fig. 3). The mean GH peak responses to the acute GHRH challenge were 2.1 ± 0.9 ng/ml before hexarelin treatment (P<0.01 compared with baseline), 12.3 ± 3.2 ng/ml after the 3rd week and 2.8 ± 1.1 ng/ml after the 6th week of treatment (P<0.01 compared with baseline) (Fig. 3). In all dogs, the GH peak value was reached between 15 and 30 min after GHRH injection, and plasma GH concentrations remained increased at 60 min.

Hexarelin treatment induced, after the 3rd week, a significant increase in the GH responses to GHRH (AUC₀₋₉₀ increased from 111.1 ± 31.1 ng/ml per h to 361.9 ± 87.7 ng/ml per h, P<0.01), but, after the 6th week, the GH responses to GHRH had declined to become almost superimposable on those present before hexarelin treatment (AUC₀₋₉₀ 137.0 ± 38.9 ng/ml per h, P = NS) (Fig. 2).

Somatostatin infusion

Baseline GH values before hexarelin or after the 3rd and 6th weeks of hexarelin treatment did not differ significantly (time 0 min: 0.5 ± 0.1, 0.6 ± 0.1, 0.5 ± 0.1 ng/ml, respectively; P = NS) (Fig. 4). Before hexarelin
treatment, termination of the somatostatin infusion was followed by a small, but unequivocal, increase in plasma GH (mean GH peaks: $3.4 \pm 0.8$ ng/ml; $\text{AUC}_{0-90} 106.2 \pm 12.3$ ng/ml per h) (Figs 2, 4). After the 3rd week of treatment, there was a consistent enhancement of the increase in GH that occurred after withdrawal of somatostatin (mean GH peaks: $7.1 \pm 1.9$ ng/ml, $P < 0.01$; $\text{AUC}_{0-90} 283.8 \pm 51.1$ ng/ml per h; $P < 0.01$) (Figs 2, 4), but no increase in GH occurred after termination of the somatostatin infusion at the 6th week of treatment (mean GH peaks: $0.8 \pm 0.0$ ng/ml; $\text{AUC}_{0-90} 39.4 \pm 2.9$ ng/ml per h compared with before hexarelin treatment; $P < 0.01$) (Figs 2, 4).

**Serum IGF-I concentrations**

During treatment, serum concentrations of IGF-I did not change significantly from pretreatment (data not shown).

**Figure 2** Areas under the curve (0 and 90 min) of the GH responses after an acute challenge with hexarelin (HEXA) or GHRH and of the rebound increase in GH after the withdrawal of somatostatin (SS) infusion in six beagle dogs, before hexarelin or at the end of the 3rd or 6th week of hexarelin treatment. Means $\pm$ S.E.M. of six determinations made in duplicate. *$P < 0.01$ compared with baseline.

**Figure 3** GH responses after an acute challenge with GHRH (2 μg/kg i.v.) in six beagle dogs treated for 6 weeks with hexarelin in a daily dose of 250 μg/kg s.c. three times a day, before hexarelin (●) or at the end of the 3rd (○) or 6th (▲) week of hexarelin treatment. Means $\pm$ S.E.M. of six determinations made in duplicate. *$P < 0.01$ compared with baseline.

**Figure 4** Rebound increase in GH concentration after withdrawal of the infusion of somatostatin (4 μg/kg per h) in six beagle dogs treated for 6 weeks with hexarelin in a daily dose of 250 μg/kg s.c. three times a day, before hexarelin (●) or at the end of the 3rd (○) or 6th (▲) week of hexarelin treatment. Means $\pm$ S.E.M. of six determinations made in duplicate. *$P < 0.01$ compared with baseline.
Food intake
During the pretreatment phase, acute administration of hexarelin increased food intake, an effect first evident at 60 min. The cumulative amounts of food eaten by dogs at 60, 90, 120 and 150 min corresponded respectively to 37.4 ± 22.3%, 38.7 ± 23.0%, 38.8 ± 23.0% and 38.9 ± 23.0% of the preweighed quantity after saline injection and to 48.6 ± 21.3%, 51.0 ± 20.0%, 51.0 ± 20.1% and 51.0 ± 20.1% after hexarelin injection (P < 0.01) (Fig. 5). At the 3rd week, the stimulatory effect on food intake was not evident, the cumulative amounts of food eaten by dogs now being superimposable on that elicited by the peptide before treatment (Fig. 5).

Cortisol concentrations
Pretreatment plasma cortisol concentrations were 0.6 ± 0.1 µg/dl. They were unaltered at the 1st and 2nd weeks of treatment (0.7 ± 0.2 µg/dl and 0.6 ± 0.1 µg/dl, respectively; P = NS). Plasma cortisol titres then progressively decreased, reaching nadir values at the 3rd and 6th weeks (0.4 ± 0.0 µg/dl and 0.3 ± 0.0 µg/dl respectively; P < 0.01 compared with pretreatment) (data not shown).

Adverse effects
There were no signs of discomfort or overt adverse reactions after acute or repeated peptide treatment.

Discussion
We have previously shown that in old dogs exposed chronically to high doses of hexarelin, the peptide restores the GH secretory profile to normal (18). In this study, although we did not examine the GH secretory profiles, we demonstrated that, in young dogs, hexarelin is a very effective GH releaser that primes the pituitary to its own GH-releasing effect during the first 3 weeks of treatment. At the 6th week, however, these effects had subsided. In keeping with all our data in dogs (this study and (18)), Rahim et al. (27) showed that, in healthy elderly subjects, during the course of a 16-week treatment with hexarelin there was, at the 4th week of treatment, a decline in the GH response to an acute hexarelin challenge, which had returned to normal 4 weeks after completion of the peptide therapy.

We are currently unable to identify precisely either the site(s) or the mechanism(s) responsible for the blunting of the GH response to the peptide – that is, whether it occurred directly at GHRP receptors located on GHRH neurones in the arcuate or ventromedial nuclei of the hypothalamus (21, 28, 29) or, alternatively, at central nervous system (CNS) or pituitary GHRP receptors downregulated by increased circulating GH titres (30, 31). In this context, involvement of the feedback inhibitory action of circulating IGF-I (32, 33) is unlikely, as plasma IGF-I titres did not change significantly under treatment and, conversely, in the study by Lieber et al. (34), the GH response to L-163,255, a non-peptidyl GHRP mimetic, did not subside in dogs treated for 15 weeks, despite the increase in circulating GH and IGF-I concentrations. An enhanced hexarelin-induced production of tissue IGF-I and a paracrine/autocrine action at the hypothalamo–pituitary system cannot, however, be ruled out in our study (18).

Whichever the cause of the rather delayed decline of the GH responsiveness to hexarelin, this phase was preceded, in the first 3 weeks of treatment, by a striking increase in the GH responsiveness to the peptide. A similar pattern had been demonstrated in the early phases of hexarelin administration in old dogs, in which, however, even during the off-periods of the peptide treatment, baseline GH concentrations remained higher than pretreatment values (18). In infant GHRH-deprived rats, treatment with hexarelin for 3 or 5 days increased pituitary GH mRNA (35), whereas, given for 5–10 days to young ‘intact’ adult rats, it was ineffective in this context (36). Also worth noting in this context is that, in young GHRH-deprived rats, a 10-day treatment with hexarelin enhanced hexarelin-induced production of tissue IGF-I and a paracrine/autocrine action at the hypothalamo–pituitary system cannot, however, be ruled out in our study (18).

Figure 5 Food intake in six dogs after administration of hexarelin (250 µg/kg s.c.) or saline (0.1 ml/kg s.c.). Food intake measurements were performed before hexarelin (○, saline; ■, hexarelin) or at the end of the 3rd (●) or 6th (◇) week of hexarelin treatment. Means ± S.E.M. *P < 0.01 compared with baseline.
The enhanced GH responsiveness to hexarelin, present in both rats (35) and dogs (this study and (18)) during the initial phase of treatment has not been reported previously in humans (27, 37, 38). Although difficult to interpret, this discrepancy may be due to the higher sensitivity of humans than rats and dogs to the GH-releasing activity of the GHRPs, an event for which no clear explanation is available at present. Peptide doses two orders of magnitude lower than those used in our study are active in humans, inducing a robust release of GH (2, 8, 39). Thus, in humans, the potential ability of hexarelin to prime the pituitary may be obscured by the occurrence of early receptor or post-receptor changes (27, 40).

Parallel evaluation of the GH response to an acute GHRH challenge during the course of treatment revealed its enhancement at the 3rd week, an event temporally fitting well with the increased response to the acute hexarelin challenge. At the 6th week, the GH response to GHRH was attenuated, but was still comparable to that induced by GHRH at pretreatment. Similar, but not superimposable, results were obtained in our study by evaluating, albeit inferentially, hypothalamic GHRH neurones freed from the inhibitory influences of somatostatin (22–24). Following somatostatin infusion, the rebound increase in GH was enhanced at the 3rd week, but completely abolished at the 6th week. This finding is of particular interest when viewed in relation to the preserved GH response to GHRH at this time. These observations would suggest that a sustained treatment with hexarelin desensitized the hypothalamic GHRH mechanism(s), but preserved the pituitary competence to respond to CNS-driven stimuli. As an alternative possibility, chronic administration of the peptide might have decreased a putative endogenous hypothalamic non-GHRH ligand (U factor), involved in GHRP stimulatory effects (1). A note of caution in interpreting these findings is necessary, however, as, in dogs under baseline conditions, the pituitary responsiveness to the heterologous GHRH is poor (authors’ unpublished results, and this study), so that differences encountered at the different time periods were not so clear cut.

Further support for the view that the tolerance following sustained hexarelin treatment is a centrally-mediated event was provided by the results regarding food intake, a behavioural function mainly driven by the CNS (41, 42). Before treatment, the acute hexarelin challenge induced a clear-cut, though transient, increase in food intake, thus for the first time extending to dogs previous findings obtained with GHRPs in rats (13–15). In the present study, however, the temporal pattern of the behavioural response was divorced from that of the neuroendocrine response: the feeding response was not enhanced at the 3rd week and, unexpectedly, had returned to the original response by the 6th week of treatment. We do not know, at present, the underlying cause of this temporal pattern: however, it supports the proposition of the existence of different receptor subtypes engaged in the manyfold actions of GHRPs (43–45). There are now peptidyl mimetics of GHRPs that stimulate food intake and lack neuroendocrine effects, and vice versa (15).

Studies in both animals (10) and humans (12) have reported the ability of hexarelin, acutely administered, to increase plasma ACTH and cortisol concentrations. In our study, however, plasma cortisol concentrations were unaltered during the early phase and were reduced during the late phase of treatment. Reduction of cortisol concentrations recalls the occurrence of tolerance of the GHRP stimulatory effect on the hypothalamo–pituitary–adrenal axis (11), although an initial stimulatory phase in our study was lacking.

In all, these data indicate that a 6-week hexarelin treatment initially primes the pituitary to its own acute administration and to acute administration of GHRH. This is followed by downregulation of the GH response to hexarelin and preservation of the GH response to GHRH. It would seem, therefore, that GHRRH can be helpful in triggering GH release from a pituitary made unresponsive to hexarelin.

The orexigenic response to hexarelin has a temporal pattern that is distinct from that of the neuroendocrine response, implying the existence of different subtypes of GHRP receptors and the potential for their therapeutic exploitation. Obviously, the use of other GHRP agonists – for example MK 677 (9, 11) – would be of aid in establishing the existence of distinct GHRP receptor subtypes.

References
GH releasing and orexigenic effects of hexarelin

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