Mechanism of action of Hexarelin. I. Growth hormone-releasing activity in the rat

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We have reported Hexarelin (HEXA), an analog of growth hormone-releasing peptide 6 (GHRP-6), potently stimulates growth hormone (GH) secretion in infant and adult rats. This study was undertaken to further investigate Hexarelin’s mechanisms of action. In 10-day-old pups, treatments with HEXA (80 μg/kg, b.i.d.) for 3–10 days significantly enhanced, in a time-related fashion, the GH response to an acute HEXA challenge. Qualitatively similar effects were elicited in pups passively immunized against growth hormone-releasing hormone (GHRH) from birth. In adult male rats, a 5-day pretreatment with HEXA (150 μg/kg, b.i.d.) did not enhance the effect of the acute challenge, and the same pattern was present after a 5-day pretreatment in male rats with surgical ablation of the mediobasal hypothalamus (MBH-ablated rats). In addition, in adult sham-operated rats, Hexarelin (300 μg/kg, iv) induced a GH response greater (p < 0.05) than that induced by GHRH (2 μg/kg, iv). However, in MBH-ablated rats 7 days after surgery, GHRH was significantly (p < 0.05) more effective than HEXA, and 30 days after surgery HEXA and GHRH evoked similar rises of plasma GH. Finally, the in vitro Hexarelin (10⁻⁶ mol/l) effect was transient while GHRH (10⁻⁸ mol/l) induced a longer lasting and greater GH release. Three different mechanisms, not mutually exclusive, are postulated for Hexarelin stimulation of GH secretion in vivo: a direct action on the pituitary, through a minor relevance; an indirect action that involves release of GHRH, of relevance only in adult rats; and an action through the release of a still unknown hypothalamic “factor”, which in infant and adult rats elicits GH release acting synergistically with GHRH.

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It is now generally accepted that the secretion of growth hormone (GH) from the pituitary is primarily controlled by two hypothalamic neuropeptides: growth hormone-releasing hormone (GHRH) and somatostatin (SRIF), the former stimulating and the latter inhibiting the release of GH (1). In addition, several neurotransmitters and neuropeptides regulate GII release either by modulating the secretion of GHRH and SRIF from the hypothalamus and/or acting directly at the pituitary (1). In recent years considerable attention has been focused on a family of synthetic peptides, the growth hormone-releasing peptides (GHRPs), which potently stimulate GH secretion in mammals (2–4). These peptides appear to activate the receptor(s) for a still unknown endogenous ligand. It is generally agreed that the effects of GHRP-6, one of the most investigated GHRPs, are exerted through specific receptor sites; specific binding sites for GHRPs have been characterized in both the hypothalamus and the pituitary of the rat (5, 6). Nonetheless, the mechanism of action of GHRP-6 is still not completely understood. It is known that it acts at a pituitary site using intracellular messenger pathways that are different from those of GHRH (7–10). However, GHRP-6 is more potent in vivo than in vitro, suggesting that it may be capable of modulating GHRH and SRIF secretion (7, 11–13). Studies from our laboratory have shown that GHRP-6 is also capable of stimulating GH synthesis and secretion in infant rats passively immunized against GHRH (14).

In the last few years, several analogs of GHRP-6 have been synthesized with the aim of developing more effective and selective GH-secretagogues. Among them is the hexapeptide Hexarelin (His-Pro-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH₂; HEXA), which was shown to be very effective in stimulating GH secretion (15).

The aim of the present study was to characterize further the GH-releasing effect of Hexarelin in the rat and thus inferentially accrue understanding of the mechanism(s) underlying GHRP action. First, we evaluated the effect of acute- and short-term treatments with Hexarelin in rat pups, an animal model well suited for evaluating GH-releasing stimuli (16–18). Further, we studied the involvement of the hypothalamic input mediating the action of Hexarelin by assessing the
effects of Hexarelin in pups deprived of endogenous GHRH since birth. We then compared the GH-releasing activity of Hexarelin and GHRH in control adult rats and rats with surgical ablation of the medio-basal hypothalamus. Finally, the GH-stimulating effect of HEXA was studied on pituitary cell monolayers and compared to that of GHRH.

Materials and methods

Animals

Male and female 10-day-old Sprague-Dawley rats weighing about 25 g and young adult male rats weighing about 250 g (Charles River, Calco, Italy) were used. Rat pups were received on the day of birth and all rats were housed in our facilities under controlled conditions (22 ± 2°C, 65% humidity and artificial light from 06.00 to 20.00 h). A standard dry diet and water were available ad libitum to the dams and the adult male rats. One hour before the experiments, pups were separated from their respective dams and were divided randomly into groups of eight each. All the experiments were performed in accordance with the Italian Guidelines for the Use of Animals in Medical Research.

Antiserum to GHRH

The antiserum against GHRH (GHRH-ab) was prepared and validated by one of us (WBW) and has been found repeatedly to slow growth in rats (19–22). In a previous work (20), we evaluated the effect on GH secretion of GHRH-ab (50–200 µl/rat, sc) injected into 10-day-old pups. Based on those studies, a dose of 100 µl/rat was chosen, because it reproducibly elicited maximal inhibition of GH secretion.

Methods

In vivo experiments

Infant rats. Pups were given GHRH-ab starting from day 1 and then on days 2, 4, 6, 8 and 10, or received isovolumetric amounts of normal rabbit serum (NRS; 100 µl/rat). Individual groups of 16 infant rats were treated daily with Hexarelin (80 µg/kg, sc, b.i.d.) or physiological saline for 10 days (days 1–10 of age). Five days (days 6–10 of age) and 3 days (days 8–10 of age). Twelve hours after the last administration of Hexarelin or saline, eight pups were acutely challenged with Hexarelin (80 µg/kg, sc) and the other eight with saline, and killed by decapitation 20 min later. Trunk blood was collected and centrifuged immediately. Plasma samples were stored at −20°C until assayed for the determination of plasma GH concentrations. The dose of 80 µg/kg Hexarelin was chosen because in preliminary experiments it proved to be the lower dose that reproducibly elicited maximal GH release after sc administration in 10-day-old rats.

Adult rats. Complete ablation of the medio-basal hypothalamus (MBH) was accomplished as described previously (23). Briefly, after 1 week of acclimatization in our facility, rats were anesthetized with ketamine hydrochloride (Inoketam, 58 mg/kg, ip; Virbac, Milan, Italy) and xylazine (Rompun, 12 mg/kg, ip; Bayer, Milan, Italy). After deep anesthesia was induced, the animal’s head was mounted in a stereotaxic device with the incisor bar positioned 2 mm below the interaural line. A midline incision of the scalp was made and a 5 mm diameter window was made in the cranium. A stirrup-shaped knife 3.5 mm wide was then lowered at the midline and 1 mm posterior to bregma to the base of the brain and rotated 360° several times. At the conclusion of the experiments, MBH-ablated rats were killed by decapitation and the brains were removed for histological examination of the lesions. The lesion was considered to be complete if all MBH structures, including the arcuate nucleus, were deafferentated. Rats whose lesion did not fulfill the above criteria were not included in the results. Sham-operated (sham-op) rats served as controls; for sham operation, the knife was lowered to 2 mm above the base of the brain and was not rotated.

Seven and 30 days after the surgical procedure, eight MBH-ablated and six sham-op rats were anesthetized with urethane (1.5 g/kg, ip). Blood was sampled (0.3 ml) 60 min later from an exposed jugular vein for the determination of baseline GH levels. Immediately thereafter, 2 ml/kg saline, 300 µg/kg Hexarelin or 2 µg/kg GHRH was injected into the jugular vein and additional blood samples were obtained 10 and 20 min later from the same route.

In another study beginning on day 9 after surgery, eight MBH-ablated and six sham-op rats were treated with Hexarelin (150 µg/kg, sc, b.i.d.) and another eight MBH-ablated and six sham-op rats were treated with isovolumetric amounts of saline for 5 days. Twelve hours after the last treatment, conscious rats were all acutely challenged by the subcutaneous route with the same dose of Hexarelin 15 min before killing. Trunk blood was collected for the determination of GH concentrations.

Blood samples were centrifuged immediately and the plasma was separated and kept frozen at −20°C until assayed. The dose of 150 µg/kg Hexarelin was chosen because in preliminary experiments it proved to be the lower dose that reproducibly elicited maximal GH release after sc administration in rats of this age.

In vitro experiments

Adult male rats were killed by decapitation and the pituitaries were rapidly dissected. Pituitary tissue used for cell dissociation included only the anterior lobe.
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(80\,\mu g/kg, NRS) HEXA in medium Briefly, saline with < 5 ml and (A) Effects of day rat orGH vs then GHRH-ab±Sal; < saline saline saline with physiological vs saline 0.1% BSA only, or HEXA (10^{-6}\text{ mol/l}) or GHRH (human GHRH-44, 10^{-8}\text{ mol/l}; Bachem). Media collected at the end of the incubation periods were immediately frozen and stored at −20°C until assayed for GH content.

**Growth hormone assay**

Growth hormone concentrations in plasma and in the pituitary culture media were measured by RIA using materials kindly provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health. Values are expressed in terms of NIDDK-rat-GH-RP-2 standard (potency 2.1U/mg) as \( \mu g/l \) plasma or medium. The minimum detectable value of rat GH was 1.0 \( \mu g/l \) and intra-assay variability was 6%. To avoid interassay variations, samples from each experiment were assayed in only one assay.

**Statistical analysis**

Statistical differences between control and experimental groups were evaluated by Dunnett’s t-test for multiple comparisons, preceded by ANOVA; a \( p < 0.05 \) was considered significant.

**Results**

**Infant rats**

Preliminary experiments had indicated that, in rat pups, plasma GH levels peaked 20 min after the sc injection of Hexarelin (15). Therefore, in these studies all blood samples were collected at that time. All pups were killed on postnatal day 10.

In control pups treated with physiological saline for 3, 5 and 10 days, acute administration of Hexarelin (80 \( \mu g/kg, \) sc) elicited a significant \( p < 0.05 \) rise in plasma GH concentrations (the results obtained in these three groups were very similar and have been pooled). Daily treatments with Hexarelin (80 \( \mu g/kg, \) sc, b.i.d.) for 3, 5 and 10 days did not induce any modification of basal GH levels but a progressive increase in the plasma GH rise was evoked by the acute challenge with Hexarelin (Fig. 1A). The effect of the acute challenge

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**Fig. 1.** Effects of pretreatment with Hexarelin (HEXA) on the stimulation of GH secretion induced by an acute challenge with HEXA in rat pups. Neonatal rats were given normal rabbit serum (NRS) (A) or GHRH-ab (100\,\mu l/rat) (B) starting on day 1 and then on days 2, 4, 6, 8 and 10. Groups of 16 pups were given HEXA (80\,\mu g/kg, sc, b.i.d.) or physiological saline for 3, 5 and 10 days. On postnatal day 10, in each treatment group eight pups were challenged with HEXA (80\,\mu g/kg, sc) and the other eight pups with physiological saline and then killed 20 min later. Plasma GH concentrations were determined in trunk blood. The experiment was repeated three times. (A) \#p < 0.05 vs NRS + Sal; \#p < 0.05 vs NRS + HEXA. (B) \#p < 0.05 vs GHRH-ab + Sal; \#p < 0.05 vs GHRH-ab + HEXA; \#p < 0.05 vs respective NRS group.

Briefly, pituitary glands were collected in sterile F-10 medium (Sigma, St Louis, MO, USA), cut into small fragments and incubated twice for 15 min at 37°C in 5 ml of F-10 medium containing 6% fetal calf serum and collagenase (2.5 mg/ml) (Boehringer, Mannheim GmbH, Germany). Fragments were then washed in Dulbecco’s PBS and Ca^{2+} and Mg^{2+}-free medium (Sigma) and mechanically dissociated by trituration with a siliconized glass pipette. Single-cell suspensions were plated onto 24-well culture plates (5 \times 10^5 cells/well). The cells were incubated in 1 ml of F-10 medium supplemented with 10% horse serum, 4% fetal calf serum and gentamycin (25 \( \mu g/ml \)) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After 3 days, the medium was removed and the cells washed twice with serum free F-10, then incubated with 1 ml of F-10 containing 0.1% BSA only, or HEXA (10^{-6}\text{ mol/l}) or GHRH (human GHRH-44, 10^{-8}\text{ mol/l}; Bachem). Media collected at the end of the incubation periods were immediately frozen and stored at −20°C until assayed for GH content.
Fig. 2. Comparison of Hexarelin and GHRH stimulation of GH secretion in adult sham-operated rats. Adult male rats were given Hexarelin (HEXA, 300 µg/kg, iv) or GHRH (2 µg/kg, iv) and blood samples collected at the indicated times. Results are the mean ± SEM of 6–8 determinations; *p < 0.05 vs respective control value and #p < 0.05 vs GHRH at the same time.

was in fact increased significantly by 37%, 156% and 291% (p < 0.05) over control levels after 3, 5 and 10 days of Hexarelin pretreatment, respectively.

In pups passively immunized against GHRH since birth and treated with physiological saline for 3, 5 and 10 days (Fig. 1B), the acute challenge with Hexarelin induced a rise in plasma GH that was significantly lower (p < 0.05) than that previously observed in control rats (see Fig. 1A); as for the NRS-treated pups, the results obtained in the saline-treated groups were similar and have thus been pooled. Also in the GHRH-ab group, Hexarelin pretreatment for 3, 5 and 10 days did not induce any modification in basal secretion but significantly enhanced the effect of the acute Hexarelin challenge (67%, 410% and 209% increases, respectively; p < 0.05) (Fig. 1B). However, all stimulated GH values remained significantly lower (p < 0.05) than in the respective control group (see Fig. 1A).

Adult rats

In the first study, the effects of the acute stimulation with Hexarelin and GHRH were compared in sham-op and MBH-ablated rats either 7 or 30 days after surgery.

In adult sham-op rats, Hexarelin (300 µg/kg, iv) induced a clear-cut GH response 10 and 20 min after injection that was significantly greater (p < 0.05) than that induced by GHRH (2 µg/kg, iv) (Fig. 2). When the same experiment was performed in MBH-ablated rats 7 days after surgery, GHRH increased GH secretion in a fashion similar to sham-op rats. However, in this animal model GHRH was significantly (p < 0.05) more effective than Hexarelin both 10 and 20 min after injection (Fig. 3). The GH response to Hexarelin measured in MBH-ablated rats 7 days after surgery was about one-third of that elicited in sham-op rats.

Thirty days after surgical ablation of the MBH, Hexarelin and GHRH evoked a similar rise in plasma GH levels (Fig. 4).

In the second study, the effect of Hexarelin stimulation
was evaluated in sham-op and MBH-ablated rats after 5 days of HEXA pretreatment. Fourteen days after surgery, in adult sham-op rats, the 5-day treatment with Hexarelin did not potentiate the effect of the acute challenge with Hexarelin (Fig. 5). Similarly, in MBH-ablated rats the plasma GH response to the acute challenge with Hexarelin was of the same extent in saline or 5-day Hexarelin-pretreated rats (Fig. 5).

In vitro studies
Hexarelin (10^-6 mol/l) significantly stimulated GH release from primary pituitary cell cultures at 15 and 30 min (52.6% and 24.7% increase over control secretion, respectively; p < 0.05) but was ineffective at any of the longer incubation times (Fig. 6). In contrast, GHRH (10^-7 mol/l) evoked a GH secretion significantly greater (p < 0.05) than that in control and Hexarelin-stimulated cells at all times tested, with the only exception being at 15 min, when Hexarelin was more active than GHRH (Fig. 6).

Discussion
Hexarelin has been developed as a GHRP-6 analog in which u-Trp is substituted with u-2-methyl-Trp (15). This substitution is believed to confer higher chemical stability and a more lipophilic character to the hexapeptide. When compared to GHRP-6 in adult and infant rats, Hexarelin proved to be a more effective GH-releaser than GHRP-6 (15, 24).

We have shown recently that Hexarelin markedly stimulates GH secretion in infant and adult rats (15). The present study was undertaken to gain more insight into the mechanism(s) underlying Hexarelin stimulation of GH secretion. In infant pups short-term treatment with Hexarelin enhanced progressively the GH response to an acute challenge with the hexapeptide. In fact, by increasing the length of Hexarelin treatment from 3 to 10 days, peak GH secretion was augmented from 37% to 291% over that observed in control rats. These data clearly demonstrate that in rat pups Hexarelin is capable of priming its GH-stimulating activity. A proper interpretation of this phenomenon is difficult at present. It is tempting to speculate that, because of the structural similarity between Hexarelin and GHRP-6, the two hexapeptides bind to the same
receptor(s) and stimulate GH secretion through an identical mechanism of action (see below). Studies performed in the adult rat have suggested that GHRP-6 enhances GH secretion by stimulating GHRH and/or inhibiting SRIF secretion and/or pituitary actions (13, 25–27). The overall evidence includes:

(i) in the adult rat, passive immunization against GHRH produces partial suppression in GHRP-6 activity (26, 28);
(ii) GHRP-6 administration activates a subpopulation of neurons in the rat arcuate nucleus (25);
(iii) in the sheep, the increase in plasma GH concentration elicited by Hexarelin is associated with an enhanced GHRH release into hypophysial portal blood (29);
(iv) infusion of GHRP-6 in conscious male rat disrupts the spontaneous episodic GH secretion and abolishes the cyclic refractoriness to GHRH (26);
(v) the GH response to GHRP-6 is increased after passive immunization against SRIF (13).

In contrast, other observations suggest that GHRP-6 stimulates GH secretion independently from endogenous GHRH and/or SRIF. For instance, it has been shown in vitro that competitive receptor antagonists of GHRP-6 and GHRH fail to inhibit the stimulation of GH secretion induced by the other peptide (7, 13). In humans, the acute GH stimulation elicited by GHRP-6 is fully operational even during the infusion of GHRH at a dose that totally desensitizes pituitary somatotrophs to GHRH (30). Moreover, we have shown in adult rats that neither cysteamine depletion of hypothalamic SRIF stores (31) nor acute passive immunization against GHRH and/or SRIF in rat pups (14) induces any alteration of the GH-releasing effect of GHRP-6.

We report here that, in rat pups, in spite of passive immunization against GHRH since birth, a 3–10-day treatment with Hexarelin was still capable of priming the pituitary. In GHRH-deprived pups, however, stimulation of GH secretion was always significantly lower than in NRS-treated pups. Two possible explanations are:

(i) Hexarelin stimulates GH secretion independently from GHRH; the lower GH stimulation observed in GHRH-deprived pups is due to reduced pituitary GH synthesis (14);
(ii) Hexarelin stimulates GHRH secretion to such an extent as to overcome the blockade effect exerted by the GHRH-ab.

The latter hypothesis seems unlikely, because in 10-day-old pups GHRP-6 stimulation of GH secretion was not altered even after doubling the amount of GHRH-ab (14).

On the other hand, it was reported previously that 36 h after surgical ablation of the MBH the somatotroph responsiveness to GHRP-6 was significantly greater than in sham-operated rats, while 7 days after surgery the response to GHRP-6 was dramatically reduced but still present (32). Similarly, our Hexarelin data obtained in MBH-ablated rats point to a mechanism that is independent of GHRH: the GH surge evoked by Hexarelin was blunted but not abolished 7 and 30 days after surgical ablation of the MBH. These observations can only be explained by a direct action of Hexarelin and GHRP-6 on the pituitary or by the existence of an extra-GHRH “peripheral” mediator of Hexarelin action. Supporting a direct pituitary site of action for Hexarelin are our findings on pituitary cell monolayers and similar in vitro observations made using GHRP-6 (7, 11, 14).

It is noteworthy, in this context, that both Hexarelin and GHRP-6 stimulate GH secretion in vitro with kinetics which are different from that of GHRH. Both Hexarelin (using cells from adult rats; present study) and GHRP-6 (using cells from infant rats (14)) induce a peak stimulation of GH release at 15 min which is no longer evident at 30 min. The action of GHRP-6 is longer lasting and of strikingly higher efficacy. These results further support the view that GHRH and the GHRPs in part activate different pituitary receptors and intracellular messenger pathways. It is widely recognized that while GHRH stimulates GH release by a cAMP-mediated intracellular pathway (33–35), GHRP-6 effects are predominantly mediated by protein kinase C (36).

Comparison of the results obtained in control and MBH-ablated rats suggests that about one-third of the in vivo actions of Hexarelin result from a direct effect on the pituitary, while about two-thirds of its effect is mediated by one or more hypothalamic effectors. However, this relative quantitation of the effect exerted at the hypothalamic and pituitary sites shall be considered as only indicative. In fact, it has been reported previously that urethane anesthesia may greatly reduce GHRP-6 stimulation of GH secretion (26), an effect that in our experimental conditions was only possible in control but not MBH-ablated rats. Consideration should also be given to the possibility that the pituitary of MBH-ablated rats contained a smaller amount of readily releasable GH than that of controls. The latter explanation is, however, at least in part weakened by the results showing that 7 days after surgery the challenge with GHRH stimulated a very similar GH response in both MBH-ablated and sham-op rats. Recently it has been reported that, in adult rats, passive immunization against GHRH significantly reduced Hexarelin stimulation of GH secretion (24). These data may indicate either that GHRH plays a role in mediating Hexarelin action or that the occupancy of GHRH receptors is a prerequisite for the full expression of Hexarelin effects. These two hypotheses have also been formulated to explain the effect of passive immunization against GHRH on GHRP-6-induced GH secretion (26, 28). It has also been proposed that GHRP stimulation of GH secretion may involve...
uncharacterized hypothalamic factors different from the classic hypothalamic hormones (13). However, further evidence supporting this hypothesis is lacking.

It is of interest that both intact and MBH-ablated adult rats a 5-day treatment with Hexarelin did not prime the pituitary response to acute Hexarelin stimulation. These data contrast with those obtained in infant rats. However, it has been observed that some stimuli induce different effects in infant and adult rats (16, 37, 38). For example, GHRH and clonidine stimulate GH biosynthesis in the pituitary of infant rats but not adult rats (17).

We do not know the reason(s) for the age-dependency of Hexarelin effect on GH release. Neonatal somatotrophs might be more sensitive to hypophysiotropic stimuli (16) or, in adult rats, the activation of feedback mechanism(s) (1) may completely obscure the effects of Hexarelin administration. The lack of priming effects with Hexarelin in adult MBH-ablated rats is worth further consideration. In fact, putative feedback effects of GH and/or IGFs on the central nervous system are abolished by surgical ablation of the hypothalamus. It is therefore suggested that priming is not an effect induced only when Hexarelin action on the pituitary is unopposed.

In summary, this study shows that in the infant rat Hexarelin is capable of priming its own stimulation of GH secretion. Because priming was also present in GHRH-deprived neonatal rats, it is likely to be independent from endogenous GHRH. However, the lack of priming in control and MBH-ablated adult rats strongly suggests that it is an age-dependent phenomenon that mainly involves the hypothalamus. We propose that in vivo Hexarelin may stimulate GH secretion through three different putative mechanisms: a direct effect on the pituitary, though of minor relevance; an indirect action which involves endogenous GHRH, of relevance only in adult rats; and through the release of a still unknown hypothalamic factor which, in adult and infant rats, acts sinergically with GHRH to elic its GH release. The relative weight of such pathways, being different in infant and adult rats, may account for the age-dependent effects observed herein.

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