In vivo effects of the GH-releasing heptapeptide GHRP-1 in lambs

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Original article

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Abstract – The novel synthetic growth hormone-releasing heptapeptide GHRP-1 is reported to be more potent than growth hormone-releasing hormone (GHRH) in eliciting GH release in vivo in rats and man. However, in ovine pituitary cells in primary culture in a perifusion system, GHRP-1 was 10-fold less active than GHRH. The purpose of this work was to study the effect of GHRP-1 in sheep in vivo. Ovine GH release stimulated by either GHRP-1 or GHRH(1-29)NH_2, in eight pre-ruminant lambs, was determined. GHRP-1 was administered at doses of 1.2, 2.4 and 6 nmole/kg by i.v. bolus, and GHRH(1-29)NH_2 at 0.3 nmole/kg. Mean ± s.e.m. peak GH levels in the plasma after injection of saline, 1.2, 2.4, 6 nmole/kg GHRP-1 and 0.3 nmole/kg GHRH were 2.2 ± 0.9, 9.3 ± 2.5, 8.8 ± 2.4, 35.1 ± 5.8 and 51.6 ± 10.5 ng/mL, respectively. As spontaneous 20 ng/mL peaks were observed, only peaks above this level can be considered as significant. The highest dose of GHRP-1 (6 nmole/kg) elicited oGH release, but its action was surpassed by GHRH 0.3 nmole/kg. Furthermore GHRP-1 and GHRH appear to behave inversely when response amplitudes are considered. Animals exhibiting a strong reaction to GHRH-1, show a correspondingly weak reaction to GHRH and vice-versa. This may reflect differences in intracellular mechanisms at the pituitary level. Our data support the results in vitro that in sheep GHRP-1 is a weaker stimulant of GH secretion than GHRH. © Inra/Elsevier, Paris

GHRP-1 / GH-release / ovine

Résumé – Effets in vivo du facteur de libération de l'hormone de croissance, l'heptapeptide GHRP-1, chez l'agneau. Le nouvel heptapeptide GHRP-1 s'est révélé plus puissant que GHRH pour provoquer la décharge de GH in vivo chez le rat et l'homme. Cependant, dans un système

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de périfusion de cellules hypophysaires ovines en culture primaire, GHRP-1 s'est montré dix fois moins actif que GHRH. Le but de ce travail est d'étudier l'effet de GHRP-1 chez les ovins in vivo. La sécrétion d'oGH stimulée soit par GHRP-1 soit par GHRH(1-29)NH_2 a été déterminée chez huit agneaux préruminants. GHRP-1 a été administré aux doses de 1.2, 2.4 et 6 nmole/kg en bolus i.v. et GHRH(1-29)NH_2 à 0.3 nmole/kg. La valeur moyenne ± s.e.m. du pic de GH plasmatique aprés injection d'une solution saline, de 1.2, 2.4 ou 6 nmole/kg de GHRP-1 ou de 0.3 nmole/kg de GHRH a été respectivement de 2.2 ± 0.9, 9.3 ± 2.5, 8.8 ± 2.4, 35.1 ± 5.8 et 51.6 ± 10.5 ng/mL. Des pics spontanés atteignant 20 ng/mL ayant été observés, seuls les pics dépassant cette valeur peuvent être considérés comme étant provoqués. Dans ces conditions seule la dose de 6 nmole/kg de GHRP-1 s'est montrée efficace, moins cependant que celle de 0.3 nmole/kg de GHRH. Par ailleurs, les amplitudes de réponses aux deux sécrétagogues tendent à s'opposer : les animaux qui répondent bien à l'un réagissent mal à l'autre, et inversement, reflet possible de mécanismes intracellulaires différents au niveau de l'hypophyse. Ces résultats étayent ceux établis in vitro, montrant que chez les ovins GHRP-1 est un sécrétagogue de GH moins puissant que GHRH. © Inra/Elsevier, Paris

GHRP-1 / sécrétion GH / ovins

1. INTRODUCTION

The first synthetic growth hormone (GH)-releasing peptides (GHRP) were reported in 1977 [6, 7], several years before the isolation and sequencing of the physiological human growth hormone-releasing hormone (GHRH) by Guillemin et al. [25] and Rivier et al. [40] in 1982. The first pentapeptides synthesized were not very potent in vitro and inactive in vivo. Bowers et al. [8] constructed a potent growth hormone releasing peptide (GHRP), the hexapeptide His-DTrp-Ala-Trp-DPhe-Lys-NH_2, which specifically elicited release of GH in vitro and in vivo. In man, a low dose of GHRP (0.1 μg/kg) injected intravenously (i.v.) causes release of GH [11]. GHRP also stimulates release of GH in rat, primate, chick, porcine, ovine and bovine species [8, 10, 19, 20, 31]. Furthermore the peptide is efficient if orally administered in rats, dogs and monkeys [43], and in man [14, 27]; however, its effect is about 300-fold less potent than when administered i.v.

Both GHRP and GHRH act directly on pituitary somatotrophs, but different intracellular mechanisms may be involved. Although both act synergistically [4, 9–11, 17], a GHRP antagonist inhibits the in vitro response to GHRP but not to GHRH [11, 17]. GHRH raises the intracellular level of cAMP in the pituitary, whereas GHRP does not [17]. GHRP receptors are different to GHRH and opiate receptors, in the pituitary and the hypothalamus [9, 18, 39]. GHRP and GHRH also elicit different patterns of GH secretion [36].

The mechanism of action of GHRP is complex in that it does not stimulate endogenous GHRH release [41] or inhibit SRIF release [13]. Bowers et al. [13] postulated that GHRP releases an unidentified hypothalamic factor (U-factor) which interacts synergistically with GHRH and stimulates the pituitary to release GH. The GH-releasing action of GHRP would then depend on the presence of GHRH. This has also been suggested by Bercu et al. [3] who showed that in rats endogenous GHRH contributes to full expression of exogenous GHRP activity in vivo.

A second generation GH-releasing peptide appeared a few years ago, the heptapeptide Ala-His-DBNal-Ala-Trp-DPhe-Lys-NH_2 (GHRP-1), three times more potent than GHRP in rat and man, GHRP

itself being more potent than GHRH(1-44)NH_2 [12]. Like GHRP, GHRP-1 is active in man when administered orally [12], and its action is not consistent with inhibition of SRIF release. Human and animal data indicate that GHRP-1 acts in the same way as GHRP. The GHRPs act on different receptors to GHRH and have distinct endocrine and probably molecular mechanism [15, 32, 34].

In rat pituitary monolayer cell cultures, Akman et al. [1] reported that GHRP-1 treatment leads to an increase in $[Ca^{2+}]_i$. The same result was reported using GHRP-6 [16, 28]. The rise in $[Ca^{2+}]_i$ provokes GH release by a cAMP-independent mechanism. Furthermore GHRP-1-induced $[Ca^{2+}]_i$ increase, and GH release, are inhibited by somatostatin, whereas cAMP elevating agents have an additive effect on the GHRP-1-stimulated GH release. This indicates that these cAMP elevating agents stimulate GH release by a distinct mechanism to GHRP-1. In a continuous perifusion system of ovine pituitary cells where GH was released at a constant rate, its secretion was increased by GHRH or GHRPs [46].

GHRH and the GHRPs stimulate GH release by mechanisms involving a common step: an increase in calcium influx. This confirms the data of Akman et al. [1] on the rat. However, the GHRPs do not act on the GHRH receptors.

The ability of GHRH and GHRPs to stimulate GH release in ovine pituitary cells was compared. The small peptides were found to be ten-fold less potent than GHRH. The maximal effects of the GHRPs were similar, but significantly less than the maximal effect of GHRH [46, 47]. These results are in contrast to the observations in rats and humans, where GHRP is more effective. This study in lambs investigates the action of the novel peptide GHRP-1 in vivo.

2. MATERIALS AND METHODS

2.1. Animals

Eight Lacaune lambs were trained to live in individual metabolic boxes for 2 weeks before the experiment. At the beginning of the injection period they weighed 16.5 ± 0.3 kg and 18.2 ± 0.2 kg on the last day of injection. They were fed ad libitum with a standard reconstituted milk, and injected every 2 or 3 days. They were weighed prior to injection and the dose was calculated for each individual weight. On the day prior to the experiment one catheter was implanted in an external jugular vein for test administration, and another in the contra-lateral jugular, for blood collection.

2.2. Experimental design

Each animal received a dose of GHRP-1 equivalent to 1, 2 or 5 μg active peptide/kg (≅ 1.2, 2.4 or 6.0 nmole/kg), or saline (controls).

To avoid day-to-day variations, lambs were allocated at random to a double Latin square (2 × 4 lambs, 4 treatments, 4 experimental days).

An additional series of injections was set up with GHRH(1-29)NH_2 1 μg/kg (≅ 0.3 nmole/kg) for comparison.

All injections were performed at 10.00 a.m.

We have reported that response to GHRH in lambs is very rapid (1 min; [2]), but nothing is known about the in vivo response to GHRP-1 in this species. We chose a sampling period of up to 5 h after injection to allow the detection of any late response or rebound, as occurs in bovines [37, 38].

Blood samples were collected in heparinized tubes containing iniprol (a protease inhibitor) 10 min prior to and immediately before injection. This sampling schedule has been shown in our previous works to reflect valid mean basal levels [2, 33]. Samples were then taken at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150, 180, 240 and 300 min post-injection, placed on ice, centrifuged and the plasma kept at –20 °C until assayed.

2.3. GH assay

GH levels were measured using a standard homologue double-antibody RIA. Ovine growth hormone (NIDDK oGH-1-4, lot # AFP-8758 C) and anti-oGH rabbit serum (NIDDK-ANTI-oGH-2, lot # AFP-C0123080) were kindly provided by the NHPP. Ovine GH was ^{125}I-labelled by the chloramine-T method, followed by a two-step purification by gel chromatography on G-50 and G-100 Sephadex columns. ^{125}I (ref. IMS 30) was purchased from Amersham, Les Ulis, France. The second antibody, anti rabbit gamma-globulin sheep antiserum was a gift from Dr J.P. Dulor, Inra, Montpellier.

To eliminate inter-assay variation, all samples were run in duplicate in the same assay. The sensitivity of the assay was 0.5 ngGH/mL and the intra-assay coefficient of variation at 10 ngGH/mL was 5 %.

2.4. Statistics

Individual responses to GH-releasing peptides were compared using the paired Student's *t*-test. The response curves from 10 to 50 min after injection of both peptides were compared for each animal, and correlation indices for peak responses of each animal to both peptides were calculated.

3. RESULTS

The average basal level of oGH was 5.8 ± 0.4 ng/mL, with individual average basal levels varying between 2.1 and 10.4 ng/mL. Ovine GH levels ranging between 0.5 and 20.2 ng/mL were detected in individual animals. Since spontaneous GH pulses of up to 20 ng/mL were recorded, only peaks above this level were considered significant. Lambs respond to GHRP-1 by releasing GH (*figure 1*); however, the response depended on the dose. The low doses were inefficient, and only the highest dose (6 nmole/kg) led to GH release, but did not elicit as high a peak as 0.3 nmole/kg GHRH 1-29. GH peaks generally occurred within 10 min of injection, and the time taken to return to normal levels depended on the height of the peak. Since doses 1.2 and 2.4 nmole/kg did not induce GH curves above the controls (*figure 2*), only the responses to 6 nmole/kg GHRP-1 were compared to GHRH. The mean peak value of 35.1 ± 5.8 ng/mL induced by 6 nmole/kg GHRP-1 is very significantly different ($P < 0.001$) to that induced by 0.3 nmole/kg GHRH, 51.6 ± 10.5 ng/mL. In all lambs but three, the response to GHRH was higher than that to the highest dose of GHRP-1. In lamb no. 8, both responses were similar, and in lambs nos 3 and 5 no response to GHRH was observed. The results in *figure 3* show maximum GH peaks within 20 min post-injection. Responses to GHRH and GHRP-1 appeared to be inversely correlated. If we exclude lamb no. 6, the highest peak in response to GHRH in lambs nos 7 and 4 corresponded to the lowest responses to GHRP-1, and conversely the highest response to GHRP-1 in lambs nos 3 and 5 corresponded to the lowest reaction to GHRH (*figure 3*). For each animal the response curve to GHRP-1 during the effective period (10–50 min post-injection), was plotted against that for GHRH, using the paired Student's *t*-test. A significant inverse correlation exists at the $P = 0.05$ level ($r = -0.76$) if data on lamb no. 6 are excluded. However, if these data are included, the inverse correlation is no longer significant.

4. DISCUSSION

As in humans and rats, i.v. bolus injection of GHRP-1 induces a rapid pituitary GH release in pre-ruminant lambs. However, sensitivity to the peptide varies greatly between species and according to the experimental conditions. In some species the small synthetic peptides are more potent than GHRH. For instance, in young barrows when an equal amount of either GHRP or GHRH(1-29)NH_2 was administered by either s.c. or i.v. the hexapeptide elicited a higher GH response [20];

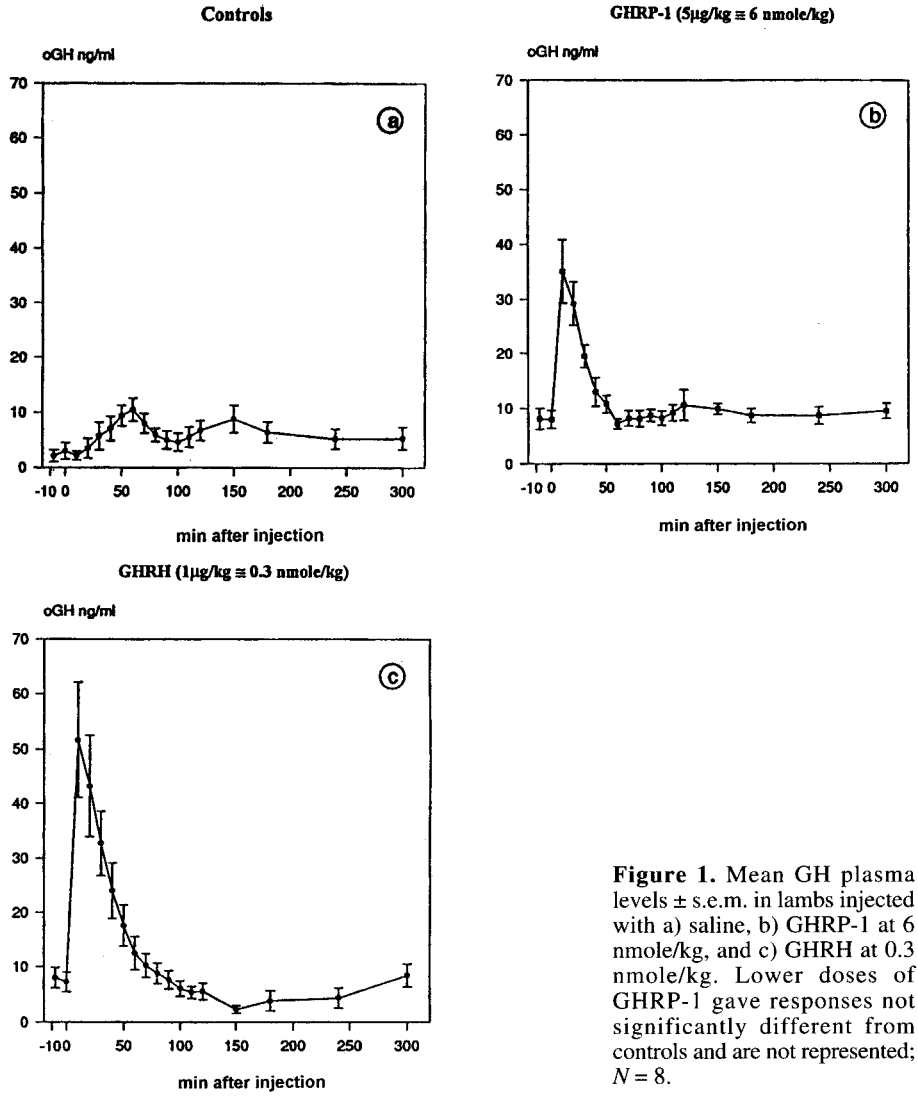

Figure 1. Mean GH plasma levels ± s.e.m. in lambs injected with a) saline, b) GHRP-1 at 6 nmole/kg, and c) GHRH at 0.3 nmole/kg. Lower doses of GHRP-1 gave responses not significantly different from controls and are not represented; $N = 8$.

however, no correction was made for the molar ratio. In monkeys, orally administered GHRP was reported to be 5–20 times more effective than in rats or dogs [43]. The data of Wu et al. [46], taken with our current results, show that GHRP-1 is much less effective in ovine species than in rats or humans. In rat pituitary cell culture, maximal GH release by the same molar concentration of GHRH and GHRP-1 was similar. The increase in intracellular $[Ca^{2+}]_i$ was also comparable in both cases [1]. By contrast in a similar system GHRP was far less potent (ED_{50} at 9 nM) than GHRH (1-44)NH2 (ED_{50} at 1.6 nM) to releasing GH [42].

In humans in vivo, the same i.v. dose (1 µg/kg) of GHRP-1 was three times more potent than GHRP, which was in

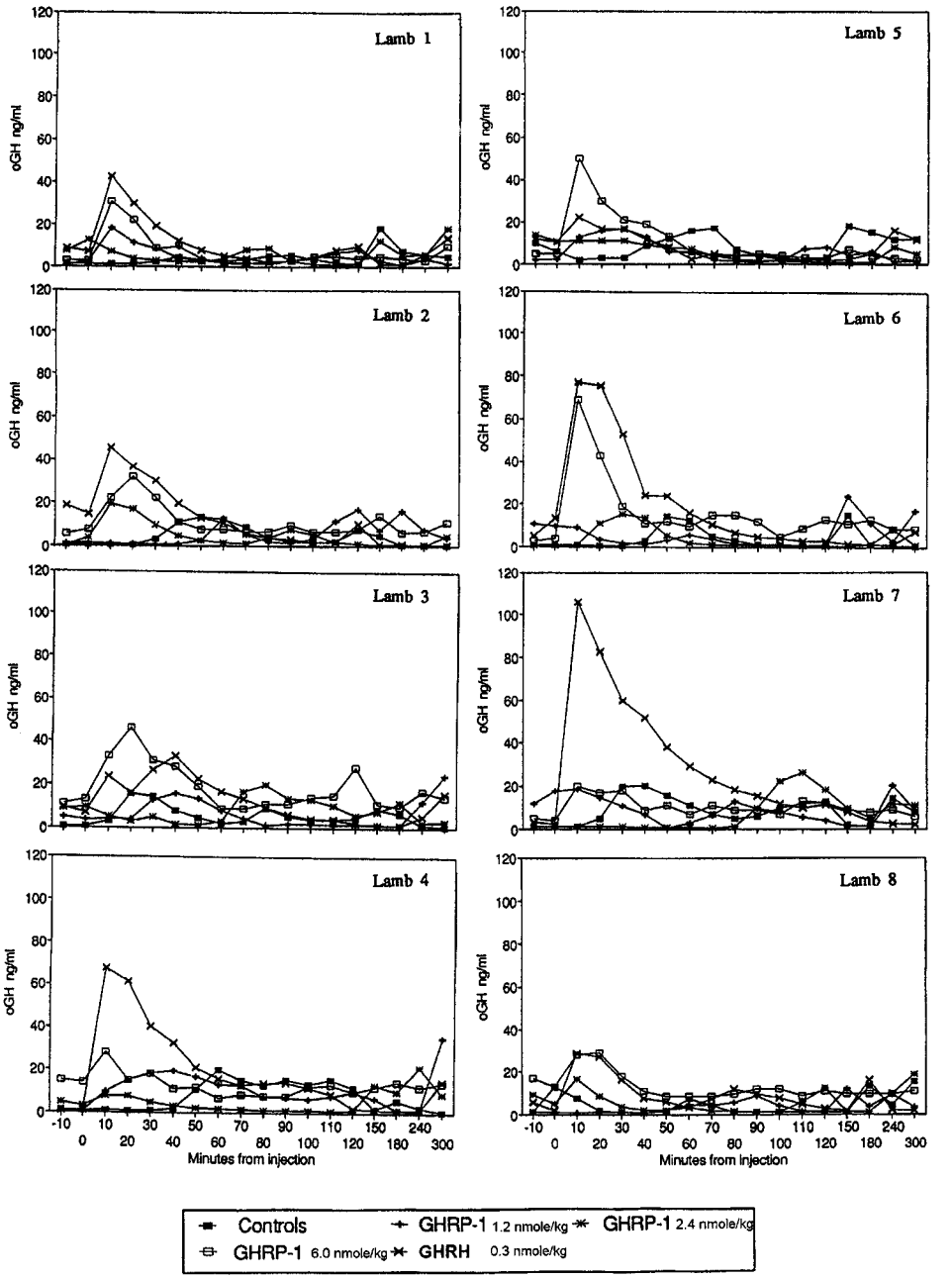

Figure 2. Individual responses of eight lambs to a single injection of: saline, 1.2, 2.4 or 6 nmole/kg of GHRP-1 or 0.3 nmole/kg GHRH 1-29.

turn twice as potent as GHRH (1-44)NH_2 in stimulating GH release [12]. Thus, the dose of 1 μg/kg GHRP-1 was approximately six-fold more potent than 1 μg/kg of GHRH(1-44)NH_2. These comparisons were based on weight, and should be corrected for the molar ratio, the GHRPs (six or seven amino acid residues) being six- to seven-fold smaller than native GHRH (44 residues). Even after such a correction, GHRP-1 is at least as potent as GHRH.

In contrast, in lambs GHRP-1 is much less active than GHRH(1-29)NH_2. This cannot be attributed to a difference in potency between the native GHRH and its analogue 1-29, because on a molar basis, GHRH(1-44)NH_2 and GHRH(1-29)NH_2 are equipotent in ovine species both in vitro [5] and in vivo [2]. This is further supported by the finding that all GHRH analogues greater than the 1-27 sequence are as potent as the native GHRH(1-44)NH_2 [26, 35, 44].

The mean peak values of 35.1 ± 5.8 ng/mL for 6 nmole/kg GHRP-1 and 51.6 ± 10.5 ng/mL for 0.3 nmole/kg GHRH show that the heptapeptide is less potent than GHRH in this experiment. This is confirmed by statistical analysis: individual responses from 10 to 50 min after injection are significantly greater (P = 0.0016) with GHRH than with GHRP-1. Therefore, our in vivo results reinforce those of Wu et al. [46] who found that in ovine pituitary cells in primary culture the half-maximal effective dose (ED_{50}) of GHRP-1 was one order of magnitude higher than that of GHRH. They also reported that the maximal response to GHRH in GH release was significantly greater than that to the heptapeptide, and that GHRH and GHRP-1 do not act synergistically, as they do in the rat. Furthermore, different GHRPs act via different mechanisms, suggesting the existence of different subtypes of GHRP receptor in the rat and sheep [46]. Ovines differ from

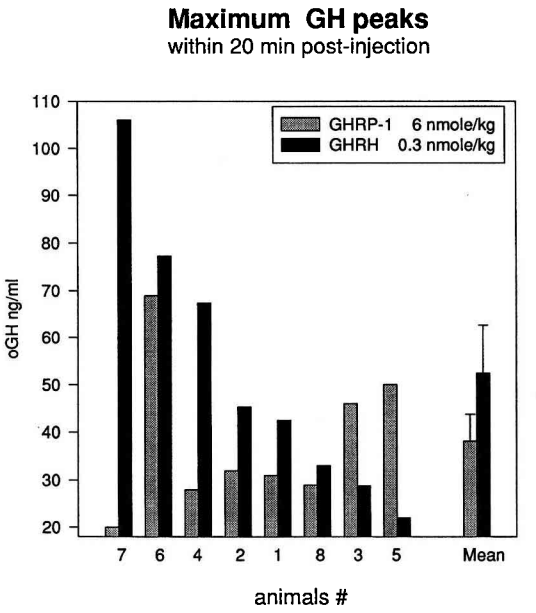

Figure 3. Maximum GH peaks within 20 min post-injection in lambs injected with secretagogues. Peak values are given in a decreasing order of response to GHRH. For the means, s.e.m. is given. Animal identification is indicated on the abscissa.

other species studied in this respect. Wide variations are commonly observed in basal GH levels in ovine species not only between animals but also from one day to the next in the same lamb [33], and wide variations in spontaneous or GH-releasing peptide induced secretory patterns of GH were also observed [2]. The tendency of responses to be opposed (high with GHRH and low with GHRP-1, or inversely, *figure 3*), might reflect a more sensitized intracellular mechanism of response to GHRH than to GHRP-1 (lamb nos 7, 4, 2 and 1) or inversely (lamb nos 5 and 3), since it is thought that they work independently. A case such as lamb no. 6, which responds to both GHRP-1 and GHRH may have an hypophyseal/hypothalamic equipment sensitive to both secretagogues. The amplitude of the peaks observed in this experiment is significantly less than that reported by Bowers et al. [8] with the hexapeptide GHRP; however, the doses differ greatly: 6 nmole/kg ≅ 5 μg/kg versus 2 mg/kg (ratio 1:400). With higher doses, we may have obtained higher and more sustained responses.

In older ovines, Guillaume et al. [24] reported that the GH blood level and GHRH portal level were stimulated less than 2.5-fold by 25 μg/kg (about 30 nmole/kg) hexarelin (a methyl derivative of GHRP-6), though this peptide was a potent stimulator of GH secretion in rats and humans at much lower doses [22, 30, 45]. These results further support the evidence that ovines are less sensitive to GHRPs than many other species. With bolus injections of 10 μg GHRP-6/kg, Fletcher et al. [21] obtained a rise in GH plasma levels in only two thirds of ewes, which did not follow either the GHRH or SRIF pathway. And Howard et al. [29] have recently reported the existence of an endogenous system, distinct from GHRH and somatostatin, that participates in the regulation of GH release. These data suggest that the GHRPs act via specific receptors, and the more recent data strongly suggest the existence of a natural GHRP-like ligand which has not yet been identified [23].

In conclusion, GHRP-1 was less potent in the lambs investigated here than GHRH(1-29)NH_2 in inducing the release of pituitary GH. Whether it is active per os in ovines is not yet known, but considering the need to multiply the dose by 300 to obtain the same response as that following injection, suggests that this is not an appropriate method for routine use. Thus, its practical interest in the field of agronomy and veterinary science for promoting or improving the main GH effects, such as growth or defatting of farm animals, must be questioned, at least in ovines.

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