Growth hormone responses to growth hormone-releasing hormone and hexarelin in fed and fasted dogs: effect of somatostatin infusion or pretreatment with pirenzepine

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Abstract

Using unanesthetized young male and female beagle dogs, before and after a 2-day fast, we studied the effect of an i.v. infusion of 0.9% saline (5 ml/h), somatostatin (SS, 4 or 8 µg/kg/h) or pretreatment with pirenzepine (PZ, 0.6 mg/kg i.v.), a muscarinic cholinergic antagonist which allegedly releases SS, on the GH release evoked by acute administration of GHRH (2 µg/kg i.v.), hexarelin (HEXA), a member of the GH-releasing peptide family (250 µg/kg i.v.) or GHRH plus HEXA.

In fasted dogs, GHRH delivered during saline infusion induced a clear-cut rise in plasma GH levels, significantly higher than that which it induced in fed dogs. In contrast, HEXA, although very effective in causing the release of GH, only slightly increased GH secretion in fasted dogs over that which it induced in fed dogs. Co-administration of GHRH plus HEXA into fed dogs induced a synergic GH response that further increased with fasting.

The action of GHRH in fed dogs was abolished by the lower dose of SS, whereas SS at either dose was ineffective in suppressing the GH-releasing effect during fasting. Infusion of the lower dose of SS failed to counter the action of HEXA, either before or during fasting, whilst the higher SS dose partially reduced it in both conditions.

In contrast to SS, PZ reduced the GH-releasing effect of GHRH and HEXA, both in the fed state and, though to

a lesser extent, during fasting. Pirenzepine only slightly reduced the robust GH rise elicited by GHRH plus HEXA in fed dogs. The suppressive effect of PZ on the GH response to combined administration of the peptides was lowest in fasted dogs.

These data show that: (1) fasting augmented the GH response to GHRH and (to a lesser degree) to HEXA; (2) SS inhibited the GH response to GHRH in the fed state, but not in the fasted state; (3) only the higher dose of SS partially reduced the GH stimulation by HEXA in either the fed or the fasted state; (4) PZ lowered the GH response to GHRH and to HEXA in both the fed and (to a lesser degree) the fasted state; (5) PZ did not modify the GH release due to the combined administration of GHRH and HEXA.

It is suggested that: (1) during fasting the greatly enhanced GH response to GHRH alone or GHRH plus HEXA probably reflects an augmented GHRH secretion; (2) somatotrope refractoriness to SS may contribute to the enhanced GH secretion in states of calorie deprivation; (3) in contrast to a general belief, muscarinic cholinergic antagonists, e.g. PZ, do not act exclusively via release of SS, but probably also through inhibition of GHRH function.

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Introduction

Growth hormone (GH) secretion is under the regulatory control of the central nervous system (CNS) through two specific hypothalamic neuropeptides, GH-releasing hormone (GHRH) and somatostatin (SS). In addition, a complex network of neurotransmitters and hormonal, metabolic, nutritional and environmental stimuli regulate GH secretion by modulating either GHRH or SS secretion from the hypothalamus or by acting directly on the pituitary (Müller 1987).

Besides GHRH, a new class of small GH-releasing peptides (GHRPs) has been characterized (Bowers *et al.*

1984, 1990, 1992, Ilson *et al.* 1989, Walker *et al.* 1990, Hartman *et al.* 1992a). One of the most effective GHRPs is hexarelin (HEXA), which stimulates GH secretion *in vitro* and *in vivo* in a number of animal species, including man (Deghenghi *et al.* 1994, Ghigo *et al.* 1994, Cella *et al.* 1995).

Experimental evidence supports the existence of different regulation for GHRH and GHRPs. It includes: different receptors (Goth *et al.* 1992) and post-receptor mechanisms (Lussier *et al.* 1991, Akman *et al.* 1993) at the pituitary level; opposite GH responses following estrogen or dexamethasone treatment (Molla *et al.* 1993); different responsiveness to SS (Arvat *et al.* 1995); diverging

modulation by indirect cholinergic agonists (Arvat *et al.* 1995). The mechanism(s) underlying such differences are, however, still obscure.

Both protein calorie restriction and fasting induce a GH hypersecretory state (Müller *et al.* 1995), which is suited to unravel the different regulation and the roles of GHRH and GHRPs. Reportedly the GHRH-induced GH response is greatly enhanced in humans (Rolla *et al.* 1986) and dogs (Arce *et al.* 1991) during calorie restriction. Thus, we first sought to probe the GH releasing effect of acute administration of GHRH, HEXA or GHRH plus HEXA in fed or short-term fasted dogs. We then studied the postulated defect of the hypothalamic somatostatinergic function (Müller *et al.* 1995) or a possible alteration of the pituitary responsiveness to SS (Bruno *et al.* 1994) during fasting, by testing GHRH and/or HEXA action in fed and fasted dogs under SS infusion.

Previous studies in calorie-restricted dogs (Arce *et al.* 1991) or patients with anorexia nervosa (AN) (Tamai *et al.* 1990, Rolla *et al.* 1991) had shown partial preservation of the GH response to GHRH despite pretreatment with pirenzepine (PZ), a muscarinic cholinergic antagonist and alleged hypothalamic SS releaser (Müller 1987). Thus, we finally evaluated the GH responses to GHRH and/or HEXA in fed and fasted dogs following PZ pretreatment.

Materials and Methods

Animals

Ten young beagle dogs (2 to 3 years old, 4 male and 6 female) were used in this study. They were well trained to lie on a comfortable pad in the laboratory for up to four hours at a time. Animals were fed normal dry food (Diete Standard, Charles River, Calco, Italy) once a day at 1600 h, with water available *ad libitum*. They were kept under a 12-h light:12-h darkness regimen, with lights on at 0700 h. At the beginning of the study body weights of all dogs were stable and they had no observable disease.

Since no differences were found in the GH responses between male and female dogs, data were pooled irrespective of sex.

Experimental protocol

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

The GH-releasing effect of GHRH (GRF(1–29)-NH₂, Geref, Serono, Rome, Italy) or HEXA (EP 23905, Europeptides, Argenteuil, France) was tested in each dog during an infusion of SS (Stilamin, Serono) or saline or after an i.v. dose of PZ (Gastrozepin, Boehringer-Ingelheim, Florence, Italy).

Every test was performed in the same dogs under fed or fasting conditions, following a randomized order with an interval of at least one month between two successive tests.

After the first test in fed dogs had been performed (see below), a 2-day complete fast with water available *ad libitum* was imposed and, on the morning of the 3rd day, when dogs were still fasting, the previous test was repeated.

The following tests were performed.

Saline or SS and saline, GHRH, HEXA or GHRH plus HEXA

Starting at 0900 h on the experimental day, an indwelling, non-thrombogenic intravenous catheter (Venisystem Butterfly-10, Abbott Ireland Ltd, Sligo, Republic of Ireland) was positioned in the cephalic vein and fixed with an adhesive bandage.

A solution of 0.9% saline was infused (5 ml/h) from 0900 h to 1230 h. In other studies, a solution of 0.9% saline was delivered (5 ml/h) from 0900 h to 0930 h, then an i.v. infusion of 4 or 8 µg/kg/h SS was started (5 ml/h) and continued until 1230 h. During saline or SS infusion, at 1130 h, GHRH (2 µg/kg i.v.) or HEXA (250 µg/kg i.v.) or GHRH plus HEXA was administered.

Doses were selected on the basis of previous dose-response experiments with either GHRH (S G Cella, unpublished results) or HEXA (Cella *et al.* 1995).

Because the GH response to GHRH was completely inhibited in fed dogs by the lower dose of SS (see below), the infusion of the higher SS dose and the ensuing GHRH test were performed only in fasted dogs.

For GH evaluation, blood samples (1 ml) were withdrawn every 30 min from 0900 h to 1130 h, then every 15 min from 1130 h to 1230 h. For SS evaluation, blood samples (5 ml) were withdrawn at 0900 h, 0930 h, 1130 h and 1200 h. In each dog, a total of about 30 ml blood was removed.

PZ and GHRH and/or HEXA

PZ (0.6 mg/kg i.v.) was administered 5 min before GHRH (2 µg/kg i.v.), HEXA (250 µg/kg i.v.) or GHRH plus HEXA. Blood samples (1 ml) were collected before GHRH or HEXA or GHRH plus HEXA (– 15 and 0) and every 15 min until 60 min post-peptide injection. In each dog a total of about 6 ml blood was removed.

Metabolic clearance rate of somatostatin

The metabolic clearance rate (MCR) of SS in all dogs in fasting and fed states was calculated by the method of Tait (1963) from the following equation: $MCR = (\text{infusion rate of SS}) / ((\text{steady-state levels of SS}) - \text{preinfusion levels})$. Preinfusion levels of SS were determined as the mean of SS concentrations in the 0900 h and 0930 h samples and

steady-state levels of SS were determined as the mean of SS concentrations in the 1130 h and 1200 h samples, i.e. 120 and 150 min after starting the infusion. The infusion rate of SS was 4 µg/kg/h.

GH radioimmunoassay

Blood was collected into tubes containing EDTA and immediately chilled. Plasma was frozen until assayed for GH by a double-antibody radioimmunoassay. Highly purified canine GH (batch AFP 1983b, Pituitary Hormones and Antisera Centre, Torrance, CA, USA), obtained through the courtesy of Dr A F Parlow, was used for radioiodination and as a standard. The assay sensitivity was 0.5 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.

SS radioimmunoassay

Blood samples were collected in 1.4 mg/ml Na₂EDTA and 1000 kIU/ml aprotinin (Antagosan, Istituto Behring, Scoppito, Italy). Octadecylsilylsilica cartridges (Sep-Pak C₁₈, Waters Co. Inc., Milford, MA, USA) were prepared by washing with 5 ml acetonitrile (Sigma Chemical Co., St Louis, MO, USA) followed by 5 ml water. Dog plasma (2.0 ml) was applied. Weakly bound plasma components were eluted with 5 ml water followed by 5 ml 0.1% trifluoroacetic acid (TFA, Aldrich Chemical Co., Milwaukee, WI, USA). Somatostatin was eluted with 2.0 ml 80:20 (v/v) acetonitrile:0.1% TFA. The eluate was promptly shell frozen, lyophilized and stored at -80 °C until subsequent assay.

The lyophilized eluates were reconstituted in 2.0 ml phosphate buffer 0.05 M, pH 7.2, containing 0.01 M EDTA and 0.3% BSA. Somatostatin RIA was performed using radiolabeled SS (3-[¹²⁵I]iodotyrosyl¹¹; Tyr¹¹-somatostatin-14), rabbit antiserum to SS (code N. 1611) and standard SS (somatostatin-14) supplied from Amersham Italia s.r.l., Milan, Italy. The assay sensitivity was 20 fmol/ml and the intra-assay variation was 8%. To avoid interassay variation all samples were assayed simultaneously.

Statistical analysis

GH values were expressed either as mean area under the plasma concentration vs time curves (AUC₀₋₆₀; ng/ml/h) ± S.E.M., calculated by the trapezoidal integration, or as absolute mean values (ng/ml) ± S.E.M. Somatostatin concentrations were expressed as absolute mean values (fmol/ml) ± S.E.M. Since no differences in hormonal levels between male and female dogs were observed in the different experimental conditions, data were pooled.

Figure 1 Integrated GH responses to saline, GHRH, HEXA and GHRH plus HEXA during saline or SS infusion (4 µg/kg/h) in 10 beagle dogs before and after a 2-day fast. See text for further details. # $P < 0.05$ vs GHRH in the fed state; ° $P < 0.01$ vs HEXA in the fed state; ** $P < 0.01$ vs GHRH in the fed state; ▲ $P < 0.01$ vs GHRH in the fed state; ++ $P < 0.01$ vs SS+GHRH in the fed state; \$ $P < 0.05$ vs HEXA in the fed state; Δ $P < 0.01$ vs HEXA in the fasting state; + $P < 0.01$ vs GHRH in the fasting state; * $P < 0.01$ vs GHRH+HEXA in the fed state.

Statistical comparisons of the mean values were performed by the *t*-test for unpaired or paired (where necessary) data, preceded by analysis of variance (ANOVA). A *P* value < 0.05 was taken to be statistically significant.

Results

Saline

Fed state During saline infusion the plasma GH concentrations remained stable over the entire experimental period and were near the detection limit of our assay (peak plasma GH, 0.7 ± 0.2 ng/ml) (Figs 1 and 2).

Fasting state Fasting increased, though not significantly, the plasma GH levels (peak plasma GH, 1.6 ± 0.6 ng/ml; $P = \text{NS}$ vs saline in fed state) (Figs 1 and 2).

GHRH

Fed state Administration of GHRH was followed by an unequivocal rise in plasma GH (peak plasma GH, 8.3 ± 1.5 ng/ml). Plasma GH concentrations peaked at about 15 min and then decreased to baseline within 60 min.

Figure 2 Integrated GH responses to saline, GHRH, HEXA and GHRH plus HEXA in 10 beagle dogs during saline or after pretreatment with PZ, before and after a 2-day fast. See text for further details. § $P < 0.05$ vs GHRH in the fed state; # $P < 0.01$ vs HEXA in the fed state; * $P < 0.01$ vs PZ+GHRH in the fed state; ° $P < 0.01$ vs PZ+HEXA in the fed state; △ $P < 0.01$ vs PZ+GHRH+HEXA in the fed state.

Fasting state The GHRH bolus induced a rise in plasma GH levels, significantly higher than that observed during the fed state (peak plasma GH, 12.5 ± 1.7 ng/ml; $P < 0.01$) (Fig. 1).

SS and GHRH

Fed state Infusion of the lower dose of SS suppressed the GH-releasing effect of GHRH in all dogs (peak plasma GH, 2.5 ± 0.8 ng/ml; $P < 0.05$ vs GHRH in fed state) (Fig. 1).

Fasting state When the lower dose of SS was infused, administration of GHRH was followed in all dogs by a robust GH burst (peak plasma GH, 11.4 ± 2.6 ng/ml), which was significantly higher than that observed in the same dogs when fed *ad libitum* ($P < 0.01$) (Fig. 1) and superimposable on that induced by GHRH alone during fasting ($P = \text{NS}$) (Fig. 1). The GH response to GHRH was still of the same magnitude when the higher dose of SS was infused (peak plasma GH, 9.6 ± 2.3 ng/ml; $P = \text{NS}$ vs GHRH in fasting state; $P = \text{NS}$ vs the lower dose of SS and GHRH in fasting state) (data not shown).

PZ and GHRH

Fed state GHRH bolus injection did not induce any GH response when dogs were pretreated with PZ (peak plasma GH, 2.2 ± 0.4 ng/ml; $P < 0.05$ vs GHRH in fed state) (Fig. 2).

Fasting state GHRH bolus injection was followed by a small, but unequivocal GH rise in dogs pretreated with PZ ($P < 0.01$ vs PZ and GHRH in fed state) (Fig. 2). Plasma GH concentrations peaked at about 15 min and then decreased to baseline values within 60 min (peak plasma GH, 7.8 ± 1.1 ng/ml).

HEXA

Fed state Administration of HEXA was followed by a striking rise in plasma GH concentrations (peak plasma GH, 59.3 ± 3.3 ng/ml). The mean plasma GH response (Fig. 1) was significantly higher after HEXA than after GHRH administration. Plasma GH concentrations peaked at about 15 min and then decreased to baseline values within about 60 min.

Fasting state Administration of HEXA in the fasted state induced only a slight increase in plasma GH concentrations over that which it elicited in the fed state ($P < 0.05$) (Fig. 1). The peak plasma GH value was 60.6 ± 2.3 ng/ml.

SS and HEXA

Fed state The lower dose of SS failed to suppress the GH response to HEXA (peak plasma GH, 91.4 ± 11.1 ng/ml; $P = \text{NS}$ vs HEXA in the fed state) (Fig. 1), while the higher SS dose only partially did so (peak plasma GH, 38.8 ± 11.3 ng/ml; $P < 0.01$ vs HEXA in the fed state) (Fig. 3).

Fasting state The lower dose of SS failed to suppress the GH-releasing effect of HEXA (peak plasma GH, 83.8 ± 20.9 ng/ml; $P = \text{NS}$ vs HEXA in the fasting state; $P = \text{NS}$ vs the lower SS dose and HEXA in the fed state) (Fig. 1), whereas the higher SS dose reduced, but did not abolish, the GH response to HEXA (peak plasma GH, 33.0 ± 9.6 ng/ml; $P < 0.01$ vs HEXA in the fasting state) (Fig. 3).

PZ and HEXA

Fed state Pretreatment with PZ markedly reduced the GH response to HEXA (peak plasma GH, 20.0 ± 6.6 ng/ml; $P < 0.01$ vs HEXA in the fed state) (Fig. 2).

Fasting state The inhibition exerted by PZ on the GH-releasing effect of HEXA was markedly reduced by

Figure 3 Integrated GH responses to HEXA during saline or SS infusion (8 µg/kg/h) in 10 beagle dogs before and after a 2-day fast. See text for further details. * $P < 0.01$ vs HEXA in the fed state; ° $P < 0.01$ vs HEXA in the fasting state.

fasting ($P < 0.01$ vs PZ and HEXA in the fed state) (Fig. 2). The peak plasma GH value was 52.7 ± 13.8 ng/ml at 15 min.

GHRH plus HEXA

Fed state Co-administration of HEXA plus GHRH induced a higher GH response (peak plasma GH, 119.2 ± 7.9 ng/ml) than HEXA alone ($P < 0.01$) or GHRH alone ($P < 0.01$) and also higher than the sum of the individual GH responses ($P < 0.01$) (Fig. 1).

Fasting state The GH response to combined administration of the peptides further increased during fasting (peak plasma GH, 153.1 ± 16.8 ng/ml; $P < 0.01$ vs GHRH plus HEXA in the fed state; $P < 0.01$ vs GHRH in the fasting state; $P < 0.01$ vs HEXA in the fasting state; $P < 0.01$ vs the sum of the individual GH responses to GHRH alone and HEXA alone in the fasting state) (Fig. 1).

PZ and GHRH plus HEXA

Fed state Pretreatment with PZ only slightly inhibited the robust GH burst elicited by combined administration of GHRH plus HEXA (peak plasma GH, 66.49 ± 6.2 ng/ml; $P < 0.01$ vs PZ and GHRH in the fed state; $P < 0.01$ vs

PZ and HEXA in the fed state; $P = \text{NS}$ vs GHRH plus HEXA in the fed state) (Fig. 2).

Fasting state Pretreatment with PZ failed to suppress the GH response to combined administration of GHRH plus HEXA (peak plasma GH, 142.5 ± 25.5 ng/ml; $P < 0.01$ vs PZ and GHRH plus HEXA in the fed state; $P < 0.01$ vs PZ and GHRH in the fasting state; $P < 0.01$ vs PZ and HEXA in the fasting state; $P = \text{NS}$ vs GHRH plus HEXA in the fasting state) (Fig. 2).

Metabolic clearance rate of somatostatin

Calculation of the MCR of SS showed no significant differences in dogs during the fed or fasting state (96.8 ± 8.9 vs 94.2 ± 9.8 ml/min/kg, $P = \text{NS}$).

Adverse effects

No particular adverse effects were noticed in any test except for a slight tachycardia in dogs pretreated with PZ.

Discussion

GH secretion is increased by fasting in mammals, including man (Ho *et al.* 1988, Hartman *et al.* 1992b), the only notable exception being the rat in which GH secretion decreases with fasting (Tannenbaum & Rorstad 1979).

In young beagle dogs, we have previously demonstrated that a 5-day fast increased GH peak frequency and amplitude, integrated concentration of GH and total peak area evaluated during a 6-h period (Cella *et al.* 1989). An increased frequency of GHRH release with prolonged periods of reduced SS secretion was surmised from the profile of GH secretion in animals (Cella *et al.* 1989, 1996a) and humans (Hartman *et al.* 1992b) during fasting.

Serum insulin-like growth factor (IGF)-I levels were unchanged in young dogs undergoing a 5-day fast (Cella *et al.* 1989). It must be recalled, however, that levels of IGF-binding protein-1 are increased by fasting (Busby *et al.* 1988, Murphy *et al.* 1991) and this may decrease the bioavailable portion of IGF-I, thus reducing IGF-I negative feedback on GH secretion (Müller 1987), before changes in total serum levels of IGF-I may occur.

The functional activation of somatotrophic function elicited by fasting may be relevant for providing information on the still elusive mechanism(s) underlying the strong GH-releasing effect of HEXA (Bowers *et al.* 1991, Korbonits & Grossman 1995). This discussion may, additionally, encompass some aspects of somatotrophic regulation in AN, a disease which, from a purely metabolic viewpoint, can be reminiscent of the dog model of calorie restriction (Müller *et al.* 1995).

In our study, as expected, a 2-day fast strikingly enhanced the GHRH-induced GH release in young dogs; this pattern was not mimicked by HEXA, the administration of which was followed by a GH rise similar to that present in the fed state. A priming action exerted by secretory bursts of GHRH of enhanced amplitude/frequency, coupled to a diminished somatostatinergic tone (Ho *et al.* 1988, Hartman *et al.* 1992b) may readily account for the exaggerated GH response to GHRH in fasted dogs.

The 'reduced' increase in the GH response to HEXA is more difficult to interpret. HEXA proved capable of priming its own GH-releasing action in an early phase of a long-term treatment in old dogs; this initial priming was then followed by a drop in HEXA-induced GH release below pretreatment values (Cella *et al.* 1996b). The most likely hypothesis was that chronic administration of the peptide had down-regulated GHRP receptors (Howard *et al.* 1996). Based on these observations, it is tempting to speculate that, for analogy, the relatively weak increase in the HEXA-stimulated GH rise in fasted dogs may result from down-regulation resulting in sustained release of an endogenous GHRP ligand (Korbonits & Grossman 1995, Howard *et al.* 1996). Interestingly, an absolute or relatively weaker GH-releasing activity of HEXA compared with GHRH has been reported recently in two independent studies in patients with AN (Foppiani *et al.* 1996, Scacchi *et al.* 1996).

Coupling of endogenous GHRH secretion with the exogenously administered GHRH would also explain why the GH response to GHRH plus HEXA of fed dogs (Bowers *et al.* 1990, Cella *et al.* 1995) was enhanced by fasting.

In fasted dogs (Cella *et al.* 1989, 1996a, Arce *et al.* 1991) or in patients with AN (Müller *et al.* 1995), increased GH secretion may be the result not only of an enhanced GHRH and/or diminished somatostatinergic tone (see above), but complete or partial pituitary refractoriness to SS, released from the hypothalamus, may also contribute. This hypothesis was substantiated in our study by the finding that SS, infused at a dose which reduced the GHRH-induced GH rise in fed dogs by about 50%, failed to do the same in fasted dogs given a GHRH bolus. Similar SS metabolic clearance rates in fed and fasted dogs make it unlikely that different pharmacokinetics of the infused peptide are involved.

In fasting rats a significant decrease of SS receptor mRNA expression, which sensitizes the pituitary responsiveness to an ensuing GHRH stimulus, has been reported (Tannenbaum *et al.* 1989, Bruno *et al.* 1994). This event, however, is probably due to a fasting-induced activation of hypothalamic somatostatinergic function and down-regulation of pituitary receptors, since in the rat fasting suppresses GH secretion (Tannenbaum & Rorstad 1979, Bruno *et al.* 1994). Such a mechanism is unlikely to occur in the dog and other mammals, including humans, where fasting induces GH hypersecretion (see above).

To the best of our knowledge, this is the first report of somatotrope refractoriness to SS in the GHRH test during fasting, a finding corroborated by the use of SS at doses able fully to counteract the GH rise induced by GHRH (Cella *et al.* 1996a). Irrespective of the underlying mechanism(s) of action, this refractoriness may contribute to GH hypersecretion and the enhanced somatotrope responsiveness to GHRH of fasting mammals (Cella *et al.* 1989, Arce *et al.* 1991) or AN patients (Müller *et al.* 1995).

In agreement with previous data in humans (Arvat *et al.* 1995), the GH-releasing activity of HEXA was partially refractory to modulation by SS in both fed and fasted dogs. In fact, in both conditions only the higher dose of SS reduced the GH response to HEXA.

At present we do not know why, in fasted dogs, the higher SS dose was still capable of reducing the GH response to HEXA, but not that to GHRH. Irrespective of a sound interpretation of this finding, this pattern once again underlines the different mechanisms of actions of the two peptides.

Based on both animal (Casanueva *et al.* 1983, Locatelli *et al.* 1986, Torsello *et al.* 1988) and human studies (Massara *et al.* 1986, Ross *et al.* 1987) it is now a tenet of the GH neuroendocrine control that muscarinic cholinergic agonists and antagonists act to inhibit or stimulate, respectively, hypothalamic somatostatinergic function and, in this way, modulate the action of CNS- or pituitary-acting GH secretagogues. Thus, in humans atropine, a muscarinic cholinergic antagonist, or PZ, a more selective antagonist of muscarinic M1 receptors (Watson *et al.* 1983), were able to suppress the GH release elicited by insulin hypoglycemia (Blackard & Waddell 1969), arginine (Casanueva *et al.* 1984) or GHRH (Massara *et al.* 1984), while conversely, pyridostigmine, an acetylcholinesterase (ACh-E) inhibitor, greatly enhanced the GH release elicited by GHRH (Massara *et al.* 1986, Ross *et al.* 1987). Studies in both animals (Torsello *et al.* 1988) and humans (Ross *et al.* 1987) have also disclosed an important cholinergic link in the feedback action of GH, involving SS release.

However, some observations in the sheep, where a single administration of neostigmine, an ACh-E inhibitor, stimulated GHRH release into hypophyseal portal blood without changes in SS levels (Magnan *et al.* 1993, 1995), challenge the view of a reciprocal and univocal relationship between SS function and the hypothalamic cholinergic system.

Further uncertainties in this context also stem from our study. Although, as already reported in calorie-restricted dogs (Arce *et al.* 1991) and in patients with AN (Tamai *et al.* 1990, Rolla *et al.* 1991), PZ failed to inhibit completely the GHRH-induced rise as it did in fed controls, there was, nevertheless, with PZ in the fasted dogs of our study a consistent suppression of the GHRH-induced GH release. This contrasted sharply with the total

ineffectiveness of a sustained infusion of SS to alter, under the same experimental conditions, the stimulatory effect of GHRH.

These data do not exclude the possibility that PZ may act via endogenous release of the SS-28 from the hypothalamus, which is more effective in portal blood, compared with the SS-14 given peripherally (Srikant & Patel 1982). However, SS and PZ effects were even more divorced in the HEXA study: in contrast to the limited ability of SS to suppress the HEXA-elicited GH rise, PZ vigorously counteracted the GH rise induced by the hexapeptide.

Although PZ may possibly act via stimulation of somatostatinergic function, our findings imply that it may also inhibit GHRH neurones. It is of note in this context that PZ was less effective in inhibiting the GH response to HEXA during fasting or to combined administration of HEXA plus GHRH in the fed and fasting states. In such experimental conditions, GHRH function was either endogenously activated (fasting) or was provided exogenously (HEXA plus GHRH in the fed state).

Consistent with our last results, Peñalva *et al.* (1993) have shown in normal volunteers that atropine was able to prevent the GH responses to GHRH or GHRP-6 alone, but did not completely abolish the GH response to combined administration of GHRH and GHRP-6. Coexistence of Ach and GHRH in nerve terminals of the rat median eminence (Meister 1989) are supportive of our view.

In conclusion, evaluation of the GH-releasing activity of GHRH, HEXA or either peptide in fed and fasted young beagles evidenced profound differences in the way they influence somatotrophic function under physiological conditions or in response to pharmacological stimuli. This has to be considered when devising therapeutic strategies with GHRH alone, HEXA alone or combined peptide administration.

In addition, the different ability of PZ and SS in inhibiting GHRH action challenges the tenet that muscarinic cholinergic compounds modulate exclusively somatostatinergic function; it implies, instead, an action also mediated by GHRH.

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