EXPERIMENTAL STUDY

Growth hormone (GH) rebound rise following somatostatin infusion withdrawal: studies in dogs with the use of GH-releasing hormone and a GH-releasing peptide

Antonello E Rigamonti, Guido Cavallera, Sara Bonomo, Romano Deghenghi1, Vittorio Locatelli, Silvano G Cella and Eugenio E Müller

Department of Medical Pharmacology, Chemotherapy and Toxicology, University of Milan, Milan, Italy, and 1Europeptides, Argenteuil, France

(Correspondence should be addressed to Eugenio E Müller; Department of Medical Pharmacology, Chemotherapy and Toxicology, University of Milan, via Vanvitelli 32, 20129 Milan, Italy; Email: eugenio.muller@unimi.it)

Abstract

Objective: Evidence has been presented that in both animals and humans the rebound secretion of growth hormone (GH) following withdrawal of an infusion of somatostatin (SS) is due to the functional activation of the hypothalamic GH-releasing hormone (GHRH) neurons of the recipient organism. Based on this premise, this study has sought to assess the existence of functional interactions between endogenous GHRH released by a SS infusion withdrawal (SSIW) and growth hormone-releasing peptides (GHRPs), a class of compounds allegedly acting via GHRH.

Methods: Five young dogs (3 to 4 years old, 2 male and 3 female) were administered, on different occasions, three consecutive intravenous bolus of physiological saline (0.1 ml/kg), or GHRH (2 μg/kg), or EP92632 (125 μg/kg), a GHRP compound, or GHRH plus EP92632 at the end of three cycles of 1-h SS infusions (8 μg/(kg×h)) or during a 6-h infusion of saline.

Results: Under saline infusion (SALI), plasma GH levels were unaltered, whereas each SSIW cycle was followed by similar GH secretory episodes. Administration of the first GHRH bolus under SALI induced a rise in plasma GH concentrations slightly higher than that induced by the first cycle of SSIW, but the GH response to the second and third GHRH bolus was similar to that after SSIW. Following SSIW, the response to the first bolus of GHRH was higher than that during SALI, but the second and third cycles of SSIW induced GH responses similar to those evoked by the GHRH bolus. During SALI, administration of the first bolus of EP92632 induced a rise in plasma GH which was higher than that induced by the first GHRH bolus, the second bolus elicited a GH peak of lesser amplitude and there was a partial restoration of the GH response to the third peptide bolus. SSIW strikingly enhanced the GH release to the first EP92632 bolus, a pattern also present, although to a lesser extent, with the second and third cycles of SSIW. Under SALI, combined administration of GHRH and EP92632 had a synergistic effect on GH release, but a progressive reduction was present in the GH response to the second and third GHRH plus EP92632 bolus. SSIW increased only weakly the GH response to the first co-administration of the peptides over that present after administration of EP92632 alone, and did not induce a GH response higher than that present during SALI when the second bolus of the peptides was administered; after the third SSIW a GH rise higher than that present during SALI was elicited by the combined administration of the peptides.

Conclusions: (i) the uniformity of the GH rebound responses to multiple cycles of SSIW may indicate that the latter activate a physiological mechanism which mimics that normally controlling GH pulse generation; (ii) EP92632 elicits, under our experimental conditions, a plasma GH rise higher than that induced by GHRH; (iii) SSIW enhances the GH response to EP92639 alone, to an extent reminiscent of that following combined administration of GHRH and EP92632. This pattern reinforces the view that SSIW elicits release of endogenous GHRH, and infers that the GHRP challenge after SSIW may be exploited in humans to distinguish between healthy and GH-deficient adults.

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Introduction

A great many studies in animals and humans have provided evidence that somatostatin (SS) and growth hormone-releasing hormone (GHRH) are essential regulators of the secretion of growth hormone (GH), and that hypothalamic SS tone dictates the pituitary responsiveness to repeated GHRH challenges (1–4). The GH secretory burst generated appears to be important in enhancing SS release, particularly during...
the trough period encountered in spontaneous GH secretory studies (negative GH auto-feedback) (5–6).

New complexity to our understanding of the neuroregulation of GH has been added by the development of a new class of peptides, the GH-releasing peptides (GHRPs) (7–11). They are potent GH releasers (12), act via specific receptor sites in both the hypothalamus and the pituitary (13–15) (divorced from GHRH receptor sites (16)) and for which an endogenous ligand, Grelin, has recently been identified (17–19). Several lines of evidence have indicated that in vivo and in vitro GH responsiveness to GHRPs is dependent on GHRH function (6, 12), whereas the functional interactions of SS with GHRPs have not been so extensively studied and appear more elusive. Somatostatin could inhibit GH response to GHRP by a hypothalamic (20) as well as a pituitary action (21, 22), and SS and GHRPs could act at either site as mutual functional antagonists (7, 23).

In recent years, studies in animals and humans have provided evidence that the rebound GH rise which follows withdrawal of an infusion of SS is due, at least in part, to the functional activation of GHRH neurons of the recipient organism (24–29). Thus, SS infusion withdrawal (SSIW) may represent a test to probe, inferentially, endogenous GHRH function, and may provide a potential tool in the diagnosis of growth disorders due to GH hyposecretory states (30) or to assess the declining GHRH function in ageing (29).

The rebound GH rise which follows SSIW could be magnified by the administration before SS withdrawal of GHRH (27, 28, 31), implying that the SSIW approach might also be exploited to investigate in vivo the functional interaction between endogenous GHRH and GHRPs.

With this in mind, we studied the functional interactions between endogenous GHRH and GHRPs in the dog, a species which behaves like humans in most aspects of GH neuroregulation (32). Dogs were exposed to three consecutive SSIW cycles and were kept at rest in the laboratory for at least 1 h. Experiments on each dog were scheduled in a randomized order, at least 1 week apart, with continued training.

All the experiments were performed in accordance with protocols previously authorized by the Committee on Animal Care and Use of the University of Milan.

Study design

Following an overnight fast, two indwelling intravenous cannulae were inserted in the forearms at 0830 h (t–30). One cannula was used for slow intravenous infusion of saline or SS (see below), which was commenced at t0, and the other for bolus administration of saline or compounds and for the collection of blood samples. Two groups of studies were performed.

Saline infusion

The aim of these studies was to determine the effect on GH release of three consecutive administrations of saline. GHRH, EP92632 (Ala-His-2Me-Trp-Ala-Trp-D-Phe-Lys-NH2), a member of the GHRP family, or GHRH plus EP92632 during a continuous 6-h saline infusion (SALI). The present unavailability of hexarelin (9) dictated the use of a new GHRP, EP92632, which has a lower efficacy than but a similar potency to hexarelin (E max = 32 ng/ml and ED50 = 145 μg/kg vs E max = 287 ng/ml and ED50 = 169 μg/kg for EP92632 and hexarelin respectively; A E Rigamonti, unpublished results). Saline (4 ml/h) was infused intravenously from t–30 to t160, and a bolus of saline (0.1 ml/kg), GHRH (2 μg/kg; Geref, Serono, Rome, Italy), EP92632 (125 μg/kg; Europeptides, Argenteuil, France) or GHRH plus EP92632 was administered intravenously at t60, t180 and t360 Blood samples for measurement of plasma GH concentrations were collected at t–30, t0, and then at 15- to 30-min intervals up to 360 min.

Somatostatin infusion

The aim of these studies was to determine the effects of three SS infusions withdrawals (SSIWs), consecutively performed, on the GH response to saline, GHRH, EP92632 or GHRH plus EP92632. Somatostatin (8 μg/(kg x h)) was infused intravenously for 60 min, from t0 to t60, from t120 to t240 and from t300 to t360. At the termination of each SS infusion it was replaced by an infusion of saline, and a bolus injection of saline, GHRH, EP92632 or GHRH plus EP92632 was delivered, according to the above reported schedule. Blood samples for measurement of plasma GH were collected at t–30 and at 30-min intervals during SS infusion and at 15-min intervals during SALI up to 360 min.

Materials and methods

Animals

Five young well-trained beagle dogs (3–4 years old, 2 male and 3 female), weighing between 8–15 kg, were used. They were exercised routinely and were fed normal dry food (Diete Standard, Charles River, Calco, Italy) once a day at 1600 h, with water available ad libitum. They were on a 12-h light:12-h darkness regimen, with lights on at 0700 h. At the beginning of the study, body weights of the dogs were stable and they had no observable diseases. All experiments were carried out in conscious animals. Before the experiments, animals were kept at rest in the laboratory for at least 1 h.

Experiments on each dog were scheduled in a randomized order, at least 1 week apart, with continued training.

All the experiments were performed in accordance with protocols previously authorized by the Committee on Animal Care and Use of the University of Milan.
Blood samples were collected in tubes containing 0.15 mol/l EDTA and immediately chilled. Plasma was frozen until assayed for canine GH (cGH) by a double-antibody RIA. Highly purified cGH (Pituitary Hormones and Antisera Center, Torrance, CA, USA), obtained together with the specific antibody anti-cGH through the courtesy of Dr A F Parlow, was used for iodination and as a standard. The sensitivity of the assay was 0.39 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.

**Statistical analysis**

GH values were expressed either as absolute mean±S.E.M. values (ng/ml) (see Figs. 1–4) or as mean±S.E.M. area under the plasma concentration vs time curve (AUC\(_{60-120}\) SALI; AUC\(_{180-240}\) SALI; AUC\(_{100-360}\) SALI for saline infusion studies; AUC\(_{60-120}\) SSIW; AUC\(_{180-240}\) SSIW; AUC\(_{300-360}\) SSIW for SS infusion withdrawal studies; ng/ml/min), calculated by the trapezoidal integration method (see Results and Table 1).

Since no differences in hormone levels between male and female dogs were observed in the different experimental conditions as was the case in other studies (9), the data were pooled.

Statistical evaluation of differences in absolute GH concentrations and mean values of AUCs among the different experimental conditions were performed by the Student-Newman-Keuls test, preceded by one-way ANOVA. \( P < 0.05 \) was taken to be statistically significant.

**Results**

Profiles of mean plasma GH concentrations during the intravenous infusion of saline or SS, and bolus administration of saline or the compounds under study are shown in Figs. 1–4.

Table 1 depicts schematically the extent of the GH responses to three cycles of SSIW or during SALI after application of GH secretagogues. In spite of the variability of standard errors of AUCs (Table 1), the overall results obtained are sound.
Figure 2 Plasma GH concentration profiles (means±S.E.M., ng/ml) from 5 dogs administered three consecutive boli of GHRH (2 μg/kg iv).

Table 1 Areas under the curve (AUC) of plasma GH concentrations versus time (means±S.E.M. ng/ml/min) from 5 dogs administered three consecutive boli of saline (0.1 ml/kg), GHRH (2 μg/kg iv), EP92632 (GHRP) (125 μg/kg iv), and GHRH plus EP92632, during a continuous 6-h saline infusion or three 1-h cycles of SS infusion alternated with saline infusions of the same duration.

<table>
<thead>
<tr>
<th>AUC60–120</th>
<th>AUC180–240</th>
<th>AUC300–360</th>
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<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALI</td>
<td>14.9±8.6</td>
<td>10.9±6.2</td>
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<tr>
<td>SSIW</td>
<td>114.5±14.4</td>
<td>111.7±10.8</td>
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<td>GHRH</td>
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<tr>
<td>SALI</td>
<td>222.4±98.2</td>
<td>81.7±59.5</td>
</tr>
<tr>
<td>SSIW</td>
<td>532.1±100.2</td>
<td>165.8±73.9</td>
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<tr>
<td>GHRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALI</td>
<td>940.6±136.9</td>
<td>418.0±105.2</td>
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<tr>
<td>SSIW</td>
<td>1790.5±162.7</td>
<td>736.4±118.7</td>
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<tr>
<td>GHRH+GHRP</td>
<td></td>
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<tr>
<td>SALI</td>
<td>2363.3±339.8</td>
<td>2210.9±151.6</td>
</tr>
<tr>
<td>SSIW</td>
<td>2946.0±280.0</td>
<td>1934.3±303.8</td>
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a P < 0.01 vs the respective AUC value (in the same cycle and with the same bolus injection) during saline infusion; b P < 0.01 vs the AUC value (in the same cycle) during saline infusion and saline bolus injection; c P < 0.01 vs the AUC value during saline infusion and GHRH bolus injection; d P < 0.01 vs the AUC60–120 value during saline infusion and GHRP bolus injection; e P < 0.01 vs the AUC value during saline infusion and GHRH plus GHRP bolus injection; f P < 0.01 vs the AUC60–120 value during saline infusion and GHRP bolus injection; g P < 0.01 vs the AUC180–240 value during saline infusion and GHRH plus GHRP bolus injection; h P < 0.05 vs the respective AUC value during saline infusion; i P < 0.01 vs the respective AUC value during SS infusion and GHRP bolus injection.
The first SSIW, concomitant with the bolus injection of saline, elicited a clear-cut plasma GH rise, whereas during SALI the same bolus injection failed to increase plasma GH titers (AUC_{60–120; SSIIW} = 114.5 ± 14.4 ng/ml/min vs AUC_{60–120; SALI} = 14.9 ± 8.6 ng/ml/min, P < 0.01). Similar significant GH increments were observed after the second and third SSIWs (AUC_{180–240; SSIIW} = 111.7 ± 10.8 ng/ml/min vs AUC_{180–240; SALI} = 10.9 ± 6.2 ng/ml/min, P < 0.01; AUC_{300–360; SSIIW} = 108.5 ± 12.1 ng/ml/min vs AUC_{300–360; SALI} = 8.4 ± 5.5 ng/ml/min, P < 0.01). No statistically significant differences were found between the plasma GH rebound rises after each SSIW (P = NS) (Fig. 1; Table 1).

No spontaneous GH peak was observed during 6-h SALI after administration of saline boli, a finding in agreement with previous studies (33, 34), since the experiments were performed at times unfavorable for spontaneous generation of GH peaks (35).

Intravenous administration of the first GHRH bolus during SALI induced a rise in plasma GH concentrations higher than that elicited by the bolus injection of saline (AUC_{60–120; SALI} = 222.4 ± 98.2 ng/ml/min vs AUC_{60–120; SSIIW} after saline bolus; P < 0.01). Under the same experimental conditions, the second GHRH bolus elicited a lower GH response (AUC_{180–240; SALI} = 81.7 ± 59.5 ng/ml/min vs AUC_{60–120; SSIIW}, P < 0.01), while the amplitude of the third GHRH-evoked GH response was intermediate between the extent of the first and second GHRH boli (AUC_{300–360; SSIIW} = 532.1 ± 100.2 ng/ml/min vs AUC_{60–120; SSIIW}, P = NS).

SSIW induced a marked increase in the GH response to GHRH (AUC_{60–120; SSIIW} = 165.8 ± 73.9 ng/ml/min vs AUC_{180–240; SSIIW}, P = NS; AUC_{300–360; SSIIW} = 228.7 ± 91.3 ng/ml/min vs AUC_{300–360; SALI}, P = NS) (Fig. 2; Table 1).

EP92632, administered during SALI, induced a rise in plasma GH concentrations which was higher than those occurring after saline or GHRH bolus (AUC_{60–120; SALI} = 940.6 ± 136.9 ng/ml/min vs AUC_{60–120; SSIIW}, P < 0.01, and vs AUC_{60–120; SALI} after GHRH bolus, P < 0.01). The second bolus of the peptide was followed by a GH peak of lesser amplitude (AUC_{180–240; SALI} = 418.0 ± 105.2 ng/ml/min vs AUC_{60–120; SALI}, P < 0.01), whereas there was a partial restoration of the GH response to the third EP92632 bolus which, however, did not reach statistical significance compared with the response to the second bolus (AUC_{300–360; SALI} = 580.3 ± 119.1 ng/ml/min.

Figure 3 Plasma GH concentration profiles (means ± S.E.M., ng/ml) from 5 dogs administered three consecutive boli of EP92632 (GHRP; 125 μg/kg iv).
SSIW at the time of the first EP92632 bolus injection strikingly enhanced the GH release elicited by the peptide (AUC 60–120; SSIW = 1790.5 ± 162.7 ng/ml/min vs AUC 60–120; SALI, P < 0.01). This pattern was also present, although to a lesser extent, with the second and third SSIWs (AUC180–240; SSIW = 736.4 ± 118.7 ng/ml/min vs AUC180–240; SALI, P < 0.01; AUC300–360; SSIW = 890.5 ± 70.8 ng/ml/min vs AUC300–360; SALI, P < 0.05) (Fig. 3, Table 1).

Combined administration of GHRH and EP92632 during SALI induced a synergistic effect on GH release, which overrode any effect of previous GHRH or EP92632 results (AUC60–120; SALI = 2363.3 ± 339.8 ng/ml/min vs AUC60–120; SALI after GHRH, P < 0.01; vs AUC60–120; SALI after EP92632, P < 0.01). A progressive reduction in the extent of the GH response was present after the second and the third GHRH plus EP92632 bolus injections (AUC180–240; SALI = 2210.9 ± 151.6 ng/ml/min vs AUC180–240; SALI, P < 0.01; AUC300–360; SALI = 1353.8 ± 248.4 ng/ml/min vs AUC180–240; SALI, P < 0.01).

The GH response to the first co-administration of the peptides was slightly increased after SSIW (AUC60–120; SSIW = 2946.0 ± 280.0 ng/ml/min vs AUC60–120; SALI, P < 0.05). In contrast, SSIW did not affect the GH rise evoked by the second bolus of GHRH plus EP92632 (AUC180–240; SSIW = 1934.3 ± 303.8 ng/ml/min vs AUC180–240; SALI, P = NS), and a rather similar pattern was present after SSIW in the third GH response, although the difference compared with the results elicited during SALI was significant (AUC300–360; SSIW = 2378.1 ± 534.0 ng/ml/min vs AUC300–360; SALI, P < 0.01) (Fig. 4; Table 1).

No adverse side-effects were recorded during or after SS infusion or administration of GH secretagogues either alone or combined.

**Discussion**

An optimal pulsatile GH release requires the combination of pulsatile GHRH stimulation and a cyclic variation in the SS tone both to prevent leakage of pituitary GH and to maximize the GH pulse that can be secreted in a short time following the next GHRH pulse (28). Reportedly, SSIW either in animals (24–27) or humans (28–30) elicits a rebound GH rise which has been referred to a hypothalamic component, i.e. disinhibition of GHRH neuronal function (2, 4, 24, 26, 27, 29).

In our study, the repetition of SSIW was followed by equivalent GH secretory episodes, suggesting that this mechanism could have activated a physiological
mechanism which mimics that which normally controls GH pulse generation (28, 36). Exposure to three SS cycles of 1-h duration presumably simulates endogenous SS secretion and allows sufficient GHRH to be synthesized at the hypothalamic level. Release of the same GHRH quantum induced by SSIW would be responsible for the uniform episodes of GH secretion detected at each SSIW cycle.

Based on the observation that the second and third GHRH boli delivered during SALI failed to elicit any sound GH rise (Fig. 2) – as already reported in in vitro (37, 38) and in vivo (39, 40) studies – the uniformity of GH release at each SSIW cycle appears to be a very interesting finding.

For GHRH, it has been argued that pituitary desensitization to repeated boli is responsible for the state of refractoriness (41), although depletion of a readily releasable pool sensitive to GHRH cannot be excluded (42). A more likely explanation, however, rests on the induction of a GH negative auto-feedback elicited by the GH rise following the first GHRH bolus. The increase in plasma GH concentrations would enhance hypothalamic SS tone (and probably reduce concomitantly the activity of GHRH-secreting neurons) (43–47), thus blunting the subsequent responsiveness to GHRH. Supporting this proposition is the finding that a cholinergic drug, pyridostigmine, which inhibits SS release (48), given before the second GHRH bolus, reinstates GH responsiveness to the peptide in humans (49, 50).

In the SSIW experiments in which GHRH was administered at the end of the first cycle, the GH rebound was greater than in the saline-GHRH experiments. This was related, presumably, to the activation of GHRH pituitary ‘spare’ receptors (28) rather than to the release of a maximal quantum of endogenous GHRH following SSIW. At the following SSIW cycles, the GH responses to GHRH were blunted and similar to those of the saline-GHRH experiments.

EP92632, a synthetic GHRP heptapeptide, exhibited a GH-releasing activity which was greater than that of GHRH; when combined with GHRH a striking synergistic effect was induced, the GH release following administration of both compounds being higher than the arithmetic sum of the GH rises induced by each compound given separately. Paradoxically, although there were unequivocal GH rises after each bolus of EP92632 alone, the GH-releasing effects of the second and third EP92632 boli were merely blunted, whereas the smaller GH response to GHRH had been nearly abolished (see above). Also, the GH responses to combined administration of EP92632 and GHRH were only slightly attenuated by the previous bolus.

Taken together, these data indicate that EP92632 probably counteracted the negative GH auto-feedback which activates somatostatinergic neurons, a proposition in keeping with the antagonistic action of GHRP on SS function (11, 51, 52).

In addition, inferential evidence has also been presented that GHRPs may play a role in SS action, functioning as SS antagonists at the pituitary level (53). For instance, in conscious rats, continuous subthreshold infusion of GHRP-6, a GHRP compound, together with repeated injections of GHRH, induced GH responses that were uniform and greater in magnitude than those of rats given GHRH alone. Interestingly, interpeak serum GH concentrations were high between repeated GHRH boli, suggesting that GHRP-6 had reduced SS inhibitory influences on the pituitary (52). Also, under our experimental conditions an antagonistic action of EP92632 on SS function at the pituitary level cannot be ruled out.

Regardless of the fact that the validity of these propositions needs to be verified, and in contrast with the scarce reproducibility of the GHRH challenge (54, 55), our study suggests that the repeatability of the GH response to multiple boli of a GHRP may be exploited clinically in GH hyposecretory states.

The most peculiar finding of this study was the clear-cut enhancement of the EP92632-stimulated GH response present after each SSIW cycle. This fact, in view of the known functional interactions between GHRPs and GHRH, reinforces the proposition that SSIW disinhibits hypothalamic GHRH neurons, allowing a synergy of the exogenously administered GHRP with endogenously released GHRH (31, 56). Since, reportedly, GHRPs promptly stimulate hypothalamic GHRH neurons (11, 57, 58), the biological effects of the endogenous GHRH release following SSIW could be further magnified.

In this context, it is noteworthy that co-administration of GHRH and EP92632 at the time of interruption of SS infusion barely increased the extent of the GH response over that following the combined administration of GHRP and GHRH during SALI, which would indicate that endogenous GHRH release triggered by SSIW was nearly maximal.

Recently, Cappa et al. (30) have demonstrated that SSIW elicits a significant GH rise in normal control children (NC), but not in GH deficient children, regardless of the underlying etiology, i.e. GH deficiency (GHD) or GH neurosecretory dysfunction (GHND). This approach allowed complete discrimination of NC from GHD or GHND, but not of GHD from GHND children. Also Popovic et al. (59) have recently shown that combined administration of GHRH plus GHRP-6 only distinguishes healthy from GHD (and GHND) adults.

In patients with hypothalamic–pituitary disconnection, hexarelin, a potent GHRP in both animals and men (9, 60–64), failed to elicit a GH response, whereas it stimulated GH secretion in patients with GHND, with a lower inter- and intra-individual variability than that occurring with GHRH (65, 66).

In view of the results of our study, one wonders whether combined SSIW and GHRP injection may be a valid tool to distinguish GHD of hypothalamic origin.
from a combined hypothalamic/pituitary or a primary pituitary impairment. In fact, in GHD of hypothalamic origin SSIW plus GHRP bolus would induce a GH release intermediate between the one elicited in normal control children and that of children with hypothalamic/pituitary or primary pituitary impairment. Granted that the synergy of SSIW-exogenous GHRP is defective in hypothalamic subjects, GHRP still has the potential to stimulate specific GHRP receptors (13), to inhibit SS function (7, 23) and to act directly at the pituitary level (14, 15). GH release following SSIW and GHRP should be minimal or even absent in subjects with combined hypothalamic/pituitary or primary pituitary impairment.

If the findings of our study could be extrapolated to humans, the GHRP challenge after SSIW, because of its effectiveness and safety, procedural simplicity and economy, might be a useful diagnostic tool in GH-dependent growth disorders.

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