

Recruiting of somatotroph cells after combined somatostatin, GHRH and growth hormone (GH) secretagogue stimulation in a study of pituitary GH reserve in prepuberal female rats

L. Jiménez-Reina¹, E. García-Martínez², J.P. Rojas¹, M.D. Cañete², G. Bernal¹ and R. Cañete²

¹Department of Morphological Sciences, Section of Human Anatomy. School of Medicine, Córdoba, Spain and

²Department of Pediatrics, Pediatric Endocrinology Unit, School of Medicine, Córdoba, Spain

Summary. Diagnostic confirmation of growth hormone (GH) deficiency in children and adults is based on stimulation tests designed to assess the pituitary reserve by measuring the amount of GH released into the bloodstream; however, the results obtained by this means cannot provide any direct indication of the amount of GH actually produced by pituitary somatotroph cells. The present paper sought to test the hypothesis that release of GH following administration of specific stimuli does not accurately reflect the somatotroph cell response, and that the amount of GH released into the bloodstream may often be greater or smaller than the amount synthesized. GH release and changes in the proportion of somatotroph cells were charted in prepuberal female Wistar rats, following administration of several different GH stimuli: GHRH (1 µg/kg), GHRP-6 (1 µg/kg), GHRELIN (1 µg/kg) and combined GHRH-based treatments, with or without SRIH pretreatment (1 µg/kg) 90 minutes earlier. Peak serum GH values were recorded 15 minutes after administration of GHRH+GHRELIN and GHRH+GHRP-6; maximum stimulation in terms of an increased proportion of somatotroph cells occurred 15 minutes after combined administration of GHRH + GHRELIN. SRIH pretreatment (-90 min) inhibited GH release, with a subsequent "escape" and lack of response to stimulation which lasted at least 30 minutes except following administration of GHRH. However, combined administration of GHRH+GHRELIN maintained stimulation of the somatotroph cell population. In conclusion, the results suggest that the enhanced GH release prompted by stimulation tests used to diagnose GH deficiency in prepuberal female rats does not fully

reflect somatotroph cell dynamics, and that not all the GH produced and stored by somatotroph cells is released into the bloodstream.

Key words: GH reserve, GHRH, SRIH, GHRELIN, Somatotroph cells

Introduction

Because serum GH levels vary throughout the day (pulsatile secretion), single random measurements are of limited value in determining even basal GH secretion. Determination of GH secretion is rendered particularly complex in cases of GH deficiency, both in adults and in children.

Two types of GH-stimulation tests are currently in use (Casanueva and Dieguez, 1999; Hoeck et al., 2000): those that explore the pituitary reserve by means of the administration of GHRH, either alone or in combination with GH secretagogues (Gelato et al., 1986; Mericq et al., 1995; Casanueva and Dieguez, 1999; Gasperi et al., 1999) and those which test the integrity of the hypothalamic mechanisms involved in the regulation of GH secretion through administration of substances that reduce somatostatin tone, such as pyridostigmine, arginine, or clonidine (Casanueva and Dieguez, 1999; Hoeck et al., 2000). It is reported that GH values increase following withdrawal of SRIH pretreatment, although pretreatment also temporarily blunts somatotroph responsiveness to further stimulation (Dickerman et al., 1993; Alvarez et al., 2002; DiVito et al., 2002).

Since the discovery of synthetic GH secretagogues, research has addressed their use in the diagnosis of GH deficiency. The combined administration of GHRH and the GH secretagogue GHRP-6 is one of the most potent

stimuli of GH release (Popovic et al., 2000; Leal et al., 2002). Both in animal models and in humans, comparisons of the GH response in healthy and GH-deficient humans indicate no overlap of GH values. Compared to other "gold standard" tests, the GHRH + GHRP-6 test affords greater reproducibility and reliability in stimulating GH release (Popovic et al., 2004). In this respect, the potential role of GHRELIN in combined stimulation has yet to be clarified (Kojima et al., 2001).

In any case, all pituitary reserve tests for clinical diagnosis of GH deficiency must refer to serum GH values, whose normal ranges in children and adults are well established (Shlalet et al., 1998); they cannot provide a direct indication of the amount of GH produced by somatotroph cells.

In previous papers (Jiménez-Reina et al., 2000, 2002) the authors addressed the heterogeneity of the somatotroph cell population, finding no apparent link between GH release and morphological changes in somatotroph cells. The purpose of the present study was to chart changes in the proportion of somatotroph cells in prepuberal female rats following GH stimulation tests similar to those performed when testing for GH deficiency, with a view to identifying a possible relationship between GH produced (expressed as the proportion of immunoreactive somatotroph cells), and GH released into the bloodstream.

Materials and methods

Animals

Twenty-six to twenty-eight-day-old female Wistar rats were used. Animals were given free access to rat chow (IPM R-20, Letica S.A., Hospitalet, Barcelona, Spain) and tap water. They were housed and kept under conventional conditions (temperature: $22\pm2^{\circ}\text{C}$ 12:12 h light/dark cycle with lights on at 06.30) in the animal laboratory center at the School of Medicine in Córdoba (Spain). The rats were cared for, and used, in accordance with European Council directive 86/609/ EEC (24/11/1987).

Experiment design

The rats were divided into two groups: a saline-pretreated group (saline group), and the SRIH-pretreated group (SRIH group). In the SRIH group, animals were injected intraperitoneally with 1 $\mu\text{g}/\text{kg}$ body weight of somatostatin (Serono Laboratories, Madrid, Spain) ninety minutes before stimulus. In the saline group, animals were injected intraperitoneally with saline serum ninety minutes before stimulus. Stimuli were GHRH, GHRP-6, GHRELIN, GHRH+GHRP-6 and GHRH+GHRELIN at 1 $\mu\text{g}/\text{kg}$ body weight in all cases. Trunk blood samples were taken on decapitation at -90', -15, 0, 15, 30 and 90 minutes for GH determination; for immunocytochemistry, pituitaries were removed and

posterior pituitaries were discarded.

Immunocytochemistry

Pituitaries were fixed with Bouin's solution for 24 hours and subsequently embedded in paraffin; sections 7-9 μm thick were immunostained for GH, using the extravidin-peroxidase method (EXTRA-3, Sigma Chemical Co). Anti-rat GH rabbit serum (Biogenesis LTD, 1:1000), was used. Endogenous peroxidase was blocked with H_2O_2 (3%). For washing and dilution of sera, phosphate buffer (PBS) was used. The reaction was developed in freshly-prepared 3,3'-diaminobenzidine (Sigma, 0.025% in PBS buffer containing 0.03% H_2O_2). The immunoreaction specificity for rat GH was measured by omission of the specific antiserum, replacing the antiserum with normal rabbit serum, and preadsorption of the specific antiserum with its homologous (rat GH) or heterologous (rat prolactin) hormone. The percentage of somatotroph cells and the proportion of strongly/weakly immunostaining somatotroph cells was then calculated on each slide (5 sections per pituitary and around 200 cells per section), using an eyepiece grid measuring 10 x 10 mm and 1.0 mm pitch on a Nikon microscope at 400 x.

GH Radioimmunoassay

Serum GH concentrations were measured by double-antibody RIA using NIDDK kits, as described previously (Jiménez-Reina et al., 2000). All samples from each experiment were measured in the same assay, and GH values were expressed in ng/ml.

Statistical analysis

Results were expressed as a mean \pm standard error of the mean (SEM). Three animals were used for each stimulus and each administration time, in both the SRIH and the saline group. Experiments were repeated three times. The statistical significance of inter-group differences was determined by ANOVA, and was accepted at $P<0.05$. The Holm-Sidak method was used after ANOVA to test for intra-group differences.

Results

GH release

A) Saline group

There was no change in serum GH levels between -90' and 0' (6.22 ± 0.53 vs 5.91 ± 0.34 ng/ml). Following stimulus (time 0') with GHRP6, GHRELIN, GHRH+GHRP6 y GHRH+GHRELIN, peak GH values were reached after 15 minutes (Fig. 1), the most potent stimuli being GHRH+GHRELIN and GHRH+GHRP6 (42.44 ± 2.41 and 38.29 ± 2.72 vs 5.91 ± 0.34 ng/ml at 0'; $P<0.0001$). With GHRH, the peak GH response was

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recorded after 30 minutes (20.08 ± 1.25 vs 5.91 ± 0.34 ng/ml; $P < 0.0001$) (Fig. 1). At 90 minutes post-stimulation with GHRH, GHRP6 and GHRELIN, serum GH levels were higher than at 0 minutes ($P < 0.05$); this was not the case following combined stimulation with GHRH+GHRP6 or GHRH+GHRELIN (Fig. 1). The area under curve (AUC) for GH released into the bloodstream was greater with all stimuli than with saline (Fig. 2); moreover, the AUC for combined stimulation with GHRH+GHRP6 and with GHRH+GHRELIN was

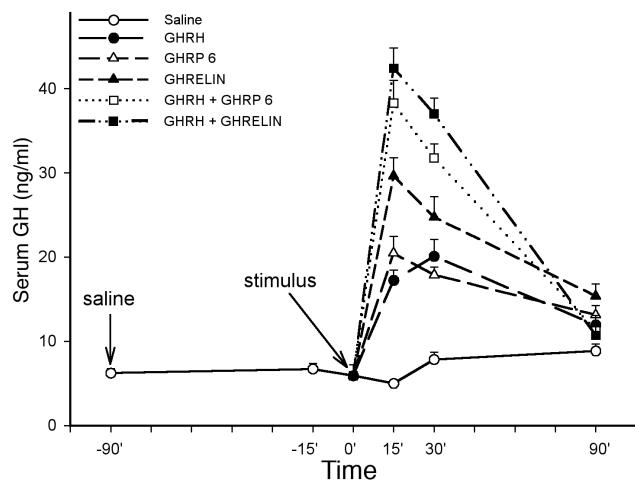


Fig. 1. Serum GH release in prepuberal female rats pretreated with saline at -90 minutes. Stimulation administered at 0 minutes. By 15 and 30 minutes, GH release was greater for all stimuli than for saline at 0 minutes ($P < 0.05$).

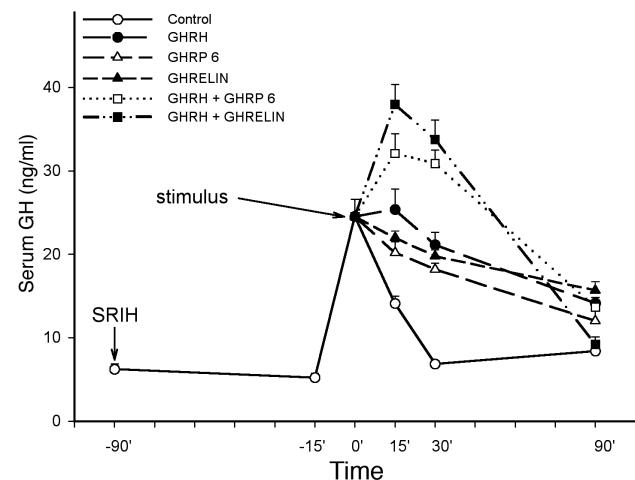


Fig. 3. Serum GH release in prepuberal female rats pretreated with SRIH at -90 minutes. Stimulation administered at 0 minutes. By 15 and 30 minutes only GHRH+GHRELIN and GHRH+GHRP6 stimulated GH release versus saline at 0 minutes ($P < 0.05$).

greater than that recorded for any of these stimuli alone (Fig. 2).

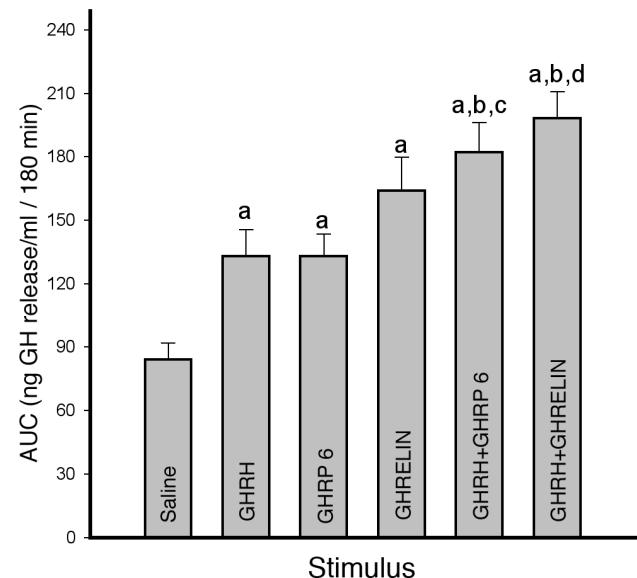


Fig. 2. Area under curve (AUC) for serum GH release over 180 minutes in prepuberal female rats pretreated with saline. Each bar indicates the stimulus administered at 0 minutes. a: $P < 0.05$ vs saline; b: $P < 0.05$ vs GHRH; c: $P < 0.05$ vs GHRP6; d: $P < 0.05$ vs GHRELIN.

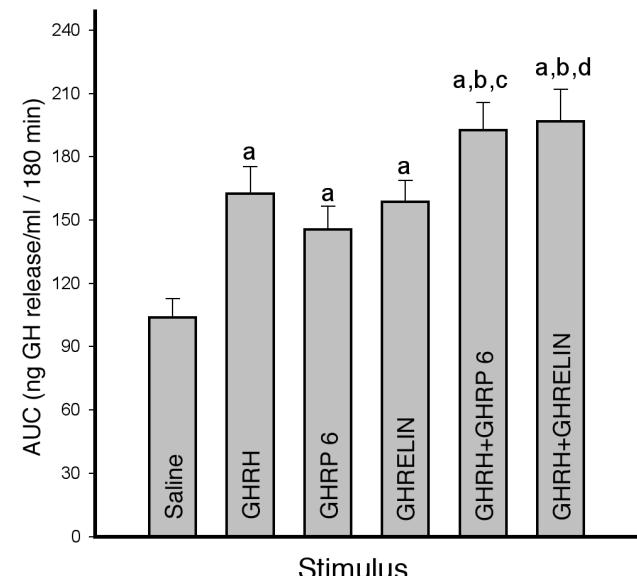


Fig. 4. Area under curve (AUC) for serum GH release over 180 minutes in prepuberal female rats pretreated with SRIH. Each bar indicates the stimulus administered at 0 minutes. a: $P < 0.05$ vs saline; b: $P < 0.05$ vs GHRH; c: $P < 0.05$ vs GHRP6; d: $P < 0.05$ vs GHRELIN.

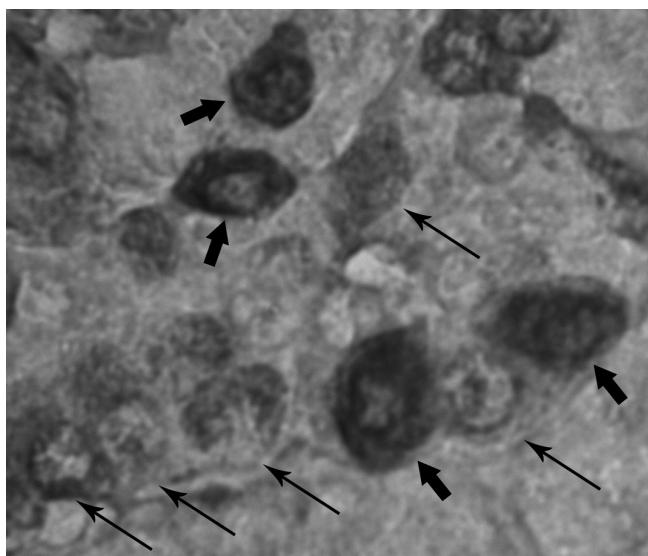


Fig. 5. GH-immunostained cells from female prepuberal rat pituitary. Staining was classed as either strong (fat arrows) or weak (thin arrows). $\times 900$

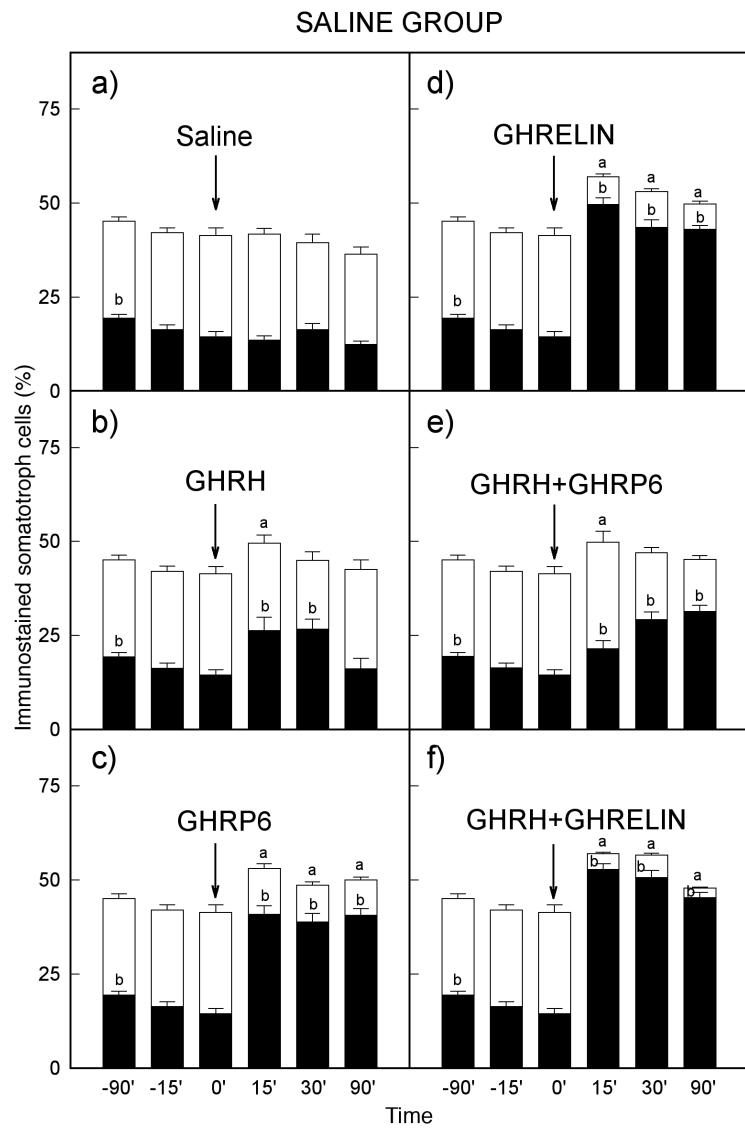


Fig. 6. Immunostained somatotroph cells (ISC) (% total pituitary) in female prepuberal rats pretreated with saline at -90 minutes. Stacked bars show the proportion of weakly-staining (white bars) and strongly ISC (black bars) for each respective bar. **a), b), c), d), e), f)** show changes in ISC proportions following different stimuli administered at 0 minutes. a: $P < 0.05$ vs ISC at 0 minutes; b: $P < 0.05$ vs strongly ISC at 0 minutes.

B) SRIH group

Administration of SRIH at -90 minutes prompted a GH release peak at 0 minutes (24.54 ± 2.08 ng/ml) (Fig. 3), the time at which stimulus was administered using saline, GHRH, GHRP6, GHRELIN, GHRH+GHRP6 and GHRH+GHRELIN. Only GHRH+GHRP6 and GHRH+GHRELIN were able to increase serum GH levels at 15 minutes (32.11 ± 2.36 and 37.98 ± 2.39 ,

respectively, vs 24.54 ± 2.08 ng/ml at 0 minutes; $P < 0.05$), and keep them high until 30 minutes (Fig. 3); the remaining stimuli prompted a drop in GH release (Fig. 3). At 90 minutes, serum GH levels were similar to those recorded following saline administration (Fig. 3). The AUC for GH released into the bloodstream was greater for all stimuli than for saline (Fig. 4). Moreover, the AUC for combined stimulation with GHRH+GHRP6 and with GHRH+GHRELIN was greater than that

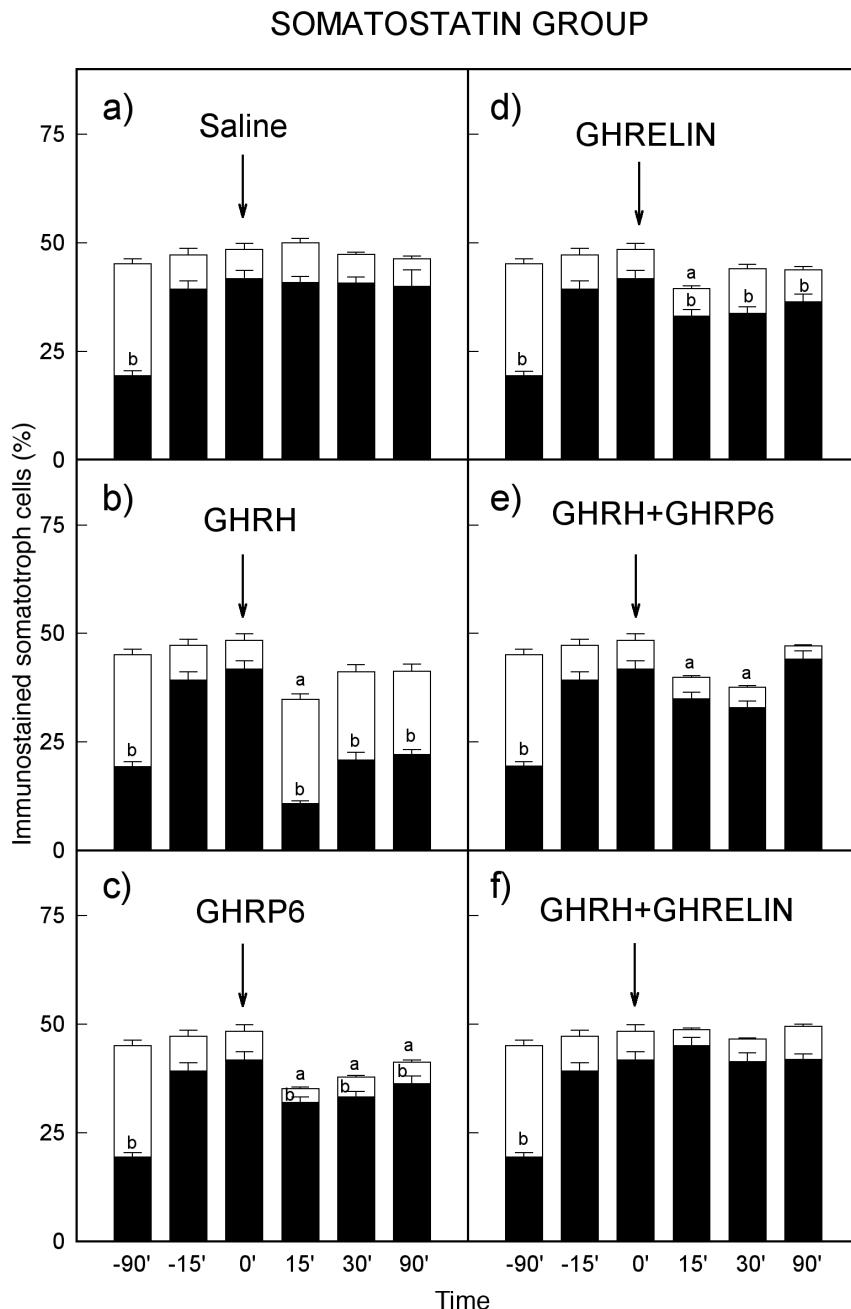


Fig. 7. Immunostained somatotroph cells (ISC) (% total pituitary cells) in female prepuberal rats pretreated with SRIH at -90 minutes. Stacked bars show the proportion of weakly (white bars) and strongly ISC (black bars) for each respective bar. **a), b), c), d), e), f)** show changes in ISC proportions following different stimuli administered at 0 minutes. a: $P < 0.05$ vs ISC at 0 minutes; b: $P < 0.05$ vs strongly ISC at 0 minutes.

recorded for any of these stimuli alone (Fig. 4).

Immunostained somatotroph cells (ISC)

Immunocytochemical analysis revealed somatotroph cells as either strongly or weakly immunostained (Fig. 5).

A) Saline group

Following saline treatment, the proportion of ISC remained virtually constant throughout the study (Fig. 6a). Stimulus with GHRH, GHRP6, GHRELIN, GHRH+GHRP6 and GHRH+GHRELIN prompted varying degrees of change in ISC proportions (Fig. 6). GHRH and GHRH+GHRP6 increased the percentage of ISC only after 15 minutes post-stimulus (Fig. 6b,e), whilst GHRP6, GHRELIN and GHRH+GHRELIN increased the percentage of ISC from 15 to 90 minutes post-stimulus (Fig. 6c,d,f); GHRELIN and GHRH+GHRELIN prompted the highest percentage of ISC at 15 minutes ($\approx 57\%$ of pituitary cells). GHRP6, GHRELIN and GHRH+GHRELIN prompted a marked increase in the percentage of strongly ISC at 15 and 30 minutes (Fig. 6c,d,f); GHRH alone prompted a more moderate increase (Fig. 6b).

B) SRIH group

Following SRIH treatment, the proportion of ISC remained virtually constant throughout the study, although the proportion of strongly ISC rose significantly from -15 minutes onwards (Fig. 7a). Stimulus with GHRH, GHRP6, GHRELIN, GHRH+GHRP6 and GHRH+GHRELIN prompted varying degrees of change in the proportion of ISC (Fig. 7). GHRH and GHRELIN caused a drop in the percentage of ISC only at 15 minutes (Fig. 7b,d), whilst GHRH+GHRELIN caused a drop at 30 minutes (Fig. 7f), GHRH+GHRP6 prompted a decrease in ISC percentage at both 15 and 30 minutes (Fig. 7e), and GHRP6 prompted a drop at 90 minutes post-stimulus (Fig. 7c). Only GHRH (Fig. 7b) was able to reduce the percentage of strongly ISC produced by pretreatment with SRIH; stimulus with GHRP6, GHRELIN or a combination of the two failed to modify the percentage of strongly ISC (Fig. 7c-f).

Discussion

Results showed that: 1) combined stimulus with GHRH+GHRP6 and with GHRH+GHRELIN had the most potent effect on GH release in prepuberal female rats; 2) pretreatment with SRIH prompted an "escape" of GH release ninety minutes after administration; at this time-point, GH release following stimulus with the combinations GHRH+GHRP6 and GHRH+GHRELIN was less marked than in the absence of SRIH pretreatment; 3) the proportion of somatotroph cells was

increased by administration of GHRH, GHRP6 and GHRELIN, either alone or in combination, due mainly to an increased proportion of strongly ISC; and 4) SRIH pretreatment provided the greatest stimulus for increasing the proportion of strongly immunostained somatotroph cells without modifying the overall percentage of GH cells.

In children, the use of GH stimulation tests based on administration of GHRH in combination with various products for the diagnosis of GH deficiency (GHD) has been widely questioned due to the poor reproducibility of results (Badaru and Wilson, 2004). However, combined stimulation with GHRH+GHRP6 for the diagnosis of GHD in adults appears to be highly reproducible (Popovic et al., 2000), a single fixed GH determination after stimulus being sufficient for GHD diagnosis (Leal et al., 2002). Due to the possible adverse effects of combined stimulation with GHRH+GHRP6, this diagnostic test should not be performed in children (Saggese et al., 1998) and therefore no data are available regarding its value. The present study sought to chart the dynamics of the GH response, as a reflection of the pituitary GH reserve, following stimulation with a combination of GHRH and GH secretagogues in prepuberal rats, and to investigate the relationship between GH production and GH release, mainly by recording the dynamics of the somatotroph cell population and their correlation with serum GH levels following stimulus.

Pretreatment with SRIH has long been known to enhance the somatotroph response to GHRH (Dickerman et al., 1993), following either single administration (Tzanelia et al., 1996) or withdrawal of continuous SRIH infusion (Cappa et al., 1999; Rigamonti et al., 2002). GH release and somatotroph cell dynamics were therefore studied with and without SRIH pretreatment. In the group pretreated with saline, both GHRH and GH secretagogues stimulated GH release, GHRELIN displaying a more marked effect than GHRH (Arvat et al., 2001). Combined stimuli act synergically (Arvat et al., 2001), as is apparent from the AUC for GH release over 180 minutes. Pretreatment with SRIH prompted an "escape" of GH release ninety minutes after administration, and only stimulus with GHRH+GHRP6 or GHRH+GHRELIN – in combination, though not alone (Tzanelia et al., 1996) – succeeded in increasing GH release. In the SRIH-pretreated group, the total amount of GH released following administration of GHRH and GHRP6 was greater than in the saline-treated group, indicating that SRIH pretreatment is a predisposing factor for increased GH release (Dickerman et al., 1993; Tzanelia et al., 1996).

Stimulation with GHRH or GH secretagogues increased the percentage of somatotroph cells in all cases, either by increasing intracellular GH content (Jiménez-Reina et al., 2000, 2002) or by prompting the recruitment of initially inactive cells (Norris et al., 2003). GH secretagogues increased the proportion of strongly ISC, while GHRH barely modified the

proportion, probably because they act on different receptors (Chen et al., 1996); GH secretagogues stimulate both the synthesis and the release of GH, while GHRH stimulates GH release more consistently than GH synthesis (Teill and Karin, 1993; Cooke and Liehaber, 1995). After SRIH pretreatment, however, stimulus of GH secretion caused a decrease in the percentage of somatotroph cells, although in most cases these became strongly ISC, except following GHRH stimulus, which permitted greater hormone release and thus an increase in weakly ISC.

The correlation between GH release and somatotroph cell dynamics after stimulation using GHRH and GH secretagogues, with or without SRIH pretreatment, suggests that despite the hypothetical benefit for peak hormone release achieved by temporary inhibition of somatotroph cells, the overall amount of GH released following subsequent stimulation was in no case greater than that obtained without SRIH pretreatment. In almost all cases there was an increase in storage of GH (i.e. increase in strongly ISC), but not in release of GH into the bloodstream, probably because the endogenous rhythmic pattern of GH production was not modified; somatotroph cells there become inactive and temporarily fail to respond to stimulation (McFerran et al., 2001; Lee et al., 2004).

It is thus felt that studies of the pituitary GH reserve provide information on only part of the GH produced (the released part), and that the somatotroph cell is able to synthesize and store a larger amount of GH, which is only available for future stimulations.

In conclusion, the results obtained suggest that after pretreatment with SRIH, the combined administration of GHRH and GH secretagogues is the most effective way of increasing the proportion of strongly immunostaining somatotroph cells, without increasing the overall proportion of ISC, thus increasing GH synthesis. Further research into somatotroph cell transcription patterns may allow some manipulation of endogenous patterns in GH-deficient patients, in order to obtain all the GH synthesized and make it available to cover the body's requirements.

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