SUMMARY

The pentapeptide Ipamorelin stimulates growth hormone (GH) release in pigs, sheep, dogs, rats and humans. In order to determine the effects of chronic treatment with Ipamorelin on the in vitro GH response, Ipamorelin was administered daily for 21 days to 60-day old female rats and the effects compared with those of growth hormone-releasing hormone (GHRH) (10 µg/kg body weight). Daily monitoring revealed a higher % weight gain in the Ipamorelin-pretreated group (IPG) and the GHRH-pretreated group (GPG) than in the vehicle-pretreated group (VPG). In 3-day pituitary cell monolayer cultures, basal GH release was greater in IPG and GPG than in VPG. After 3-day culture, basal GH release from individual cells was lower in IPG than in VPG and GPG. The intracellular GH content was lower in IPG and GPG than in VPG (P<0.05). In 3-day monolayer cultures, Ipamorelin (10^{-8} M) and GHRH (10^{-8} M) increased GH release in VPG and IPG, but not in GPG. Similarly, Ipamorelin (10^{-8} M) and GHRH (10^{-8} M) stimulated individual GH release in VPG and IPG. In conclusion, these data show that chronic administration of Ipamorelin to young female rats is effective in increasing body weight gain and enhancing in vitro basal and Ipamorelin or GHRH-stimulated GH release. In the same conditions, GHRH increases body weight gain and maintains basal GH at levels similar to those produced by Ipamorelin, but without prompting any in vitro enhancement of the GH response to subsequent stimuli. These results suggest that Ipamorelin does not lead to desensitization of the GH response in young female rats, whilst GHRH produces desensitization of GH release in vitro.

Key Words: Growth hormone secretagogues –Ipamorelin – Individual growth hormone release in vitro – Image analysis

INTRODUCTION

Over recent years, a new series of growth hormone (GH)-releasing peptide (GHRP) receptor-active GH secretagogues (GHS) have been developed (Raun et al., 1998); one of the prototypes is the pentapeptide Ipamorelin, which has been shown to be a very potent and efficacious GH secretagogue, with no apparent collateral effects on the release of other pituitary hormones. Ipamorelin also induces longitudinal bone growth and an increase in body weight (Johansen et al., 1999; Svensson et al., 2000).

In 1996, a G-protein-linked receptor cloned in the pituitary and hypothalamus was shown to be a target of GHS (Howard et al., 1996; Pong et al., 1996); an endogenous ligand specific to the GHS
receptor was later identified (Kojima et al., 1999). Ipamorelin acts in a similar way to GHRP6 through a GHS-type receptor; since GHRP 6- and Ipamorelin-induced GH release is inhibited to similar extents by GHS receptor antagonists (Raun et al., 1998). As in the case of other GHRPs, chronic treatment with Ipamorelin seems to induce partial desensitization of the GHS receptor (Johansen et al., 1999; Raun and Von Voss, 1998), thus attenuating GH response. GHS-induced GH release is due to the direct stimulation of somatotroph cells and to indirect stimulation through the hypothalamus, which at least partially involves growth hormone-releasing hormone (GHRH) neurons (Argente et al., 1996; Dickson et al., 1999; Ghigo et al., 1997; Giustina and Veldhuis, 1998; Korbonits et al., 1999a; Korbonits et al., 1999b; Laron, 1995; Raun et al., 1998; Smith et al., 1998). Therefore, it may be hypothesized that if Ipamorelin reduces GH response, this may be the result of desensitization of the GHS receptor at pituitary and/or hypothalamic level.

Currently, the potential therapeutic properties of GHRPs in patients with intact hypothalamic function are currently being studied. These GHRPs act directly on the hypothalamus and pituitary, prompting GH release; they will therefore not bypass the feedback loop controlling the GH/IGF-1 axis (Peino et al., 1999; Micic et al., 1998) and the rats were weighed daily. They were divided into three groups (n=6): the Ipamorelin-pretreated group (IPG); the GHRH-pretreated group (GPG); and the vehicle-pretreated group (VPG). In the Ipamorelin-pretreated group, animals were injected subcutaneously (sc) daily at 9 am with 100 µg/kg body weight of the diacetate salt of Ipamorelin (Novo-Nordisk, Denmark). In the GHRH-pretreated group animals were injected sc daily at 9 am with 10 µg/kg body weight of GHRH1-29 (Serono Labs., Spain). The Vehicle-pretreated group underwent the same manipulation as the above groups, but received saline serum instead of Ipamorelin or GHRH.

After the last injection, animals were decapitated at 1 pm and the pituitaries were removed. The posterior pituitaries were discarded, and the anterior pituitaries were diced into small pieces for cell dispersion.

Cell dispersion and cell culture

Pituitary cell cultures were prepared according to Jiménez-Reina et al. (2000). Briefly, pituitaries were diced into small pieces and dispersed in Erlenmeyer flasks with a mixture of 0.02 g/l collagenase (Type V, Sigma Chemical Co.) and 0.01 g/l trypsin 1:250 (Sigma Chemical Co.) in Dulbecco Modified Eagle’s Medium (DMEM) (Sigma Chemical Co.). The Erlenmeyer flask was placed in a humidified atmosphere of 5% CO₂ in air, at 37°C, for 60 minutes. The cellular suspension obtained was gently pipetted for ten minutes, centrifuged (100 g for 10 min.) and washed in DMEM, twice. The cellular pellet was washed again, centrifuged, and resuspended in 10% fetal bovine serum (FBS)-DMEM. Cell yield and viability were checked by the trypan blue exclusion method: cell yields were 1.64 ± 0.12 x 10⁶/pituitary, and cell viability was over 90% in all cell dispersions. The cellular suspension was dispensed into tissue cultures consisting of 96 wells (2 x 10⁴ cells/well/200µl 10% FBS-DMEM) and incubated in a humidified atmosphere of 5% CO₂ in air, at 37°C, for 3 days. Monolayer cultures were washed twice with DMEM and fresh serum-free DMEM was added. After a 4 hour incubation at 37°C and 5% CO₂, the medium was decanted and stored at -20°C.

MATERIAL AND METHODS

Animals

Sixty-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 individually and kept under conventional conditions (temperature: 22±2°C 12:12 h light/dark cycle with lights on at 06.30) in the laboratory animal center of the School of Medicine in Córdoba. The rats were cared for and used in accordance with European Council directive 86/609/ EEC (24/11/1987).

Experiment Design

The duration of the experiment was 21 days and the rats were weighed daily. They were divided into three groups (n=6): the Ipamorelin-pretreated group (IPG); the GHRH-pretreated group (GPG); and the vehicle-pretreated group (VPG). In the Ipamorelin-pretreated group, animals were injected subcutaneously (sc) daily at 9 am with 100 µg/kg body weight of the diacetate salt of Ipamorelin (Novo-Nordisk, Denmark). In the GHRH-pretreated group animals were injected sc daily at 9 am with 10 µg/kg body weight of GHRH1-29 (Serono Labs., Spain). The Vehicle-pretreated group underwent the same manipulation as the above groups, but received saline serum instead of Ipamorelin or GHRH.

After the last injection, animals were decapitated at 1 pm and the pituitaries were removed. The posterior pituitaries were discarded, and the anterior pituitaries were diced into small pieces for cell dispersion.
GH Radioimmunoassay

GH concentrations in culture media were measured in a 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. Using this method, the intra and inter-assay coefficients of variation were 7% and 12% respectively.

Cell immunoblot assay (CIBA)

Pieces (1.5 x 1.5 cm) of polyvinylidene difluoride transfer membrane (IMMOBILON™ Millipore) were placed in multiwell plates. Three-day monolayer cell cultures were incubated with 0.01 g/l trypsin 1:250 (Sigma Chemical Co.) 95% air-5% CO₂ at 37°C for 30 minutes in order to obtain pituitary cell suspensions, which were placed on membranes (4x10⁶ cells/50µl DMEM) and preincubated at 37°C in 95% air-5% CO₂ for 30 minutes. Then, 50 µl DMEM either alone, or with Ipamorelin (10⁻⁸ M) or with GHRH (10⁻⁸ M), was added and incubated for 4 hours. Thereafter, the transfer membranes were fixed with Bouin fluid for 30 minutes, and then immediately washed three times with PBS.

The standard curve and the estimated amount of GH released from each isolated somatotroph cell were recorded following Dobado-Berrios et al. (1992). Briefly, various concentrations of purified rat GH (GH-RP-2, NIADK-NIH) dissolved in bicarbonate buffer-NaCl (1µl drops), containing between 0.78 ng and 25 ng of rat GH (increase x2), were fixed on IMMOBILON™ membranes.

Transfer membranes were immunostained using the extravidin-peroxidase method (EXTRA-3, Sigma Chemical Co) modified from the method described by Kendall and Hymer (1987). Anti-rat GH serum (Biogenesis Ltd.) was used at a final dilution of 1:1000. The specificity of CIBA for rat GH was examined by removing the antiserum, replacing the antiserum with normal rabbit serum, and preabsorbing the antiserum with rat GH (Biogenesis Ltd.) at 4°C for 24 hours. The crossreactivity of the primary antiserum with prolactin was also checked by incubating in the presence of anti-rat GH IMMOBILON™ membranes in which 1 µl droplets contained 25 ng/µl of purified rat prolactin.

Image analysis

To ascertain possible changes in GH-immunostained cell proportions in transfer membrane cultures, a count of secreting and non-secreting GH cells (Fig. 1) was performed on the central areas of each membrane, using a gridded eyepiece.

Image analysis was performed as described previously (Jiménez-Reina et al., 2000; Dobado-Berrios et al., 1992). GH-immunostained cell blots were measured using a conventional Nikon microscope equipped with a light source stabilized at 5 volts and connected via a Hitachi television camera to an image-analysis system consisting of a computer (Pentium/Intel) with a digital card equipped with VISILOG 4.1 software (Noesis, Orsay, France) belonging to the University of Córdoba Cell Biology Department, an additional monitor (Fujitsu, Japan), and a digital board (KURTA). In each membrane, the halo secretion area, the cellular area (in µm²) and optical density of both areas (OD, in arbitrary units) of 60 GH-immunostained cell blots were measured. Measurements of standard intensities define a variable that expresses the amount of hormone immobilized per unit area or immobilization density (pg/µm²) (Dobado-Berrios et al., 1992); this enabled determination of the amount of GH released by or the GH content (in terms of pg/cell) of each somatotroph cell.

Statistical analysis

Results were expressed as means ± standard errors of the means (SEM). A minimum of six animals was used in each experimental and control group. Experiments were repeated three times with different groups of animals. One way repeated measures ANOVA was used to analyze weight gain curves. The statistical significance of inter-group differences was determined by ANOVA, and was accepted at P<0.05. The Student-Newman-Keuls test was used after ANOVA. When the normalized test failed, the Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney Rank Sum Test, were used.

RESULTS

Body weight

Figure 2 shows the percentage gain in body weight (BW) with respect to the start of treatment of every group. The effect of Ipamorelin and GHRH on BW gain was immediately appa-
rent and was more marked than in the vehicle pretreated group (VPG) (p<0.05); this difference was maintained up until day 21 (p<0.05). The greatest gain in BW due to Ipamorelin and GHRH (with respect saline serum) occurred during the first week of treatment (figure 2); BW in the second and third weeks was similar for all three groups studied (VPG, IPG and GPG).  

Monolayer culture
Cumulative basal GH release from pituitary cells in monolayer culture after 3 days was greater in IPG and GPG than in VPG (3720±251 and 3868±231 vs 2316±134 ng/ml; p<0.05 and p<0.001, respectively) (Fig. 3A). No difference was recorded for GH release between IPG and GPG.

In the three-day monolayer cultures, basal GH release over 4 hours in fresh medium (Fig. 3B) was similar for the three groups studied (VPG: 141.2±12.64; IPG: 131.57±8.74; and GPG: 141.8±8.63 ng/ml) whilst Ipamorelin (10⁻⁸M) and GHRH (10⁻⁸M) stimulated GH release in VPG (141.2±12.6 -control- vs 189.72±18.1 -Ipamorelin- p<0.05; and 255.68±16.04-GHRH-p<0.001; ng/ml) and in IPG (131.58±18.74 -control- vs 243±23.9 -Ipamorelin- and 427.1±42.8-GHRH- p<0.001; ng/ml) (Fig. 3B). In the GPG group, neither Ipamorelin nor GHRH stimulated GH release (Fig. 3B).

Cell-immunoblot assay (CIBA)
After three days in monolayer culture, individual basal GH release over 4 hours on IMMÔBILÔN™ membranes was lower in IPG than in VPG and GPG (11.2±2.58 vs 15.82±1.92 and 16.2±2.38 pg GH/cell, respectively; p<0.05) (Fig. 4A). In these conditions, Ipamorelin (10⁻⁸M) caused an increase in individual GH release (Fig. 5) in both VPG and IPG (p<0.05), but not in GPG. Similarly, GHRH (10⁻¹⁸M) stimulated individual GH release in VPG and IPG (p<0.05), but not in GPG (Fig. 4A).

The basal intracellular GH content in somatotroph cells over 4 hours in transfer membranes was lower in IPG and GPG than in VPG (6.05±0.76 and 5.75±0.63 vs 10.4±1.23 pg GH/cell; respectively; p<0.05) (Fig. 4B). In these conditions, Ipamorelin (10⁻⁸M) and GHRH (10⁻¹⁸M) only increased intracellular GH contents in IPG (6.05±0.76 -control- vs 9.41±1.15 -Ipamorelin- and 10.99±1.13 -GHRH- pg GH/cell; p<0.05) (Fig. 4B).

Individual basal GH release was heterogeneous. Roughly 90% of somatotrophs in VPG released between 0 and 25 pg GH/cell, whilst 7% released more than 40 pg/cell and 10%
released around 75 pg GH/cell (Fig. 6A). In IPG, almost 14% of somatotroph cells released more than 40 pg GH/cell (Fig. 6B). In GPG, almost 25% of somatotroph cells released between 25 and 35 pg GH/cell (Fig. 6C). With regard to intracellular GH contents, in VPG almost 7% of somatotroph cells contained between 30 and 40 pg GH/cell and the remainder (93%) contained less than 20 pg GH/cell (Fig. 6D). In IPG and GPG no somatotroph cell contained more than 20 pg GH/cell (Fig. 6E and 6F).

**DISCUSSION**

This study examined the effects of chronic treatment (21 days) with Ipamorelin or GHRH on body weight and basal and stimulated GH release in vitro in young female rats. The results obtained showed that both Ipamorelin and GHRH prompted an increase in body weight and stimulated basal GH release in vitro. GH stimulation by Ipamorelin (10^{-8} M) and GHRH (10^{-8} M) in vitro (overall and individual) was effective in the group pretreated with saline and in the group pretreated with Ipamorelin, but not in animals pretreated with GHRH.

Rats receiving Ipamorelin displayed significant increases in BW with respect to the group receiving only saline serum. An increase in BW was also observed in rats receiving 10 µg/Kg/day GHRH. The increase in BW was lower than that reported in other Ipamorelin (Johansen et al., 1999) studies using the orally-administrated derivative NN703 (Hansen et al., 1999) or G-7039, a GH secretagogue from the Genentech group (Clark, 1999). This was probably because the present experiment used young female rats, which grow faster than the adult rats used in the above studies (Johansen et al., 1999; Raun and Von Voss, 1998; Hansen et al., 1999). Recent reports have suggested that the increase in BW prompted by GH secretagogues may be due to greater food intake, with no increase in longitudinal bone growth (Lall et al., 2001). The data obtained here show that body weight gain in young female rats took place primarily during the first week of treatment with Ipamorelin or GHRH (almost 75% of total gain). We therefore believe that weight gain would be due not to increased food intake, in which case weight gain would have been constant throughout treatment, but rather to the effect of Ipamorelin on GH secretion. No data are available to show why body weight gain was limited to the first week of treatment, although it is likely that the discontinuous administration of Ipamorelin for 21 days, instead of continuous infusion, served to limit the GH response (Huhn et al., 1993).

The GH response was investigated at the end of the study using two in vitro systems to assess GH release in monolayer cultures and individual GH release using the cell-immunoblot assay method. In vivo treatment with GHRH or Ipamorelin increased basal GH release in monolayer cultures by 60% and 67% respectively with respect to saline. It is well known that repeated...
stimulation with GHRH prompts a decreased GH response (Aleppo et al., 1997), although the appearance of desensitization in the GH response to GHRH depends more on the frequency and method of administration (continuous infusion vs. repeated injection) than on the GHRH dose employed (Arsenijevic et al., 1987; Kovacs et al., 1994; Pérez-Romero et al., 1999; Pinski et al., 1996; Sato et al., 1994). In the present case, repeated administration of GHRH at 24-hour intervals maintained high levels of in vitro basal GH release, a finding similar to that reported in vivo in prepubertal female rats (Pérez-Romero et al., 1999) and in sheep (Pérez-Romero et al., 2000). Bercu and Wideman (1991) showed that chronic treatment with GHRP-6 (14 days) maintained high serum GH levels in female rats. In the present study, similarly, chronic administration of Ipamorelin increased basal GH release in vitro, suggesting that, at least in these conditions, the GHS Ipamorelin does not elicit somatotrophic desensitization.

In vitro, somatotroph cells are able to increase GH response after stimulus by GHRH or Ipamorelin when obtained from animals treated in vivo with the GHS Ipamorelin, although when they are obtained from animals treated with GHRH the same cells do not modify GH release. It is therefore surprising that pretreatment with GHRH should block the response to both Ipamorelin and GHRH. In the case of stimulus with GHRH, this might be accounted for by a down-regulation of the cAMP system (Aleppo et al., 1997), although there is no clear evidence that this system has any major influence on intracellular signalling in GHSs. Nevertheless, some research has shown that GHSs enhance cAMP activity when adenylyl cyclase activity has been previously increased; for example following prior stimulation with GHRH (Wu et al., 1996) or in human somatotropinomas with gsp mutations involving intense adenylyl cyclase activity (Adams et al., 1996). The present study did not address the intracellular mechanisms involved in

Figure 6.- Frequency distribution of individual GH release (A, B and C panels) and individual GH contents (D, E and F panels) of transfer membrane after a 4 hour incubation by rat pituitary cells previously cultured in monolayer for 3 days. A, D: Vehicle-pretreated group (VPG). B, E: Ipamorelin-pretreated group (IPG). C, F: GHRH-pretreated group (GPG).
the response to pretreatment with Ipamorelin or GHRH, but from the data reported in the literature it may be hypothesized that there could be some point of connection between the intracellular signals of the two secretagogues that would limit the stimulatory effect of the subsequent stimulus.

Using the CIBA technique described by Kendal and Hymer (1987), both the size and the optical density of GH immunoblot cells can be determined, affording a parameter for individual GH release (Jiménez-Reina et al., 2000; Dobado-Berrios et al., 1996a; Dobado-Berrios et al., 1996b), as opposed to the reverse hemolytic plaque assay technique, which only permits the measurement of plaque size. Moreover, cell-blotting ensures that paracrine interactions are minimized on two fronts (Ramirez et al., 1997). Firstly, it increases the distance between individual cells. Second, the membrane used for culture retains a putative paracrine peptide signal in the vicinity of the releasing cells, similar to that reported for the cell-secreted hormone. Third, individual GH release is heterogeneous (Frawley and Neill, 1984) and there is a small group of somatotroph cells that release more GH than the rest. Consequently, some discrepancies may be encountered between the overall GH release data (monolayer cultures) and individual GH release data (cell-blotting).

Although pretreatment with Ipamorelin prompted a lower individual GH release, the secretion pattern was not very different from that recorded in the groups pretreated with GHRH or saline. However, there was an apparent decline in the proportion of GH-secreting cells in the group pretreated with Ipamorelin (data not shown) and hence the data obtained suggest that changes in the individual basal GH response would be due to changes in the proportions of GH-releasing cells, as proposed in other experimental conditions following stimulation with GHRP-6 (Goth et al., 1992). Changes in the proportion of GH-secreting cells might account for the decline in individual basal GH release in the group pretreated with Ipamorelin, whereas cumulative basal GH release from pituitary cells in monolayer culture increased.

Changes in the amount of hormone released were accompanied by a decline in intracellular GH content in the groups pretreated with Ipamorelin or GHRH. Indeed, chronic treatment with GHS (GHRP-6) over 14 days (Berru and Weideman, 1991), or with GHRH over one week (Stefaneanu et al., 1993), prompts a decrease in pituitary GH contents, suggestive of somatotroph cell activation.

Despite GH cell activation following chronic administration of Ipamorelin, somatotroph cells in vitro responded to both Ipamorelin and GHRH, individual GH release increasing. There-


