EXPERIMENTAL STUDY

Novel ghrelin analogs with improved affinity for the GH secretagogue receptor stimulate GH and prolactin release from human pituitary cells

H Rubinfeld, M Hadani1, J E Taylor2, J Z Dong2, J Comstock2, Y Shen2, D DeOliveira2, R Datta2, M D Culler2 and I Shimon

Institute of Endocrinology and 1Department of Neurosurgery, Chaim Sheba Medical Center, Tel-Hashomer, 52621 and Sackler School of Medicine, Tel-Aviv University, Israel and 2Biomeasure Inc/IPSEN Group, Milford, Massachusetts, USA

(Correspondence should be addressed to I Shimon; Email: ilan.shimon@sheba.health.gov.il)

Abstract

Objective: Ghrelin, a recently identified 28-amino acid peptide is a potent GH secretagogue (GHS) produced predominantly by the stomach. Ghrelin stimulates GH secretion through binding to the GHS receptor in the hypothalamus and pituitary. In addition to the GH-releasing action, ghrelin has been found to be a powerful orexigenic factor. To assess the direct in vitro effects of ghrelin on human pituitary hormone secretion we have produced a panel of novel ghrelin analogs (molecular weight, 3323–3384; human native ghrelin, 3371) with enhanced affinity for the human GHS receptor (IC50 0.38–1.09 nM; human ghrelin, 1.2–2.2 nM).

Methods: The peptidic analogs were tested for their effect on GH secretion using dispersed human fetal pituitaries (21 to 23 weeks of gestation) and cultured GH- and prolactin (PRL)-secreting adenomas. The expression of the GHS receptor in normal (fetal and adult) human pituitary tissues, GH- and PRL-cell adenomas was established using RT-PCR.

Results: The effects of ghrelin, its analogs and GH-releasing hormone (GHRH) alone or in combination on GH and PRL secretion were compared at various concentrations. The ghrelin analogs stimulated GH release by 35–60% from human fetal pituitary cells (1–10 nM; P, 0.05) and by 50–75% from cultured pituitary adenomas (10 nM; P, 0.05). This releasing effect was dose-dependent, achieving maximal stimulation with analog concentrations at 100 nM. Human ghrelin was less potent as compared with its analogs in stimulating human GH, in keeping with the improved binding affinity of the analogs for the GHS-1a receptor. The ghrelin analogs and GHRH had comparable effects on GH secretion from both normal and adenomatous cells, and in combination produced an additive stimulatory effect on GH (150%; P < 0.0001). In contrast, ghrelin and its analogs induced a comparable increase in PRL release ranging between 25 and 40% (P < 0.05) from fetal cells and 30 and 70% (P < 0.001) from cultured PRL-cell and mixed GH–PRL adenomas.

Conclusions: Our results have demonstrated for the first time that ghrelin analogs with enhanced affinity for the GHS receptor are potent stimulators of GH secretion from human pituitary cells, and thus may possess potential clinical therapeutic benefits.

European Journal of Endocrinology 151 787–795

Introduction

Growth hormone (GH) release from the pituitary is regulated by hypothalamic hormones, including GH-releasing hormone (GHRH) and somatostatin, by the peripheral insulin-like growth factor-I (1), and probably autoregulated by GH itself (2). Several years ago, the GH secretagogue (GHS) receptor, a typical G-protein-coupled seven-transmembrane receptor, was cloned and identified in the pituitary and hypothalamus (3). Small synthetic hexa-peptides, such as GH-releasing peptide-6 (GHRP-6) or hexarelin, and non-peptidyl molecules with GH-releasing ability, known for over 20 years as GHRPs, stimulate GH release from the pituitary through binding to the GHS receptor (4, 5), and increasing intracellular Cu2+ concentration via inositol 1,4,5-triphosphate signaling (3). Only in 1999 was the natural endogenous ligand for the GHS receptor purified and identified in neuroendocrine cells (X/A-like cells) of the oxyntic mucosa in the rat and human stomach (6, 7). This specific ligand, named ghrelin, is a 28-amino acid peptide with a unique n-octanoyl modification at serine3, essential for its GH-releasing activity both in vitro and in vivo (6, 8). This serine3 acylation is a post-translational modification that has been identified for the first time in mammalian physiology.
and is necessary for binding and activation of the functional isoform of the GHS receptor (receptor subtype 1a; GHSR-1a) (9). Like GHRH, ghrelin stimulates secretion of GH from the pituitary upon GHS receptor ligand binding. In addition to its GH-releasing action, ghrelin has been found to be a powerful orexigenic factor, mediated by hypothalamic neurons co-expressing neuropeptide Y and agouti-related protein in the arcuate nucleus (10–12).

We have previously shown the ability of GHRP-6, a synthetic GHRP, to stimulate human GH release from cultured human fetal pituitary cells (13). In our efforts to produce therapeutically useful ghrelin-based molecules, we have produced novel analogs of human ghrelin with enhanced affinity for the human GHS receptor. In the present study, several of these ghrelin analogs were compared with native ghrelin for their ability to stimulate GH secretion from human fetal pituitary cells and cultured hormone-secreting adenomas. Our observations have demonstrated for the first time that ghrelin analogs with enhanced affinity for the GHS receptor are potent stimulators of GH secretion from human pituitary cells.

Materials and methods

Peptides, GHS receptor ligand binding and activation

Peptide compounds, including human ghrelin (h-ghrelin) and three different full-length h-ghrelin derivatives, were obtained from Biomeasure Inc/IPSEN Group (Milford, MA, USA). Specific binding affinities of the compounds for the human GHSR-1a were determined by radioligand binding studies. Membranes were prepared by homogenization of CHO-K1 cells expressing the human recombinant GHSR-1a in 20 ml ice-cold Tris–HCl (50 mM) with a Brinkman polytron (Westbury, NY, USA). The homogenates were washed twice by centrifugation (39 000 g/10 min), and the final pellets were resuspended in 50 mM Tris–HCl containing 2.5 mM MgCl₂ and 0.1% bovine serum albumin (BSA). For assay, aliquots (0.4 ml) were incubated with 0.05 nM [¹²⁵I]ghrelin, with and without unlabeled competing test peptides. After a 60-min incubation, the bound [¹²⁵I]ghrelin was separated from the free radiolabeled ghrelin. The filters were then washed and the bound radioactivity trapped on the filters was counted by gamma spectrometry. Specific binding was defined as the total [¹²⁵I]ghrelin bound minus that bound in the presence of 1000 nM unlabeled ghrelin or analogs tested. The curves shown are representative from a single experiment.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human GHS receptor affinity (IC₅₀, nM)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human ghrelin</td>
<td>1.2–2.2*</td>
<td>3371</td>
</tr>
<tr>
<td>BIM-28125</td>
<td>0.38</td>
<td>3367</td>
</tr>
<tr>
<td>BIM-28143</td>
<td>0.65</td>
<td>3379</td>
</tr>
<tr>
<td>BIM-28152</td>
<td>0.75</td>
<td>3384</td>
</tr>
</tbody>
</table>

* Values calculated from numerous experiments.

BIM-28125, -28143 and -28152 are code names for analogs under patent.
the different analogs are summarized in Table 1. All analogs used in our experiments (Table 1) were found to be GHSR-1a agonists by this assay.

Affinity for the GHSR-1a and molecular weights of the different analogs are summarized in Table 1. All three ghrelin analogs had ~3-fold increase in their affinity for the GHSR-1a, as compared with native ghrelin. Stock solutions (100 μM) of these compounds were prepared in 0.01 M acetic acid and 0.1% BSA and were stored at −20°C until used. GHRH was purchased from Sigma Chemicals Co. (St Louis, MO, USA).

**Human pituitary tissues**

Human fetal pituitary tissues of 21 to 23 weeks of gestation (males and females) were obtained after therapeutic pregnancy terminations. Studies of human fetal pituitaries followed the guidelines of the National Advisory Board on Ethics in Reproduction (14), and informed consent was obtained from pregnant subjects. Normal adult pituitary tissue was obtained during a postmortem examination. Pituitary adenoma specimens were obtained from transphenoidal surgical resections, after obtaining informed consent from the patients. Normal (fetal or adult) and adenomatous pituitary tissues were either snap-frozen (for RNA assays) or placed in culture medium for cell culture studies.

**Pituitary RNA extraction**

Normal human adult and fetal pituitaries and pituitary adenomas were harvested and kept at −70°C for RNA extraction. After homogenization, total RNA was extracted using guanidium isothiocyanate–phenol–chloroform (TRizol; Invitrogen Inc., Carlsbad, CA, USA) and aliquots of RNA were electrophoresed through Tris-borate/EDTA (TBE) gel to confirm RNA integrity.

**RT-PCR**

RT followed by PCR amplification was performed to detect GHSR-1a mRNA expression in normal and adenomatous pituitary tissues. RNA was treated with deoxyribonuclease before the RT reaction to eliminate contaminating genomic DNA. RNA was then used in a 20 μl RT reaction containing Oligo(dT)16 as a primer and SuperScript II (Life Technologies, Invitrogen Inc.). RT reactions were incubated at 42°C for 50 min and then 70°C for 15 min. The resulting cDNA and negative water control were used for subsequent PCR amplification of GHSR-1a in the presence of 2 mM MgCl2 and 5 U Taq DNA polymerase (Bioline, Randolph, MA, USA). Amplifications were carried out for 40 cycles, with an initial denaturation step at 95°C for 5 min and a final 7-min extension step at 72°C. Each cycle consisted of denaturation at 94°C, annealing at 58°C and elongation at 72°C; each step lasted 1 min. The primer set used was: 5′-TTCTGTCTCAGGTCCTCTACGTA (nucleotides 676-699, exon 1) and 3′-CCAGAAGTCGAAACTGCCCAC (nucleotides 994-1015, exon 2; GenBank accession no. U60179 for the human GHSR-1a mRNA). The PCR product thus generated was 340 bp and it was digested by Sau3AI (New England Biolabs, Beverly, MA, USA) to 221 and 119 bp restriction products and visualized with ethidium bromide after electrophoresis on 2% agarose gel.

**Human fetal pituitary and pituitary adenoma primary cell cultures**

Fetal specimens were harvested from pathologic specimens within 0.5 to 2 h of the termination procedure. Pituitary adenoma specimens were collected during transphenoidal procedures. Fetal pituitary and tumor specimens were treated similarly, and washed in low glucose Dulbecco’s minimum Eagle’s medium (DMEM) supplemented with 0.3% BSA, 2 mM glutamine and antibiotics, then minced and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase (both from Sigma Chemicals Co.) for 45–60 min. Cell suspensions were filtered through 80 μM nylon mesh (Millipore, Bedford, MA, USA) and resuspended in low glucose DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. For primary cultures, ~5 × 10^4 cells were seeded in 48-well tissue culture plates (Costar, Cambridge, MA, USA) in 0.5 ml medium, and incubated for 48–72 h in a humidified atmosphere of 95% air/5% CO2 at 37°C. Medium was then changed to serum-free defined (SDF) low glucose DMEM containing 0.2% BSA, 120 nM transferrin, 100 nM hydrocortisone, 0.6 nM tri-iodothyronine, 5 μl insulin, 3 nM glucagon, 50 nM parathyroid hormone, 2 mM glutamine, 15 nM epidermal growth factor and antibiotics. Cells were treated for 4 h with each of the ghrelin analogs (1–1000 nM), h-ghrelin (1–10 nM) or GHRH (10 nM). A single pituitary specimen (either fetal or adenoma) was divided and plated onto 60–80 wells, depending upon the age and size of the specimen. In each experiment, six to eight wells served as controls (treated with vehicle solution) and groups of six to eight wells were treated as indicated. Medium was then collected and stored at −20°C for later hormone measurements.

**Hormone assays**

Human GH and prolactin (PRL) concentrations in culture medium were measured by RIA and IRMA respectively (Diagnostic Products Corp., Los Angeles, CA, USA).

**Statistical analysis**

Results are presented as the means ± S.D. As absolute hormonal levels differed between fetal and adenoma
specimens, hormonal data are expressed as percentage of control. Data were analyzed by one-way ANOVA and P values < 0.05 were considered significant.

Results

Human pituitary GHS receptor expression

Total RNA was extracted from normal adult and fetal (21 to 27 weeks of gestation) pituitaries and from GH- and PRL-secreting pituitary adenomas. RNA was subjected to RT, followed by PCR amplification for GHSR-1a cDNA. Consistent with previous reports (13, 15–17), expression of the receptor was found in all tissues studied (Fig. 2A) including normal pituitary, fetal pituitary specimens of 21, 23 and 27 weeks (only 21 week is shown) and five GH-secreting adenomas (340 bp product; Fig. 2A). Negative control was free of PCR products (—). Specificity of PCR was confirmed by digestion with Sau3AI restriction enzyme, resulting in the expected 221 and 119 bp DNA products (Fig. 2B).

Ghrelin analogs stimulate secretion of human fetal GH and PRL

The effects of ghrelin analogs on GH secretion from dispersed human fetal pituitary cultures (21 to 23 weeks of gestation) were studied by incubation for 4 h with BIM-28125, BIM-28152, BIM-28143 (Table 1), h-ghrelin or human GHRH. The analogs tested (10 nM each) significantly increased GH secretion by 35–60% (Fig. 3). This degree of GH stimulation was higher than that evoked by h-ghrelin (up to 25%; P < 0.01; Fig. 3A and C), and was comparable with that induced by GHRH (50%; P < 0.01; Fig. 3B). When reduced to a concentration of 1 nM, BIM-28152 enhanced GH secretion by 60% whereas no significant effect on GH secretion was observed by h-ghrelin at this concentration (Fig. 3C). These results are in keeping with the 3-fold increase in affinity of BIM-28152 for the GHSR-1a, as compared with h-ghrelin. Incubation of human fetal pituitary cultures with BIM-28125 or BIM-28152 (10 nM) resulted in a 25–40% (P < 0.05) increase in PRL secretion, which was comparable with the stimulation evoked by h-ghrelin (Fig. 4).
GH induction by ghrelin analogs in GH-secreting adenomas

To extend these findings, the effect of ghrelin analogs on GH secretion was also tested on human GH-secreting tumor cells. In agreement with the results obtained in fetal pituitary cells, incubation of GH-secreting tumor cells with BIM-28125 or BIM-28152 (10 nM) also increased GH secretion up to 60% as compared with controls ($P < 0.05$; Fig. 5A), whereas treatment of cells with h-ghrelin resulted in only mild and non-significant GH stimulation (20%; Fig. 5A). The higher efficacy of the analogs is further apparent at concentrations of 1 nM, resulting in a 40–50% increase in GH by BIM-28143 and BIM-28152 ($P < 0.01$), as compared with no stimulation observed with h-ghrelin (Fig. 5B). GH induction by BIM-28152 was dose-dependent (1–1000 nM), achieving maximal stimulation at a concentration of 100 nM ($P < 0.01$; Fig. 6A). Stimulation of GH release by the ghrelin analogs was consistent in all GH-secreting adenomas studies. For example, BIM-28152 (10 nM) enhanced GH release in the range of 30–76% ($P < 0.05$; Fig. 6B) in five cultured GH-cell adenomas. All of these adenomas expressed the GHSR-1a (Fig. 2A).

Additive effects of ghrelin analogs and GHRH on GH secretion

The ghrelin analogs and GHRH produced comparable effects on GH release from cultured GH-secreting adenomas. BIM-28143 and BIM-28152, tested individually, increased GH secretion by 40% and 76% respectively, which was comparable with the increase observed with GHRH (Fig. 7). Combined administration of ghrelin analogs and GHRH (both 10 nM) produced an additive stimulatory effect on GH secretion up to 150% ($P < 0.0001$).

PRL induction by ghrelin analogs in PRL-secreting adenomas

To compare the effect of ghrelin and its analogs on PRL secretion, we used GHSR-1a-expressing, PRL-secreting macroadenoma cells (Fig. 8A). Treatment of cells

Figure 4 Human fetal PRL stimulation by ghrelin analogs. Human fetal pituitary (22 weeks of gestation) was harvested, and cells were cultured at $5 \times 10^4$ cells/well. Forty-eight hours later cultures were treated with h-ghrelin, BIM-28125 (125) and BIM-28152 (152) in SFD medium for 4 h (10 nM). Control cells were treated with vehicle solution. Medium was then collected for later PRL measurements. Each of the bars represents the mean ± S.D. PRL levels in six wells. *$P < 0.05$, **$P < 0.01$.

Figure 5 GH stimulation by ghrelin analogs in cultured GH-cell adenomas. Human GH adenomas were harvested during trans-sphenoidal procedures, and then were cultured at $5 \times 10^4$ cells/well. Forty-eight hours later cultures were treated with h-ghrelin, BIM-28125 (125), BIM-28152 (152) and BIM28143 (143) at the indicated concentrations ((A) 10 nM and (B) 1 nM), in SFD medium for 4 h. Control cells were treated with vehicle solution. Medium was then collected for later GH measurements. Each of the bars represents the mean ± S.D. GH levels in six wells in one of three independent experiments. *$P < 0.05$, **$P < 0.01$.

Figure 6 GH stimulation by the ghrelin analog, BIM-28152, in cultured GH-cell adenomas. (A) Stimulation of GH secretion by BIM-28152 is dose-dependent (1–1000 nM) as compared with controls treated with vehicle solution ($P < 0.01$) for 4 h. (B) Human GH stimulation by BIM-28152 (10 nM) in five cultured GH-cell adenomas (GH-1 to GH-5) as compared with controls treated with vehicle solution. *$P < 0.05$, **$P < 0.01$, #$P = 0.06$. 

Downloaded from Bioscientifica.com at 11/05/2018 06:26:43AM via free access
with BIM-28143, BIM-28152 and h-ghrelin resulted in comparable increases in PRL secretion (70%; \( P < 0.001 \); Fig. 8A). In contrast, GHRH induced only a moderate increase in PRL (Fig. 8A). The pattern of PRL release by ghrelin and its analogs was also compared using a mixed GH–PRL adenoma. Again, a comparable increase in PRL was induced by ghrelin and its analogs (~23–33%; Fig. 8B).

**Discussion**

This study is the first to show that novel ghrelin analogs with enhanced binding affinity for the GHSR are potent *in vitro* stimulators of human GH secretion from fetal pituitary tissues and cultured GH-secreting adenomas. The new peptide analogs stimulate human GH secretion to the same level as GHRH, and are more effective than the natural ligand in stimulating GH secretion. The results also demonstrated an additive stimulatory effect on GH secretion by the combined administration of GHRH and ghrelin analogs. Ghrelin analogs also enhanced PRL secretion from human fetal pituitary and adenoma cells.

We have previously shown the functional expression of the GHS receptor in human fetal pituitaries that responded to the synthetic hexapeptide, GHRP-6 (13). In the present study, we have shown expression of the functional isoform of this receptor in human fetal pituitaries and hormone-secreting adenomas. Several studies have already shown the expression of GHSR-1a in human secreting pituitary adenomas (15–17). Moreover, GHSR-1a expression is higher in GH-producing adenomas as compared with other types of pituitary adenomas or normal human pituitary (15–17).

Short peptides encompassing the first four or five residues of ghrelin (including Gly-Ser-Ser(n-octanoyl)-Phe) have been shown to bind and functionally activate the human GHSR-1a in HEK-293 cells over-expressing this human receptor (9), even though they had 40- to 200-fold weaker binding affinity for the GHSR-1a, as compared with the parent ghrelin molecule. However, short truncated ghrelin analogs failed to displace radiolabeled ghrelin from binding sites in membranes derived from human hypothalamus and pituitary (18). Moreover, GHSR-1a expression is higher in GH-producing adenomas as compared with other types of pituitary adenomas or normal human pituitary (15–17).

A recent study by Holst \et al. (19) reports a strong, ligand-independent signaling of the ghrelin receptor in transfected HEK-293 or COS-7 cells, as measured by inositol phosphate turnover. Interestingly, this constitutive signaling was further stimulated by ghrelin, and suppressed by the low potency ghrelin antagonist, substance P analog (19). Whether this constitutive activity plays a role in vivo is yet to be determined; however, it may explain the activity of the short peptides with low GHS receptor-binding affinity shown in transfected HEK-293 cells (9). This constitutive signaling...
from the GHS receptor may also be partially responsible for the continuous GH release from isolated cultured human pituitary cells, in which stimulators of GH secretion should not exist, although there is some evidence for the expression of GHRH mRNA in the human pituitary (20, 21) and of ghrelin mRNA in human (15, 22) and rat pituitaries (23). Our study reports for the first time the use of ghrelin peptide derivatives that are potent agonists of the human GHS receptor and are able to stimulate in vitro GH release from human pituitary cells. At a concentration of 1 nM, the ghrelin analogs exhibit GH stimulation by 40–60%, whereas h-ghrelin at this concentration had no effect on GH secretion. This higher efficacy of ghrelin analogs was consistent in both fetal and adenoma cells and may reflect both the improved affinity of the analogs for the human receptor (Table 1) and their ability to mobilize intracellular calcium. Although h-ghrelin has a strong GH-releasing activity in vivo (24–26), the lack of hypothalamic-mediated stimulation, which has been found to be a major mechanism of ghrelin action in vivo (27), and perhaps the lack of hypothalamic somatostatin inhibition (28), may explain its low hormone-releasing activity in vitro, in which it interacts directly with somatotrophs. This may, in part, explain why in vivo and in vitro studies comparing the effects of ghrelin and GHRH do not correlate. Ghrelin’s ability to induce GH release in vivo is thus stronger than that of GHRH in humans (24, 25); however, our study, performed on isolated cultures of pituitary cells, showed similar in vitro effects of ghrelin, its analogs and GHRH, whereas in vitro studies using pituitary cells from pigs (29) and rats (30) show weaker effects of ghrelin as compared with GHRH.

It has been clearly demonstrated that GHRP synergizes with GHRH in stimulating GH release in vivo (31, 32). Ghrelin was also found to act synergistically with GHRH to release GH secretion in humans in vivo (33, 34). It has been postulated that GH release from the hypothalamus induced by GHS is responsible for these synergistic effects. Synergistic GH response to combined GHRP and GHRH treatments have been reported in cultured rat pituitary cells (35–37). However, other groups have reported only additive effects with these two peptides in rat (38, 39), bovine (40), ovine (41) and human fetal pituitary cultures (13). In the present study, combined treatment with ghrelin analogs and GHRH had an additive effect on GH release from cultured human GH-secreting adenomas. This observation is in agreement with the reported additive effect of ghrelin and GHRH on GH release from pig pituitary cells (29), although neither a synergistic nor an additive GH response to combined ghrelin and GHRH was obtained in the same cells by Malagon et al. (42), or in cultured rat pituitary cells (30).

The stimulatory effect of ghrelin on PRL secretion in vivo in humans is slight (24, 25), in accordance with the previous studies of GHRPs in humans (43). PRL secretion was not induced by GHRP-6 in vitro in human fetal pituitary cells (13). We now report in vitro stimulation of PRL release by ghrelin and its analogs from human fetal cells, and cultured PRL- and mixed GH–PRL-secreting pituitary adenomas. The different effects of GHRP-6 and ghrelin on PRL secretion from human fetal cells could point to differences in the molecular mechanisms mediating the PRL-releasing activities of the two peptides. Likewise, a recent study by Malagon et al. (42) suggests that ghrelin exerts its GH-releasing activity through a unique set of multiple signaling cascades, indicating that the signaling complex activated by ghrelin is partially different from that employed by synthetic GHSs.

The effects of the ghrelin analogs on PRL secretion was similar to that of native ghrelin in all cultured tissues tested (Figs 4 and 8) and, thus, not in keeping with their improved GH-releasing activity as compared with the natural ligand. These results may suggest that the ghrelin analogs possess a higher selectivity for GH stimulation, although this must be further examined. In this study, we have evaluated the GH-releasing efficacy of several novel ghrelin analogs with high binding affinity for the GHS receptor. Our observations may have relevance to clinical situations associated with GH deficiency. As these new peptides have a remarkable improved GH-releasing capacity directly at the pituitary level, they may possess a potential role in assessing GH secretory capacity and in the treatment of subjects with GH-deficient states, including patients with an intact pituitary, but with an abnormal hypothalamic–pituitary connection. These patients may benefit from the improved GH-releasing ability of the studied analogs, demonstrated directly at the pituitary level. As this is the first study to report active ghrelin analogs, it opens new opportunities for the manipulation of GH release through ghrelin receptor ligand binding.

References


www.eje.org
13 Shimon I, Yan X & Melmed S. Human fetal pituitary expresses 
14 Cohen CB & Jonsen AR. The future of the fetal tissue bank. The 
16 Korbonits M, Jacobs RA, Aylwin SJB, Burrin JM, Dahia PLM, 

www.eje.org

794

21 Mustano A, Katakami H, Nagashima T, Teramoto A, Osamura RY & 
20 Levy A & Lightman SL. Growth hormone-releasing hormone 
18 Levy A & Lightman SL. Growth hormone-releasing hormone 
17 Monaco AM, Raff N, Beatrix L, Edward RL & Michael OT. Growth 
1663–1665. 
13 Hataya Y, Akamizu T, Takaya K, Kanamoto N, Arivusya H, Saijo M, 
12 Cheng K, Chan W, Butler B, Barreto AJ & Smith R. Evidence for a 
11 Cheng K, Chan W , Butler B, Wei L, Schoen WR, Wyvart MJ Jr, 
10 Cheng K, Chan W, Butler B, Wei L, Schoen WR, Wyvart MJ Jr, 
9 Bednarek MA, Feighner SD, Pong SS, Kulju McKee K, Hreniuk DL, 
4370–4376. 
4314–4320. 
43314–4320. 
83 174–178. 
43 1623–1630. 
54 759–768. 
83 1624–3630. 
83 4134–4320. 
81 789–794. 
81 16169–1971. 
14 101–104. 
14 2201–2210. 
17 24 1169–171. 
17 Ch 2001 17 2791–2798. 
14 Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, 
656–660. 
425–457. 
245–457. 

EUROPEAN JOURNAL OF ENDOCRINOLOGY (2004) 151 

151


Received 8 July 2004
Accepted 14 September 2004