EXPERIMENTAL STUDY

Expression and homologous regulation of GH secretagogue receptor mRNA in rat adrenal gland

M L Barreiro, I Pinilla, E Aguilar and M Tena-Sempere

Department of Cell Biology, Physiology and Immunology, Section of Physiology, Faculty of Medicine, University of Córdoba, Avda Menéndez Pidal s/n, 14004 Córdoba, Spain

(Correspondence should be addressed to Manuel Tena-Sempere; Email: fi1tesem@lucano.uco.es)

Abstract

Objective: GH secretagogues (GHSs) elicit a variety of biological effects in several endocrine and non-endocrine target tissues, including activation of the hypothalamic–pituitary–adrenal axis. The latter is mainly carried out through a central hypothalamic action; yet the possibility of additional effects directly at the adrenal level cannot be ruled out. The aims of this study were to evaluate the expression and homologous regulation of the GHS receptor (GHS-R) gene in rat adrenal and to assess the effects of synthetic (GH releasing peptide-6 – GHRP-6) and natural (ghrelin) ligands of GHS-R upon basal and ACTH-stimulated corticosterone secretion in vitro.

Design and Methods: Analysis of adrenal expression of target mRNAs (GHS-R, GHS-R1a, ghrelin, and several steroidogenic factors) was conducted by means of primer-specific, semi-quantitative RT-PCR. Evaluation of corticosterone secretion by incubated adrenal tissue was carried out by specific RIA.

Results: RT-PCR analysis demonstrated expression of the GHS-R gene, but not of the gene encoding the cognate ligand ghrelin, in rat adrenal. Moreover, expression of the mRNA coding for the type 1a GHS-R (GHS-R1a), i.e. the biologically active receptor form, was demonstrated. The adrenal expression of the GHS-R message appeared under the regulation of homologous signals in vitro, as short-term incubation of adrenal samples in serum-free medium induced a significant increase in GHS-R mRNA levels that was inhibited by exposure to different doses of GHRP-6 (10^{-9}–10^{-5} mol/l) or ghrelin (10^{-7} mol/l). Notably, an opposite pattern of homologous regulation of GHS-R gene expression was observed at the pituitary. Finally, short-term stimulation with increasing concentrations of GHRP-6 (10^{-9}–10^{-5} mol/l) or ghrelin (10^{-7} mol/l) failed to alter basal and ACTH-stimulated corticosterone secretion in vitro, neither did it modify ACTH-stimulated mRNA expression levels of several upstream elements in the steroidogenic route: the steroidogenic acute regulatory (StAR) protein, and the enzymes P450 cholesterol side-chain cleavage (P450sc) and 3β-hydroxysteroid dehydrogenase (3β-HSD).

Conclusions: Our study provides novel evidence for the expression and homologous regulation of the GHS-R gene in rat adrenal. However, our results cast doubts on the possibility of direct adrenal actions of ligands of the GHS-R in the regulation of corticosterone secretion in the rat.

European Journal of Endocrinology 147 677–688

Introduction

Growth hormone secretagogues (GHSs) are a family of synthetic peptidyl and non-peptidyl compounds with the ability to induce GH release in all species tested, including humans (1, 2). In addition, GHSs are able to elicit a number of biological responses in different endocrine and non-endocrine systems. These include modulation of food intake (3, 4), stimulation of prolactin secretion (2, 5–7), activation of the corticotropic axis (2, 5, 6, 8, 9), as well as a variety of cardiovascular effects (10, 11). Recently, an unexpected widespread pattern of GHS-binding sites in a variety of peripheral tissues has been demonstrated in humans (12), thus not only providing the basis for their currently known effects, but also strongly suggesting additional, as yet undefined, biological actions of these synthetic compounds.

The physiological relevance of the GHS-signaling system has been recently substantiated by the identification of the specific receptor for GHSs, namely GHS-R, as well as its endogenous ligand, termed ghrelin. Elucidation of the functional structure of GHS-R revealed that it belongs to the G-protein-coupled seven-transmembrane receptor superfamily (1, 13, 14); it being mainly expressed in the pituitary, hypothalamus and hippocampus (15). Interestingly, two GHS-R subtypes, generated by alternative splicing of a
single gene, have been identified: the fully functional type 1a receptor (GHS-R1a) and the biologically inactive GHS-R type 1b (GHS-R1b) (13, 14). More recently, the search for the endogenous ligand of GHS-R using an ‘orphan receptor strategy’ resulted in the identification of ghrelin (16). Ghrelin is a 28 amino acid peptide with an essential n-octanoyl modification at Ser3 that is primarily expressed in stomach and hypothalamus (16, 17). As expected for the endogenous counterpart of GHSs, this molecule has been proven to elicit GH secretion in vivo and from anterior pituitary cells in culture (16–20), and to induce food intake and adiposity in rodents (19, 21, 22).

Among the effects of GHS-R ligands unrelated to GH release, moderate but consistent elevation of serum adrenocorticotropic (ACTH) and glucocorticoid levels have been reported for a variety of GHSs in different species (2, 5, 6, 8, 9, 23). In good agreement, it was shown recently that ghrelin is able to elicit ACTH and cortisol responses in humans (18, 20), as well as ACTH and corticosterone release in rats and mice respectively (19, 24). Notably, such an activation of the hypothalamic–pituitary–adrenal (HPA) axis appears to be tightly regulated. Thus, desensitization of corticosterone responses after repeated exposure to GHSs takes place (25) whereas, in turn, glucocorticoids are able to regulate GHS-R gene expression (26). The mechanism whereby GHS-R ligands activate the HPA axis primarily involves a central action at the hypothalamic level (8, 9, 27). However, the potential contribution of additional direct effects at the adrenal level remains to be carefully evaluated. Indeed, we have recently obtained evidence of the novel expression level remains to be carefully evaluated. Indeed, we have recently obtained evidence of the novel expression of the steroidogenic acute regulatory (StAR) protein in adrenal tissue has been described in detail elsewhere (29). For comparative analysis, a group of adrenals was incubated in the presence of rat ghrelin (5 n mol/l). In addition, to test the ability of GHRP-6 and ghrelin to modulate stimulated corticosterone secretion, a similar experimental setting was carried out in the presence of ACTH (10–7 mol/l). After 90 and 180 min, 100 μl aliquots from the incubation media were taken for corticosterone measurements, as described below. Incubations were terminated at the latter time-point, when samples of adrenal tissue were frozen in liquid nitrogen and stored at −70°C until used for RNA analysis. In addition, to compare the pattern of homologous regulation of GHS-R mRNA expression between the adrenal and pituitary, a similar experimental set-up for static incubation of pituitary tissue was conducted, as described in detail previously (30).

**Materials and methods**

**Animals and drugs**

Adult, 75-day-old Wistar male rats bred in the vivarium of our Institution, and housed under constant conditions of light (14 h of light; from 0700 h) and temperature (22°C) were used. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. GHRP-6, a potent GHS, and rat ghrelin were purchased from Bachem AG (Bubendorf, Switzerland). ACTH was obtained from Sigma (Sigma, St Louis, MO, USA).

**Tissue incubations**

General procedure for static incubation of adrenal tissue has been described in detail elsewhere (29). Briefly, adrenal glands were bilaterally removed upon decapitation of experimental animals, dissected free of the surrounding fat, and divided into two pieces of approximately equal size (mean weight/piece: 24.3±0.465 mg/piece, two slices/adrenal). Adrenal slices (two slices/well) were incubated in 2 ml DMEM/F12 medium (1:1; Life Technologies, Grand Island, NY, USA) supplemented with 0.1 g/l gentamicin in a Dubnoff shaker (60 cycles/min) at 37°C under an atmosphere of 5% CO2–95% O2. After 60 min pre-incubation, the media were replaced either by fresh medium or medium containing increasing doses of GHRP-6 (10–9–10–5 mol/l). For comparative analysis, a group of adrenals was incubated in the presence of rat ghrelin (10–7 mol/l). In addition, to test the ability of GHRP-6 and ghrelin to modulate stimulated corticosterone secretion, a similar experimental setting was carried out in the presence of ACTH (10–7 mol/l). After 90 and 180 min, 100 μl aliquots from the incubation media were taken for corticosterone measurements, as described below. Incubations were terminated at the latter time-point, when samples of adrenal tissue were frozen in liquid nitrogen and stored at −70°C until used for RNA analysis. In addition, to compare the pattern of homologous regulation of GHS-R mRNA expression between the adrenal and pituitary, a similar experimental set-up for static incubation of pituitary tissue was conducted, as described in detail previously (30).

**RNA analysis by semi-quantitative RT-PCR**

Adrenal and pituitary expression of the mRNA encoding the previously cloned GHS-R (13, 14) was assessed by semi-quantitative RT-PCR. Similarly, this approach was used for analysis of relative expression levels of the messengers encoding the biologically active GHS-R1a, the cognate GHS-R ligand, ghrelin, as well as the steroidogenic acute regulatory (StAR) protein and enzymes P450 cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in adrenal samples and incubated adrenal tissue respectively. Total RNA was isolated from adrenal and pituitary samples of the different experimental settings using the single-step, acid guanidinium thiocyanate–phenol–chloroform extraction method (31). For amplification of the different signals, the primer pairs indicated in Table 1 were used. These sets of primers were synthesized according to the published rat cDNA sequences of GHS-R (14), ghrelin (16) and the factors of the steroidogenic pathway under analysis (32–34), and, whenever possible, they were selected based on previous references (26, 28, 35, 36). Amplification of a 290 bp fragment of L19 ribosomal protein mRNA
was carried out in parallel in each sample, using the primer pair and conditions indicated in Table 1, as an internal control.

For amplification of the targets, RT and PCR were run in two separate steps. In addition, to enable appropriate amplification in the exponential phase for each target, PCR amplification of specific signal and L19 ribosomal protein transcripts was carried out in separate reactions with different number of cycles (see Table 1), but using similar amounts of the corresponding cDNA templates, generated in single RT reactions, as previously described (29, 37). Briefly, equal amounts of total RNA (5 μg) were heat denatured and reverse transcribed by incubation at 42°C for 90 min with 12.5 U AMV RT (Promega, Madison, WI, USA). 20U ribonuclease inhibitor RNasin (Promega), 200 nmol/l deoxy-NTP mixture, and 1 nmol/l specific and L19 antisense primers, in a final volume of 30 μl. The reactions were terminated by heating at 97°C for 5 min and cooling on ice, followed by dilution of the RT cDNA samples with nuclease-free H2O (final volume 60 μl). For semi-quantitative PCR, 10 μl aliquots of the cDNA samples (equivalent to 650 ng total RNA input) were amplified in 50 μl of 1× PCR buffer in the presence of 2.5 U Taq-DNA polymerase (Promega), 200 nmol/l deoxy-NTP mixture, and the appropriate primer pairs (1 nmol/l of each primer; see Table 1). PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5 min, annealing for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 15 min was included. Annealing temperature was adjusted for each target: 55°C for ghrelin, 60°C for GHS-R, 62°C for GHS-R1a, and 60°C for StAR, 61°C for P450scc, and 64°C for 3β-HSD. In addition, different numbers of cycles were tested to optimize amplification in the exponential phase of PCR. On this basis, and considering our previous experimental work (28, 29), the PCR cycles indicated for each target in Table 1 were chosen for further analysis (see Results).

PCR-generated DNA fragments were resolved in Tris-borate-buffered 1.5% agarose gels, and visualized by ethidium bromide staining. Specificity of PCR products was confirmed by direct sequencing (NewBiotech Ltd, Seville, Spain). In all assays, liquid controls and reactions without RT were included, yielding negative amplification. When relevant, quantitative evaluation of RT-PCR signals was carried out by densitometric scanning using an image analysis system (1-D Manager; TDI Ltd, Madrid, Spain); the values for the specific targets being normalized to those of internal controls.

Corticosterone measurements

Corticosterone levels in static incubation media of adrenal tissue were measured using a commercial kit from ICN Biomedicals (Costa Mesa, CA, USA). The sensitivity of the assay was 2.5 ng/tube, and the intra-assay coefficient of variation was 4.5%.

Presentation of data and statistics

RT-PCR analyses were carried out in triplicate using independent RNA samples within each experimental group. When relevant, RNA data are presented as means±s.e.m. from at least three independent observations. Tissue incubations were carried out in duplicate, with a total number of 12 determinations per group. Hormonal levels are expressed as normalized values per 100 mg of incubated tissue. Quantitative results were analyzed for statistically significant differences using ANOVA, followed by Tukey’s test. P < 0.05 was considered significant.

---

**Table 1** Oligo-primer pairs used for RT-PCR amplification of GHS-R, ghrelin and steroidogenic-related factor transcripts. For each target, the primer pair used for amplification is included. In addition, the expected size of the generated cDNA products and the number of cycles selected for semi-quantitative RT-PCR analysis is indicated for each signal.

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo-primers</th>
<th>Expected size</th>
<th>PCR cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHS-R</td>
<td>GHS-R sense (5'-AGG CAA CCT GCT CAC TAT GCT G-3')</td>
<td>321 bp</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>GHS-R as (5'-GAC AAG GAT GAC CAG CTT CAC G-3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHS-R1a</td>
<td>GHS-R1a sense (5'-TTT TCT TTA CTA CGC GTC TTC TGC CTC-3')</td>
<td>261 bp</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>GHS-R1a as (5'-GGA CAC CAG GTT GCA GTA CTG GCT-3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Ghrelin sense (5'-TTG AGC CCA GAG CAG CAC AAA-3')</td>
<td>347 bp</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Ghrelin as (5'-AGT TGC AGA GGA GGC AGA AGC T-3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>StAR</td>
<td>StAR sense (5'-TGG GTG GAT GGG TCA GGT CC-3')</td>
<td>500 bp</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>StAR as (5'-GCT CAG GCA TCT CCC CAA AGT -3'),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450scc</td>
<td>P450scc sense (5'-AGA TCC CTT CCC CTT GGG TAT ATG-3')</td>
<td>509 bp</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>P450scc as (5'-CCA GGC GCT CCC CAA ATA CAA CA-3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-HSD sense (5'-CAG GAG CAG GAG GGT TTG T-3')</td>
<td>400 bp</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>3β-HSD as (5'-GTG GCC ATT CAG GAT GAT -3')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L19</td>
<td>L19-sense (5'-GAA ATC GCC AAT GCC AAC TC-3')</td>
<td>290 bp</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>L19-as (5'-ACC TTC AGG TAC AGG TG-3')</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

Optimization of semi-quantitative RT-PCR assays

Assessment of relative expression levels of the mRNAs under analysis (common GHS-R, isoform-specific GHS-R1a, ghrelin, and the steroidogenic factors StAR, P450scc and 3β-HSD) was conducted by means of RT-PCR, optimized for semi-quantitative determination. Such a method is especially useful for highly sensitive detection and discrimination between alternatively spliced species (e.g. the GHS-R1a form). To obtain optimal conditions for amplification, i.e. in the exponential phase of PCR, different numbers of cycles were tested for each target. This procedure is presented in detail for GHS-R and L19 transcripts (Fig. 1). Plotting of intensity of PCR signals (as expressed by absolute OD values) against the number of amplification cycles revealed a strong linear relationship between cycles 24 and 39 in the case of GHS-R (correlation coefficient $r^2 = 0.9909$) and cycles 17 and 26 in the case of L19 (coefficient $r^2 = 0.993$). Thus, PCR amplification of GHS-R and L19 ribosomal protein transcripts was conducted in separated reactions, using 33 and 23 amplification cycles respectively (Table 1). The validity of the above RT-PCR assays for semi-quantitative analysis is supported by (a) the selection, for each target, of amplification conditions in the exponential phase of PCR, (b) the repetitive observation of results within experimental groups (at least three assays per data point using independent RNA and tissue samples), and (c) the use of an appropriate internal control.

Expression of GHS-R gene and GHS-R1a mRNA in rat adrenal

Evaluation of GHS-R mRNA expression in rat adrenal tissue by means of semi-quantitative RT-PCR, using a specific primer pair flanking a coding area common for both forms of GHS-R, i.e. GHS-R1a and GHS-R1b (26, 36), demonstrated expression of the gene in adrenal samples from three independent adult specimens (Fig. 2A). The identity of the amplicon was confirmed by direct sequencing. Moreover, the possibility of spurious amplification of the signal was ruled out by the lack of amplification in reactions without RT and those with liquid controls. For comparison, RT-PCR amplification of the GHS-R transcript was also conducted in samples from rat pituitary, i.e. a major target tissue for GHS. Our semi-quantitative analysis demonstrated that, despite detectable expression of the gene in adrenal tissue, basal expression levels of the GHS-R mRNA are significantly higher in rat pituitary gland (Fig. 2A). In parallel, assessment of expression of the ghrelin gene in the very same adrenal samples was conducted by semi-quantitative RT-PCR, using a primer pair and conditions optimized previously.
Our analysis, however, failed to demonstrate positive amplification of ghrelin mRNA transcript in adrenal tissue, despite strong expression of ghrelin signal detected in the stomach, used as control for reaction conditions (Fig. 2B).

In addition, evaluation of the expression levels of the mRNA encoding GHS-R1a, i.e. the biologically active receptor isoform, in rat adrenal was conducted using semi-quantitative RT-PCR and a GHS-R1a-specific primer pair spanning over the single intron of the GHS-R gene (35). In keeping with analyses on the net expression of the GHS-R gene, GHS-R1a mRNA expression was detected in adrenal tissue from three independent adult specimens (Fig. 3). The identity of the amplicon was confirmed by direct sequencing. No signal was amplified in reactions without RT and those with liquid controls.

Homologous regulation of GHS-R gene expression in rat adrenal

In a next step, analysis of homologous regulation of GHS-R mRNA expression, i.e. by ligands of the cognate receptor, in rat adrenal tissue was undertaken. To this end, both synthetic (GHRP-6) and natural (ghrelin) ligands of GHS-R were used. RT-PCR assays confirmed moderate expression levels of GHS-R transcript in adrenal samples dissected out from adult rats immediately after decapitation. These values were taken as reference basal expression levels. Short-term (4-h) incubation of adrenal tissue in serum-free medium resulted in a significant 2.5-fold increase in the relative GHS-R mRNA levels in the samples. Such a response was completely blunted by 3-h incubation in the presence of increasing concentrations of GHRP-6 ($10^{-9}$ to $10^{-3}$ mol/l) or ghrelin ($10^{-7}$ mol/l). In fact, an apparent dose-dependency for the inhibitory effect of GHRP-6 upon the expression levels of GHS-R mRNA was noticed, as expression...
values of the transcript were lower in samples incubated in the presence of $10^{-5}$ mol/l GHRP-6 than in those challenged with $10^{-9} - 10^{-7}$ mol/l doses (Fig. 4).

For comparative purposes, the pattern of homologous regulation of GHS-R mRNA expression in rat adrenal was compared with that of the pituitary, using a similar experimental setting. High expression levels of GHS-R transcript in pituitary samples dissected out from adult rats immediately after decapitation were detected by RT-PCR assays, in keeping with our previous observations (see Fig. 2A). As was the case for the adrenal, these values were taken as reference basal expression levels. Short-term (4-h) incubation of pituitary tissue in serum-free medium resulted in a significant 3.0-fold decrease in the relative GHS-R mRNA levels in the samples. Such a decrease was completely prevented by 3-h incubation in the presence of GHRP-6 ($10^{-9} - 10^{-7}$ mol/l) or ghrelin ($10^{-7}$ mol/l). Indeed, these doses were able to enhance GHS-R mRNA expression levels over basal control values. Nevertheless, a partial down-regulatory response to increasing doses of GHRP-6 was observed in terms of GHS-R mRNA levels, as expression values of the transcript were lower in samples incubated in the presence of $10^{-5}$ mol/l GHRP-6 than in those challenged with $10^{-9} - 10^{-7}$ mol/l doses. However, relative GHS-R mRNA levels in the group exposed to $10^{-5}$ mol/l GHRP-6 were significantly higher than in samples incubated in the presence of medium alone (Fig. 5).

Assessment of direct steroidogenic effects of natural and synthetic GHSs in rat adrenal

Considering the proven stimulatory effects of GHS-R ligands upon the HPA axis (6, 8, 9, 18–20) and the expression of GHS-R gene in rat adrenal (our present results), the potential involvement of this signaling system in rat adrenal steroidogenesis was explored using a static in vitro system. A similar experimental setting was used recently by our group to analyze the direct adrenal actions of the adipocyte-derived hormone, leptin (29). Moreover, using an analogous experimental approach, we recently obtained evidence for a direct effect of ghrelin upon rat testicular steroidogenesis (28). Secretory responses, in terms of corticosterone release, to GHRP-6 ($10^{-9} - 10^{-7}$ mol/l) and ghrelin ($10^{-7}$ mol/l) were assessed after 90- and 180-min incubation, both under basal and ACTH ($10^{-7}$ mol/l)-stimulated conditions. As predicted, a 3-h incubation in the presence of $10^{-7}$ mol/l ACTH was able to induce a significant threefold increase in corticosterone secretion in vitro, which was considered as positive control for our incubation system. However, at both time-points tested, basal and ACTH-stimulated corticosterone secretion remained unaffected by a 3-h exposure to increasing doses of GHRP-6 or ghrelin (Fig. 6).

The above hormonal responses were correlated with the relative mRNA expression levels of several upstream
steroidogenic factors in the very same experimental samples. The targets to be analyzed were selected based on their role as common key early steps in steroid hormone biosynthesis (39, 40). For analysis, a semi-quantitative RT-PCR approach, using specific primer pairs for StAR protein, and the enzymes P450scc and 3β-HSD, was used. In good agreement with our secretory data, stimulation of adrenal tissue for 180 min with 10^{-2} mol/l ACTH induced, respectively, 3.0-, 4.0- and 2.75-fold increases in StAR, P450scc and 3β-HSD mRNA levels respectively. However, exposure to increasing doses of GHRP-6 (10^{-9} – 10^{-5} mol/l) or ghrelin (10^{-7} mol/l) failed to modify ACTH-stimulated mRNA expression levels of any of the targets under analysis (Fig. 7).

Discussion

The biological effects, known to date, of ligands of the GHS-R are assumed to be primarily carried out at central levels, i.e. the hypothalamus and/or pituitary. Among these effects, activation of the HPA axis has
been related to the regulation of the hypothalamic network controlling ACTH secretion, via stimulation of corticotropin releasing hormone and arginine-vasopressin release, rather than to the induction of ACTH secretion directly at the pituitary level (9, 27). However, the possibility of direct actions of GHS-R ligands at the adrenal level remains poorly characterized. In the present paper, we provide compelling

**Figure 7** Assessment of the effects of synthetic (GHRP-6) and natural (ghrelin) ligands of GHS-R upon ACTH-stimulated mRNA expression levels of StAR, P450scc and 3β-HSD in rat adrenal in vitro. The left panels are representative semi-quantitative RT-PCR assays of expression levels of the targets in adrenal samples incubated in the presence of medium, 10^{-7} mol/l ACTH or ACTH plus increasing doses of GHRP-6 (10^{-9}–10^{-5} mol/l) or ghrelin (10^{-7} mol/l). The right panels show compilations of semi-quantitative data on the steady-state levels of StAR, P450scc and 3β-HSD in the above experimental groups. Relative expression levels were obtained, in each sample, by normalization of absolute optical densities (OD) of the specific target to that of L19 signal. For presentation of data, expression values in the control group (samples incubated in medium alone) were taken as 100% and the others were normalized accordingly, thus allowing semi-quantitative comparison. Values are the means±S.E.M. of at least three independent determinations. Groups with different superscript letters are statistically different (P < 0.01; ANOVA followed by Tukey’s test).
Evidence for the expression of GHS-R gene in rat adrenal. These results are in keeping with previous findings on the presence of GHS binding sites in a number of peripheral human tissues, including the adrenal gland (12). Furthermore, our results are the first to demonstrate expression of the mRNA encoding the biologically active GHS-R form, i.e. GHS-R1a, in rat adrenal tissue; a phenomenon reported also very recently for human adrenal gland (41). In this sense, although direct assessment of GHS-R protein expression was not conducted, our data strongly suggest the expression of functional GHS-Rs in rat adrenal, as evidenced by the regulation of GHS-R gene expression by synthetic (GHRP-6) and natural (ghrelin) ligands, i.e. homologous regulation, which must be a receptor-mediated event.

Pituitary and hypothalamic expression of the GHS-R gene appears as a tightly regulated event, where both inhibitory and stimulatory signals interact to ensure adequate expression levels of this transcript. Indeed, pituitary and hypothalamic GHS-R mRNA levels are inhibited by GH (36, 42), whereas pituitary GHS-R expression is up-regulated by GH-releasing hormone (GHRH) (43). Additional heterologous signals involved in the regulation of GHS-R expression are sex steroids (44) and, notably, glucocorticoids (26). In contrast, little attention has been paid to homologous regulatory events. Evidence for down-regulation of pituitary GHS-R mRNA expression after exposure to the GHS L629, 585 in vivo was presented in a previous study (43). The physiological relevance of such a phenomenon has been substantiated by the recent identification of the endogenous ligand of GHS-R, ghrelin. Our results indicate that adrenal GHS-R gene expression is also under the regulation of homologous signals, and that such a phenomenon does take place in our short-term static in vitro setting, as demonstrated by semi- quantitative RT-PCR assays using primer pairs and conditions similar to those of previous studies on the hormonal regulation of net GHS-R gene expression at the pituitary and hypothalamus (26, 36, 43, 44). Interestingly, strong repression of basal adrenal GHS-R mRNA levels by endogenous ghrelin likely occurs, as evidenced by the significant increase in the expression of this transcript after 4-h incubation in serum-free medium. In contrast, dose-dependent inhibition of GHS-R mRNA expression after exposure to increasing doses of GHRP-6 was detected; an event that was mimicked by ghrelin. Notably, this phenomenon seems to be specific, as it was not observed after incubation of adrenal samples in the presence of signals unrelated to GHS-R, i.e. leptin (authors, unpublished results). From a physiological standpoint, our data indicate that expression of GHS-R gene in rat adrenal is subjected to a precise regulatory mechanism that involves the endogenous cognate ligand, ghrelin, and open up the possibility of novel, as yet undefined, roles of this molecule in adrenal function. The homologous down-regulation of adrenal GHS-R mRNA levels, reported herein, may contribute to the auto-limitation of the biological actions of ghrelin in this target tissue. Whether additional mechanisms of ligand-induced receptor desensitization, as uncoupling and internalization that take place at earlier stages following ligand–receptor interaction, also participate in this phenomenon awaits further investigation.

Our comparative analysis evidenced a strikingly different pattern of homologous regulation of GHS-R gene expression between the adrenal and pituitary glands. The pituitary is a major target for actions of both synthetic and natural ligands of GHS-R; thus, it was considered as an optimal standard tissue for comparison. In keeping with a previous report in vivo (43), pituitary GHS-R gene mRNA expression appeared under the regulation of the synthetic GHS, GHRP-6, in vitro. Moreover, a similar finding was observed for the natural counterpart, ghrelin. However, in contrast to observations at the adrenal level, short-term incubation of pituitary tissue in serum-free medium resulted in a significant 3.0-fold decrease in the relative GHS-R mRNA levels; a response that was prevented by 3-h incubation in the presence of GHRP-6 (10⁻⁹ – 10⁻⁷ mol/l) or ghrelin (10⁻⁷ mol/l). Considering that, in addition, a partial down-regulatory response was detected after exposure to increasing doses of GHRP-6 (10⁻⁵ mol/l), it is tempting to hypothesize that, rather than a simple down-regulatory event, homologous regulation of pituitary GHS-R gene expression is a dose-dependent, biphasic phenomenon. In this system, low levels of the ligand apparently up-regulate, whereas high levels down-regulate, the expression of the mRNA encoding the cognate receptor. If operative also in vivo, this model may be relevant for the proper understanding of the physiological regulation of biological actions of ghrelin in one of its major targets, the pituitary gland.

Interestingly, a pattern of homologous regulation of GHS-R mRNA expression roughly analogous to that of the pituitary has been recently observed by our group in the testis (M L Barreiro & M Tena-Sempere, in preparation), a tissue that also expresses functional GHS-Rs (28). Taken together, comparative evaluation of results from pituitary, adrenal and testis suggests that regulation of GHS-R mRNA expression by homologous signals is a rather widespread phenomenon. However, the precise nature of such an event appears to be tissue-specific. In fact, evidence for region-specific heterologous regulation of GHS-R gene expression in rat brain and pituitary has been presented recently (45). Overall, it is tempting to suggest that tissue- and region-specific regulation of GHS-R mRNA expression by homologous and heterologous signals likely reflect the divergent physiological roles of GHS-R signaling in different target systems.

On the basis of the well-documented corticotropic responses to systemic administration of GHS-R ligands...
in vitro 
atating whether synthetic and natural ligands of GHS-R
expression of GHS-R in rat adrenal, we aimed at eval-
(6, 8, 9, 18–20, 24) and our current results on the
expression of GHS-R in rat adrenal, we aimed at evalu-
worthy that we have recently obtained evidence on the
ability of ghrelin to directly regulate testosterone
secretion by testicular tissue in vitro (28). In our setting,
however, neither increasing doses of GHRP-6 (10^{-9}–
10^{-5} \text{ mol/l}) nor ghrelin (10^{-7} \text{ mol/l}) induced signif-
icant changes in the pattern of basal or ACTH-stimu-
lated corticosterone release by incubated adrenal
tissue. Such a lack of response closely mirrored the
absence of effects of GHRP-6 and ghrelin upon ACTH-
stimulated expression levels of the mRNAs encoding
StAR, P450scc and 3β-HSD, i.e. three key upstream
elements in the common steroidogenic pathway. In
this sense, regulation of steroidogenesis by various hor-
monal signals is tightly correlated with concomitant
changes in StAR and P450scc gene expression in differ-
ent experimental settings (39, 40, 46, 47). Thus, our
results cast doubts on potential direct actions of GHS-R
ligands in the regulation of adrenal steroidogenesis.
Interestingly, in a similar static in vitro system, ghrelin
was able to modulate StAR and P450scc mRNA
expression in rat testis (28). Again, these data are sug-
gestive of the tissue-specificity of the biological actions
of ligands of GHS-R. Nevertheless, further experimental
work, testing additional protocols of GHS stimulation
and target tissues, is needed to fully substantiate this
hypothesis.

Upon cloning of GHS-R and its endogenous ligand,
ghrelin, extensive experimental work has been con-
ducted to elucidate fully the physiological relevance of
this novel signaling system. Growing evidence indicates
that, in addition to their central actions in the control
of GH secretion and food intake, peripheral effects of
GHS-R ligands are likely to take place. This is suggested
by the wide range of endocrine and non-endocrine tis-
sues that possess GHS-binding sites and express the
GHS-R gene in humans (12, 41), and the novel expres-
sion of ghrelin in non-central tissues, such as placenta, kidney and pancreas (38, 48, 49). In good
agreement, we have recently gathered data on the
expression and functional role of ghrelin in rat testis
(28). Our present results extend those previous observ-
ations, and provide novel evidence for the expression
and homologous regulation of the GHS-R gene in rat
adrenal. The functional significance of this phenom-
eron remains to be determined. Previous findings
from our group regarding the tests, as another major
steroidogenic tissue, suggested that GHS-R signaling
may function as a regulator of steroid secretion (28).
However, on the basis of our current experimental
approach, the possibility of direct effects of GHS-R
ligands in the control of adrenal corticosterone
secretion appears unlikely. In addition, novel biological
actions of ghrelin, including regulation of energy
expenditure and metabolic responses to starvation as
well as modulation of cell proliferation and tumor
growth, have recently emerged (22, 49, 50). Whether
some of these effects involve direct adrenal actions
and/or take place at the adrenal level is yet to be estab-
lished. Future evaluation of additional end-points of
GHS/ghrelin action as well as identification of the site(s) of expression of GHS-R within rat adrenal will
certainly help to elucidate the role of this signaling
system in the control of adrenal function.

Acknowledgements

The authors thank Rocío Campón for her excellent
technical assistance. The authors are indebted to Dr C
Diéguez for helpful discussions during the design
and preparation of this manuscript. This work was sup-
ported by grant PM98-0163 from DGESIC (Ministerio
de Educación y Cultura, Spain) and project 1FD97-
0696-02 (FEDER).

References

1 Smith RG, van der Ploeg LHT, Howard AD, Feighner SD, Cheng K, Hickey GJ et al. Peptidomimetic regulation of growth hormone
2 Casanueva FF & Dieguez C. Growth hormone secretagogues: physiological role and clinical utilities. Trends in Endocrinology
and Metabolism 1999 10 30–38.
3 Kurtyama H, Hotta M, Wakabayashi T & Shibasaki T. A 6-day intracerebroventricular infusion of the growth hormone-releasing
peptide KP-102 stimulates food intake in both non-stressed and intermitten
4 Okada K, Ishii S, Minami S, Sugihara H, Shibasaki T & Wakabayashi I. Intracerebroventricular administration of growth
hormone-releasing peptide KP-102 increases food intake in free-
5 Korbonits M & Grossman AB. Growth hormone releasing peptide
445–460.
7 Carmignac DF, Bennett PA & Robinson ICAF. Effects of growth hormone secretagogues on prolactin release in anesthetized
8 Clark RG, Thomas GB, Mortensen DL, Won WR, Ma YH, Tomlinson EE et al. Growth hormone secretagogues stimulate the hypothala-
mic–pituitary–adrenal axis and are diabetogenic in the Zucker diabeti
9 Thomas GB, Fairhall KM & Robinson ICAF. Activation of the hypothalamo–pituitary–adrenal axis by the growth hormone
11 Tivesten A, Bollano E, Cavadhl K, Kujacic V, Sun XY, Hedner T et al. The growth hormone secretagogue hexarelin improves cardiac
GHS-receptor in rat adrenal


30 Onk RB, Krasnow JS, Beattie WG & Richards JS. Cyclic AMP dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scx) in rat ovarian granulosa cells and corpora lutea cDNA microarray and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. Journal of Biological Chemistry 1991 266 583 – 593.


39 Kineman RD, Kamegai J & Frohman LA. Growth hormone (GHRH) releasing hormone (GHRH) and the GH secretagogue (GHS), L629,585, differentially modulate rat pituitary GHS receptor and GHRH receptor messenger ribonucleic acid levels. Endocrinology 1999 140 3581 – 3586.


www.eje.org


Received 29 April 2002
Accepted 9 July 2002