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Novel Expression and Functional Role of Ghrelin in Rat Testis

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Ghrelin, the endogenous ligand for the GH-secretagogue receptor (GHS-R), is a recently cloned peptide, primarily expressed in the stomach and hypothalamus, that acts at central levels to elicit GH release and, notably, to regulate food intake. However, the possibility of additional, as yet unknown, peripheral effects of ghrelin cannot be ruled out. In the present communication, we provide evidence for the novel expression of ghrelin and its functional receptor in rat testis. Testicular ghrelin gene expression was demonstrated throughout postnatal development, and ghrelin protein was detected in Leydig cells from adult testis specimens. Accordingly, ghrelin mRNA signal became undetectable in rat testis following selective Leydig cell elimination. In addition, testicular expression of the gene encoding the cognate ghrelin receptor was observed from the infantile period to adulthood, with the GHS-R mRNA being persistently expressed after selective withdrawal of mature Leydig cells. From a functional standpoint, ghrelin, in a dose-dependent manner, induced an average 30% inhibition of human CG- and cAMP-stimulated T secretion *in vitro*. This inhibitory effect was associated with significant decreases in human CG-stimulated expression levels of the mRNAs encoding steroid acute regulatory protein, and P450 cholesterol side-chain cleavage, 3β-hydroxy steroid dehydrogenase, and 17β-hydroxy steroid dehydrogenase type III enzymes. Overall, our data are the first to provide evidence for a possible direct action of ghrelin in the control of testicular function. Furthermore, the present results underscore an unexpected role of ghrelin as signal with ability to potentially modulate not only growth and body weight homeostasis but also reproductive function, a phenomenon also demonstrated recently for the adipocyte-derived hormone, leptin. (*Endocrinology* 143: 717–725, 2002)

GHRELIN WAS RECENTLY identified as the endogenous ligand for GH secretagogue (GHS) receptor [GHS-R (1, 2)]. The GHSs are a group of synthetic compounds with ability to induce GH release in all species tested, including humans (3). The actions of GHSs are carried out through interaction with a specific G protein-coupled receptor, named GHS-R, distinct from that of GHRH (4, 5). Evidence for a GHRH-independent signaling system anticipated the existence of an endogenous counterpart of GHSs. Search for such a factor using an "orphan receptor strategy" finally resulted in the identification of ghrelin (1). Ghrelin is a 28-amino acid peptide with an essential *n*-octanoyl modification at Ser3 and is primarily expressed in stomach and hypothalamus (2). As expected for the endogenous ligand of GHS-R, this molecule has been proven to elicit GH secretion *in vivo* and from anterior pituitary cells in culture (1, 6, 7). Interestingly, besides its role in the control of GH release, ghrelin, likely from a stomach source and acting upon hypothalamic centers, has recently emerged also as an orexigenic food-intake-controlling signal (7–9).

Notably, the biological effects of ghrelin known to date are carried out at central levels, *i.e.* the hypothalamus and/or pituitary. However, additional as yet unknown peripheral actions of ghrelin cannot be ruled out. In this sense, it was shown recently that a wide range of endocrine and nonendocrine tissues possess GHS binding sites in humans (10). Moreover, novel expression of ghrelin in noncentral tissues, such as placenta and kidney, has been reported very recently (11, 12). Nevertheless, the functional roles, if any, of ghrelin in such peripheral systems remain unexplored.

The testis is a complex endocrine organ in which different cell types interplay in the fine tuning of the reproductive function under the control of a plethora of endocrine, paracrine, and autocrine regulatory signals (13). In recent years, it has become evident that different factors with key roles in the growth axis (*e.g.* GHRH and IGF-I) and body weight homeostasis (*e.g.* leptin) are potentially involved in the regulation of testicular function (14–17). The identification of ghrelin as a novel endogenous factor implicated in growth and body weight regulation (1, 2, 7–9) prompted us to evaluate whether this signal and its functional receptor are expressed in rat testis. Our current data are suggestive of a possible involvement of ghrelin signaling in the direct control of gonadal function in the male rat, underscoring an unexpected reproductive facet of this newly discovered molecule.

Materials and Methods

Animals and drugs

Wistar male rats bred in the vivarium of the University of Córdoba were used. The day the litters were born was considered d 1 of age. The animals were maintained under constant conditions of light (14 h of

Abbreviations: EDS, Ethylene dimethane sulfonate; GHS, GH secretagogue; GHS-R, GH-secretagogue receptor; hCG, human CG; HSD, hydroxy steroid dehydrogenase; P450scc, cytochrome P450 cholesterol side-chain cleavage; StAR, steroid acute regulatory.

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light, from 0700 h) and temperature (22 C) and were weaned at d 21 of age in groups of five rats with free access to pelleted food and tap water. 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. Rat ghrelin was purchased from Bachem AG (Bubendorf, Switzerland), highly purified human CG (hCG; Profasi) was obtained from Serono Laboratories, Inc. (Madrid, Spain), and dibutyryl-(Bu_2)-cAMP was supplied by Sigma (St. Louis, MO).

Experimental designs

Assessment of ghrelin expression in rat testis was carried out using different experimental approaches. First, testicular expression of ghrelin gene was evaluated by means of RT-PCR at different age points throughout postnatal development. In detail, based on previous reports on the timing of postnatal sex development in the rat (18), testis samples from 15-, 30-, 45-, 60-, and 90-d-old rats were assayed for expression of ghrelin mRNA. Secondly, expression and cellular location of ghrelin peptide within testicular tissue from adult rat specimens was studied by immunohistochemistry (see *Ghrelin immunohistochemistry*). In addition, because the latter suggested location of ghrelin protein in Leydig cells within rat testis, testicular ghrelin mRNA expression was analyzed at different time points after selective Leydig cell elimination by systemic administration of the cytotoxic drug ethylene dimethane sulfonate (EDS). In this model, mature Leydig cells are completely and selectively eliminated from the testicular interstitium within 24–48 h after administration of the toxicant *in vivo*, a phenomenon that is followed by reappearance of a newly formed population of Leydig cells in approximately 3–4 wk (Ref. 19 and references therein). Thus, this setting provides an optimal experimental background in which to test Leydig cell-specific expression of testis-derived factors (as an example, see Ref. 20).

In a second set of experiments, evaluation of testicular expression of the cognate receptor for ghrelin, *i.e.* GHS-R, was undertaken. Using an experimental approach similar to that used for analysis of the ligand, assessment of GHS-R mRNA expression was conducted in testis samples by RT-PCR at different representative age points of postnatal development: 15, 30, 45, and 75 d of age. In addition, testicular GHS-R mRNA expression was analyzed in a model of selective Leydig cell destruction. Thus, relative GHS-R mRNA levels were assayed in adult rat testis before 0, 5, 15, 20, 30, and 40 d after systemic administration of EDS.

In a third group of experiments, the potential functional role of ghrelin signaling in the control of testicular function was explored. To this end, assessment of the effect of ghrelin upon basal and stimulated T secretion *in vitro* was carried out using static incubations of adult rat testicular tissue, as described below. In addition to secretory responses, the effects of ghrelin on the mRNA expression levels of several key factors in the steroidogenic route were explored in this setting. In detail, four targets were evaluated: steroidogenic acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxy steroid dehydrogenase (HSD), and testis-specific 17β-HSD type III. They were selected given their crucial role as hormonally regulated and/or pivotal steps in T biosynthesis in rat testis (21–24).

RNA analysis by semiquantitative RT-PCR

Testicular expression of the mRNAs encoding ghrelin and its cognate GHS-R was assessed by semiquantitative RT-PCR. Similarly, this approach was used for analysis of the relative expression levels of the messages encoding StAR protein and enzymes P450scc, 3β-HSD, and 17β-HSD type III in incubated testicular tissue. Total RNA was isolated from testis samples from different experimental settings using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (25). 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 cDNA sequences of rat ghrelin (1) and GHS-R (5) and the factors of the steroidogenic pathway under analysis (24, 26–28), and whenever possible, they were selected based on previous references (11, 29). In addition, to provide an appropriate internal control, parallel amplification of a 290-bp fragment of L19 ribosomal protein mRNA was carried out in each sample using the primer pairs and conditions indicated in Table 1, as described in detail elsewhere (11, 17).

For amplification of the targets, reverse transcription and PCR were run in two separate steps. Furthermore, 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 below) but using similar amounts of the corresponding cDNA templates, generated in single RT reactions, as previously described elsewhere (17). Briefly, equal amounts of total testicular RNA (6 μg) were heat denatured and reverse transcribed by incubation at 42 C for 90 min with 12.5 U avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI), 20 U ribonuclease inhibitor RNasin (Promega Corp.), 200 nM deoxynucleoside 5′-triphosphate mixture, and 1 nM specific and L19 antisense primers in a final volume of 30 μl of 1× avian myeloblastosis virus reverse transcriptase buffer. The reactions were terminated by heating at 97 C for 5 min and cooling on ice, followed by dilution of the reverse transcriptase cDNA samples with nuclease-free H_2O (final volume, 60 μl). For semiquantitative 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 Corp.), 200 nM deoxy-nucleoside 5′-triphosphate mixture, and the appropriate primer pairs (1 nM of each primer; see Table 1). PCR consisted in 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 and StAR protein, 61 C for P450scc, 64 C for 3β-HSD, and 59 C for 17β-HSD type III. In addition, different numbers of cycles were tested to optimize amplification in the exponential phase of PCR (data not shown), and based on current data and previous references (11, 17), the PCR cycles indicated for each target in Table 1 were chosen for further analysis.

TABLE 1. Oligo-primer pairs used for RT-PCR amplification of ghrelin, GHS-R, and steroidogenic-related factor transcripts

Target	Oligo-Primers		Expected Size	PCR Cycles
Ghrelin	Ghrelin sense	5′-TTG AGC CCA GAG CAC CAG AAA-3′	347 bp	34
	Ghrelin antisense	5′-AGT TGC AGA GGA GGC AGA AGC T-3′		
GHS-R	GHS-R sense	5′-AGG CAA CCT GCT CAC TAT GCT G-3′	321 bp	34
	GHS-R antisense	5′-GAC AAG GAT GAC CAG CTT CAC G-3′		
StAR	StAR sense	5′-TGG GTG GAT GGG TCA GGT CC-3′	500 bp	36
	StAR antisense	5′-GCT CAG GCA TCT CCC CAA AGT-3′		
P450scc	P450scc sense	5′-AGA TCC CTT CCC CTG GTG ACA ATG-3′	509 bp	35
	P450scc antisense	5′-CCA GGC GCT CCC CAA ATA CAA CA-3′		
3β-HSD	3β-HSD sense	5′-CAG GAG CAG GAG GGT TTG T-3′	400 bp	34
	3β-HSD antisense	5′-GTG GCC ATT CAG GAT GAT-3′		
17β-HSDIII	17β-HSD sense	5′-CCT GAG ATC AAT GGG ACA ATG-3′	435 bp	37
	17β-HSD antisense	5′-CCC TAC TCC CGA AGA GAT A-3′		
L19	L19-sense	5′-GAA ATC GCC AAT GCC AAC TC-3′	290 bp	24
	L19-antisense	5′-ACC TTC AGG TAC AGG CTG TG-3′		

For each target, the primer pair used for amplification is included. In addition, the expected size of the generated cDNA products and the number cycles selected for semiquantitative RT-PCR analysis is indicated for each signal.

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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 (NewBiotechnic Ltd., Sevilla, Spain) or by Southern hybridization using radiolabeled nested oligonucleotide primers, as described elsewhere (11). In all assays, liquid controls and reactions without reverse transcriptase 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), with the values for the specific targets being normalized to those of internal controls.

Ghrelin immunohistochemistry

Detection of ghrelin protein was carried out in 4% paraformaldehyde-fixed sections of adult (75-d-old) rat testis using a rabbit antighrelin polyclonal antibody and the avidin-biotin-peroxidase complex method, as described in detail previously (11).

Tissue incubation and T measurements

The general procedure for static incubations of testicular tissue has been described in detail elsewhere (16, 17). In this setting, testis samples were incubated in fresh medium or medium containing increasing doses of ghrelin (10^{-9} to 10^{-7} M) alone (basal) or supplemented with human hCG (10 IU/ml; stimulated). Moreover, the ability of ghrelin to modulate cAMP-stimulated T secretion was tested in additional samples incubated with Bu_2-cAMP (10^{-4} M) alone or in combination with 10^{-7} M ghrelin. T was measured from diethyl ether extracts of incubation media, at 90 and 180 min, as described elsewhere (16). The levels of T in the media were expressed as normalized values per gram of incubated tissue. At the end of the incubation period, samples of testicular tissue from the different experimental groups were frozen in liquid nitrogen and stored at −70 C until used for RNA analysis (see *RNA analysis by semiquantitative RT-PCR*).

Presentation of data and statistics

RT-PCR analyses were carried out in triplicate using independent RNA samples. Tissue incubations were carried out in duplicate, with a total number of 12 determinations per group. When relevant, data are presented as mean ± SEM. Quantitative results were analyzed for statistically significant differences using ANOVA, followed by Tukey's test. Values of $P < 0.05$ were considered significant.

Results

Expression of ghrelin gene and protein in rat testis

Assessment of ghrelin mRNA expression by RT-PCR analysis, using a specific primer pair (11), demonstrated persistent expression of the gene in rat testis throughout postnatal development (Fig. 1). In detail, four representative stages of development were explored: infantile (15-d-old), prepubertal (30-d-old), pubertal-early adult (45-d-old), and adult (60- and 90-d-old). Quantitative analysis of the RT-PCR signals revealed, however, that the expression levels of ghrelin message in rat testis changed along the study period, with the highest values being detected during the adult period. In addition, clear-cut ghrelin immunostaining was observed in the testicular interstitium of adult rat specimens using a rabbit antighrelin polyclonal antibody. In contrast, negligible staining in the seminiferous tubules was impossible to differentiate from background and was considered negative. In the interstitial areas, ghrelin peptide was strongly located in mature Leydig cells (Fig. 2). In good agreement, testicular expression of ghrelin mRNA became undetectable 5 and 15 d after administration of the Leydig cell toxicant EDS. In this time frame, testicular interstitium is completely devoid of

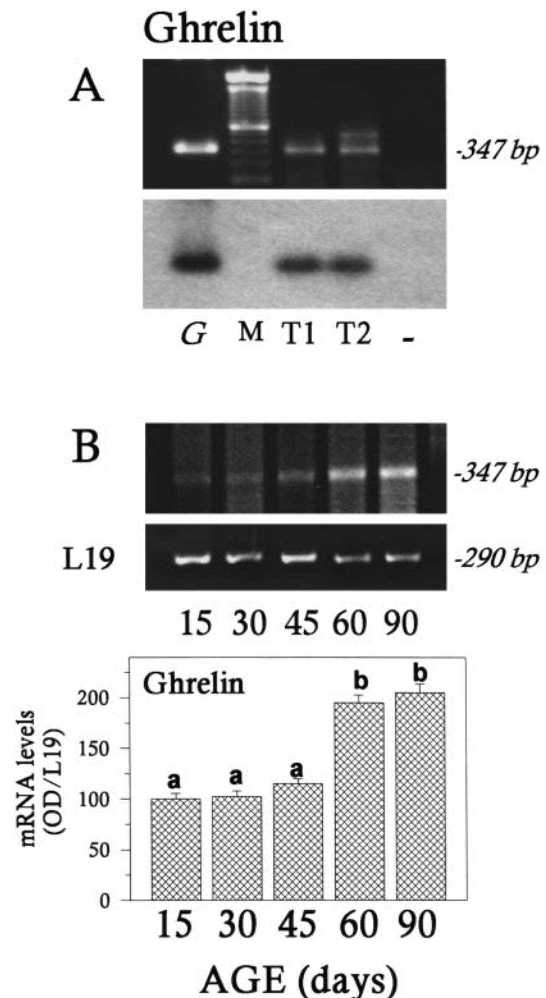

FIG. 1. Expression of ghrelin gene in rat testis. A, Representative RT-PCR assay of the expression levels of ghrelin mRNA in two independent testicular samples (T1 and T2) is presented. In addition, positive (rat ghrelin cDNA, G) and negative (liquid) controls are shown. A 100-bp mol wt marker (M) was used. Specificity of the amplicons was demonstrated by Southern hybridization using a nested oligo-primer. B, Representative RT-PCR analysis of ghrelin mRNA expression in testicular samples from 15-, 30-, 45-, 60-, and 90-d-old rats is shown. Amplification of L19 ribosomal protein mRNA served as internal control. In addition, semiquantitative data on the expression levels of ghrelin mRNA in testicular samples along postnatal development are presented. Relative expression levels were obtained, in each sample, by normalization of absolute ODs of the specific target to that of L19 signal. Values are the mean ± SEM of at least three independent determinations. *Groups with different letters above them* are statistically different (ANOVA followed by Tukey's test).

mature Leydig cells (Ref. 19 and references therein). Along with Leydig cell repopulation, reappearance of ghrelin mRNA was detected in rat testis 30 and 40 d after EDS administration (Fig. 3).

Gene expression of the cognate ghrelin receptor in rat testis

Expression of the mRNA encoding the cognate ghrelin receptor, *i.e.* the GHS-R, was evaluated by RT-PCR using a specific primer pair (29). Such an analysis demonstrated persistent expression of the message in rat testis throughout

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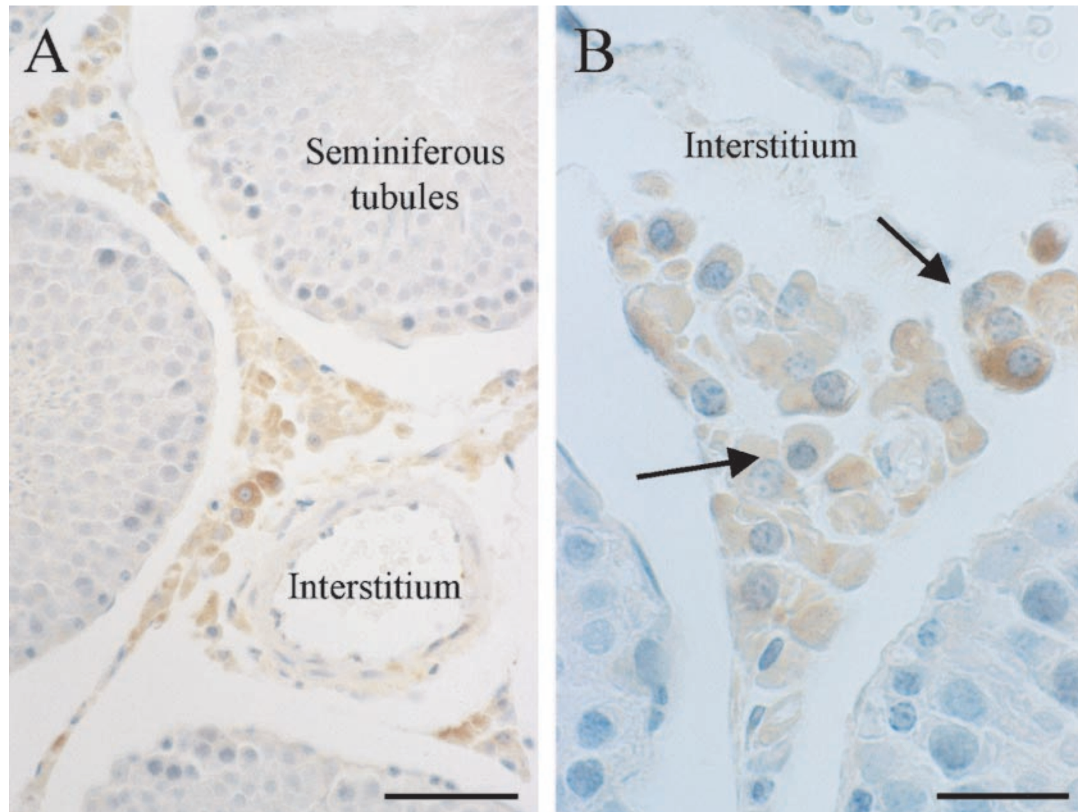

FIG. 2. Sections of adult (75-d-old) rat testis immunostained with a rabbit antighrelin polyclonal antibody and counterstained with hematoxylin. Clear immunostaining is observed in the testicular interstitium (A). Within the interstitium, Leydig cells (B, *arrows*) showed strong immunostaining. *Scale bar*, 150 μm in A and 50 μm in B.

postnatal development, from infantile period to adulthood (Fig. 4). Quantitative evaluation of the intensity of amplicons indicated maximum expression levels of GHS-R mRNA in adult samples, in keeping with above observations on the pattern of testicular ghrelin expression. However, unlike the ligand, minimum expression levels of GHS-R mRNA were detected in pubertal 45-d-old testis samples.

In addition, expression of GHS-R mRNA was assayed in testicular samples at different time points after selective Leydig cell elimination *in vivo* by EDS. In this setting, expression of GHS-R message was persistently detected at rather constant relative levels throughout the study period (Fig. 5). In detail, 5 and 15 d after EDS administration, a period when no mature Leydig cells are detected in the testicular tissue (19), positive amplification of GHS-R signal was clearly obtained, thus suggesting an alternative cell source for testicular expression of GHS-R gene.

Inhibition of stimulated T secretion by ghrelin: evidence for expression of functional ghrelin receptors in rat testis

The potential involvement of ghrelin signaling in rat testicular steroidogenesis was explored using an static *in vitro* system. First, secretory responses to ghrelin, in terms of T secretion, were assessed after 90 and 180 min of incubation. Basal T secretion by incubated testicular tissue remained unaffected in the presence of increasing doses of ghrelin (10^{-9} to 10^{-7} M). In contrast, 10 IU/ml hCG-stimulated T secretion was significantly inhibited by ghrelin, at both time-points tested, in a dose-dependent manner: 10^{-9} M was ineffective, whereas similar inhibitory responses were observed after 10^{-8} and 10^{--7} M ghrelin exposure (Fig. 6). In good agreement, 10^{-4} M cAMP-stimulated T secretion *in vitro* was significantly inhibited by coexposure to an effective 10^{-7} M dose of ghrelin (Fig. 7).

In addition, to explore further the above inhibitory action, the effects of ghrelin upon hCG-stimulated T secretion were correlated with responses in terms of mRNA expression levels of several steroidogenic key factors. The targets to be analyzed were selected based on their pivotal role as hormonally regulated and/or key steps in T biosynthesis (21–24). Stimulation of testicular tissue for 180 min with 10 IU/ml hCG induced an approximately 2.5-fold increase in StAR and P450scc mRNA levels, whereas marginal but significant approximately 50% increases in 3β-HSD and 17β-HSD mRNA levels were also detected. As was the case for the secretory responses, exposure to increasing doses of ghrelin (10^{-9} to 10^{-7} M) induced a significant inhibition of hCG-stimulated mRNA expression levels of the steroidogenic factors under analysis. In detail, dose-dependent inhibitory responses in terms of StAR and P450scc mRNAs were observed that closely paralleled those of T secretion: 10^{-9} M ghrelin was ineffective, whereas 10^{-8} and 10^{-7} M doses were similarly inhibitory. In the case of 3β-HSD and 17β-HSD, hCG-stimulated mRNA levels were inhibited by ghrelin at all doses tested (Fig. 8).

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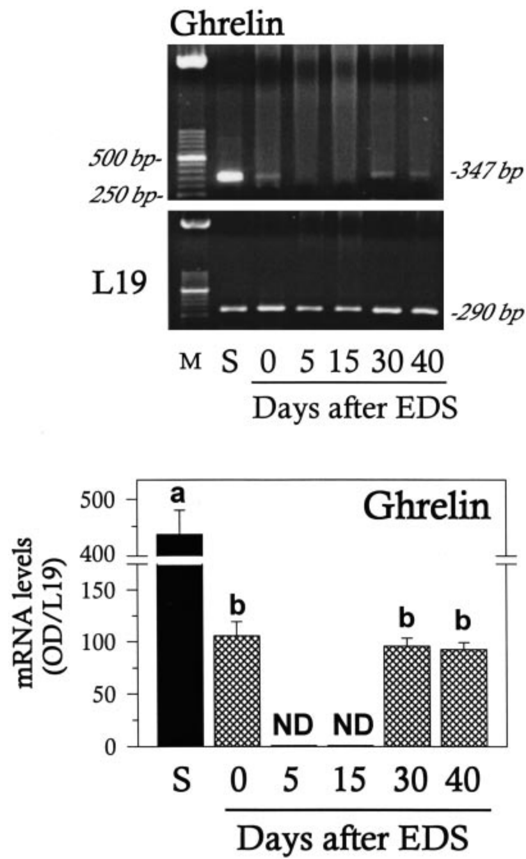

FIG. 3. Expression of ghrelin mRNA in rat testis before 0 and at different time points after administration of the cytotoxic drug EDS. In this model, mature Leydig cells are rapidly (within 24–48 h) and selectively eliminated from testicular interstitium, a response that is followed by reappearance of a population of newly formed Leydig cells within 3–4 wk. In the *upper panel*, a representative RT-PCR assay of expression levels of ghrelin mRNA in testicular samples from adult rats before 0, 5, 15, 30, and 40 d after EDS administration is presented. As positive control, amplification of ghrelin signal from rat stomach (S) is shown. A 50-bp mol wt marker (M) was used. Amplification of L19 ribosomal protein mRNA served as internal control. In the *lower panel*, semiquantitative data on the expression levels of ghrelin mRNA in the experimental groups are presented. Relative expression levels were obtained in each sample by normalization of absolute ODs of the specific target to that of L19 signal. Values are the mean ± SEM of at least three independent determinations. *Groups with different letters above them* are statistically different (ANOVA followed by Tukey's test). ND, Not detectable.

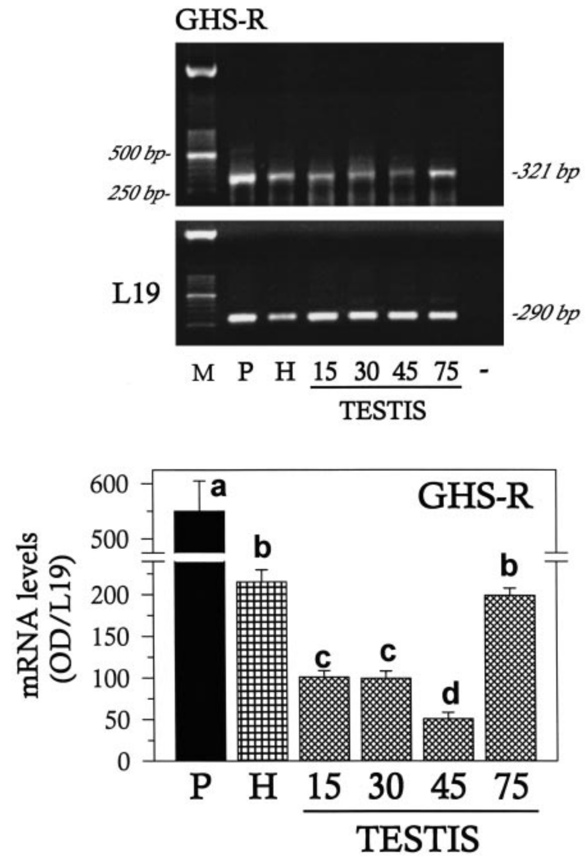

FIG. 4. Expression of the gene encoding GHS-R, *i.e.* the cognate ghrelin receptor, in rat testis. In the *upper panel*, a representative RT-PCR assay of expression levels of GHS-R mRNA in testicular samples from 15-, 30-, 45-, and 75-d-old rats is presented. In addition, positive [pituitary (P) and hypothalamus (H)] and negative (liquid) controls are shown. A 50-bp mol wt marker (M) was used. Amplification of L19 ribosomal protein mRNA served as internal control. In the *lower panel*, semiquantitative data on the expression levels of GHS-R mRNA in testicular samples along postnatal development are presented. Relative expression levels were obtained in each sample by normalization of absolute ODs of the specific target to that of L19 signal. Values are the mean ± SEM of at least three independent determinations. *Groups with different letters above them* are statistically different (ANOVA followed by Tukey's test).

Discussion

In the present work, we provide compelling evidence for the expression of ghrelin and its functional receptor in rat testis. To date, the biological effects of ghrelin have been restricted to its ability to induce GH release acting through pituitary and/or hypothalamic GHS-Rs (1, 7), and to stimulate food intake through modulation of hypothalamic NPY and/or agouti-related protein expression (7, 8). Our results are in keeping with previous findings on the expression of GHS binding sites and ghrelin itself in peripheral tissues (10–12), and to our knowledge, are the first to demonstrate a biological action of ghrelin in a noncentral endocrine organ, the testis.

Identification of testicular expression of ghrelin was accomplished by molecular (RT-PCR and Southern hybridization) and immunological approaches. Our analyses demonstrated that ghrelin gene is expressed in rat testis throughout postnatal development, although the relative mRNA levels changed sharply along the study period: the lowest expression values were detected in infantile-prepubertal testicular samples, whereas the highest levels were observed during the adult (60- and 90-d-old) period. By means of immunohistochemistry, ghrelin protein within the testis structure was located with high selectivity in interstitial Leydig cells, *i.e.* the steroidogenic cell-type of the testis. In good agreement, ghrelin mRNA expression became undetectable in rat testis after selective withdrawal of adult-type Leydig cells by administration of the cytotoxic compound EDS. Conversely, repopulation of this cell type was associated to recovery of testicular ghrelin mRNA signal. Moreover, our preliminary immunohistochemical analysis demonstrated absence of ghrelin protein in testis tissue at early stages (*i.e.* 5 and 15 d)

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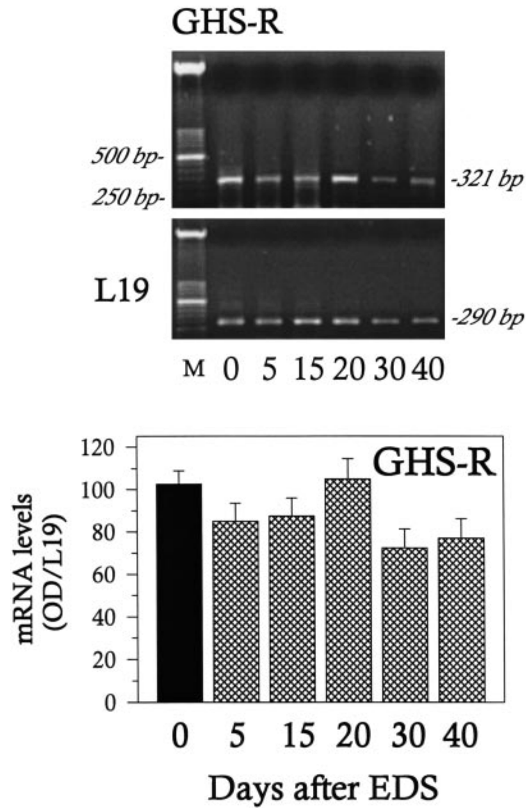

FIG. 5. Expression of GHS-R mRNA in rat testis before (0) and at different time points after Leydig cell withdrawal by administration of the cytotoxic drug EDS. In the *upper panel*, a representative RT-PCR assay of expression levels of GHS-R mRNA in testicular samples from adult rats before 0, 5, 15, 20, 30, and 40 d after EDS administration is presented. A 50-bp mol wt marker (M) was used. Amplification of L19 ribosomal protein mRNA served as internal control. In the *lower panel*, semiquantitative data on the expression levels of GHS-R mRNA in the experimental groups are presented. Relative expression levels were obtained in each sample by normalization of absolute ODs of the specific target to that of L19 signal. Values are the mean ± SEM of at least three independent determinations. No statistically significant differences between groups were detected (ANOVA followed by Tukey's test).

after EDS administration (data not shown). Overall, our present results strongly indicate that Leydig cells are the primary source of ghrelin expression in rat testis.

In addition to the cognate ligand, our current data document the expression of functional receptors for ghrelin in rat testis. In this sense, RT-PCR analysis was performed to evaluate whether the message encoding the previously cloned GHS-R (4, 5) is expressed in rat testis. Our assays demonstrated positive amplification of GHS-R signal in testicular samples at different stages of postnatal development. As was the case for the ligand, expression levels of GHS-R message changed throughout the period under analysis, with the highest expression levels being detected in adult tissue. In this sense, although subtle differences in the pattern of temporal expression of the messages encoding the GHS-R and ghrelin itself can be noted, it is apparent from our analyses that both genes are maximally expressed in rat testis at the adult age. Worthy to note, expression of GHS-R mRNA in adult testes was persistently detected after selective Leydig

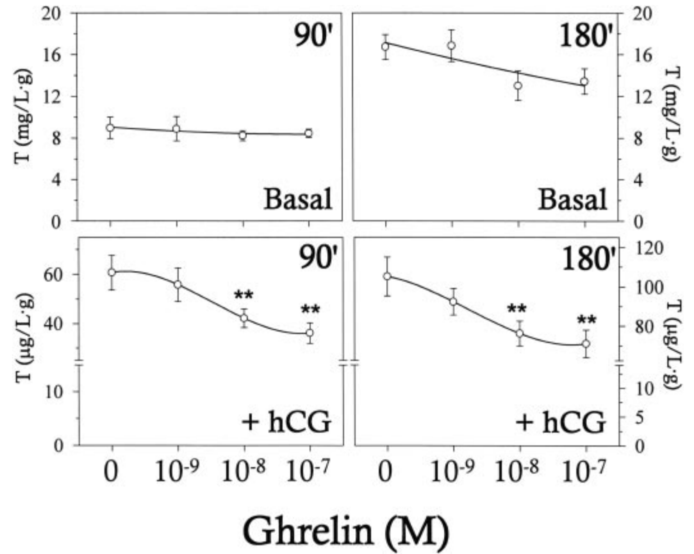

FIG. 6. Inhibition of hCG-stimulated T secretion *in vitro* by ghrelin. Testicular slices were incubated with increasing doses of ghrelin (10^{-9} to 10^{-7} M; basal secretion) or coincubated with hCG (10 IU/ml) and ghrelin (10^{-9} to 10^{-7} M; stimulated secretion). Groups of samples incubated with medium or hCG (10 IU/ml) alone served as respective controls. The pattern of hormone release after 90 and 180 min of incubation is presented. Values were normalized per gram of incubated tissue. Data are expressed as mean ± SEM (n = 10–12 samples/group). **, $P < 0.01$ *vs.* corresponding controls (ANOVA followed by Tukey's test).

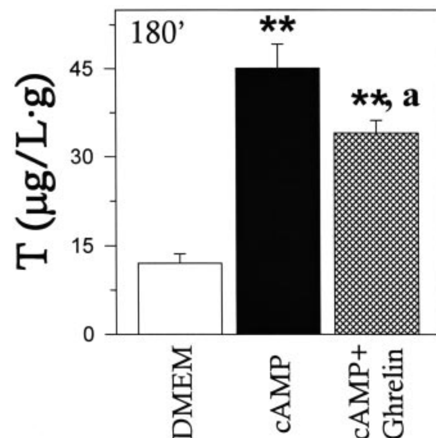

FIG. 7. Inhibition of cAMP-stimulated T secretion *in vitro* by ghrelin. Testicular slices were incubated with 10^{-4} M Bu_2-cAMP in the presence or absence of an effective dose of ghrelin (10^{-7} M). As the pattern of hormone release was similar after 90 and 180 min of incubation, only data from the latter time point are presented. Values were normalized per gram of incubated tissue. Data are expressed as mean ± SEM (n = 10–12 samples/group). **, $P < 0.01$ *vs.* controls; a, $P < 0.01$ *vs.* cAMP-treated group (ANOVA followed by Tukey's test).

cell destruction by EDS, thus suggesting that, unlike the cognate ligand, the major cellular source of testicular GHS-R signal is not Leydig cells. However, expression of GHS-R in this cell type cannot be ruled out on the basis of our current data.

Further evidence on the expression of functional ghrelin receptors in rat testis is provided by our studies using incubated testicular tissue. In this setting, basal T secretion

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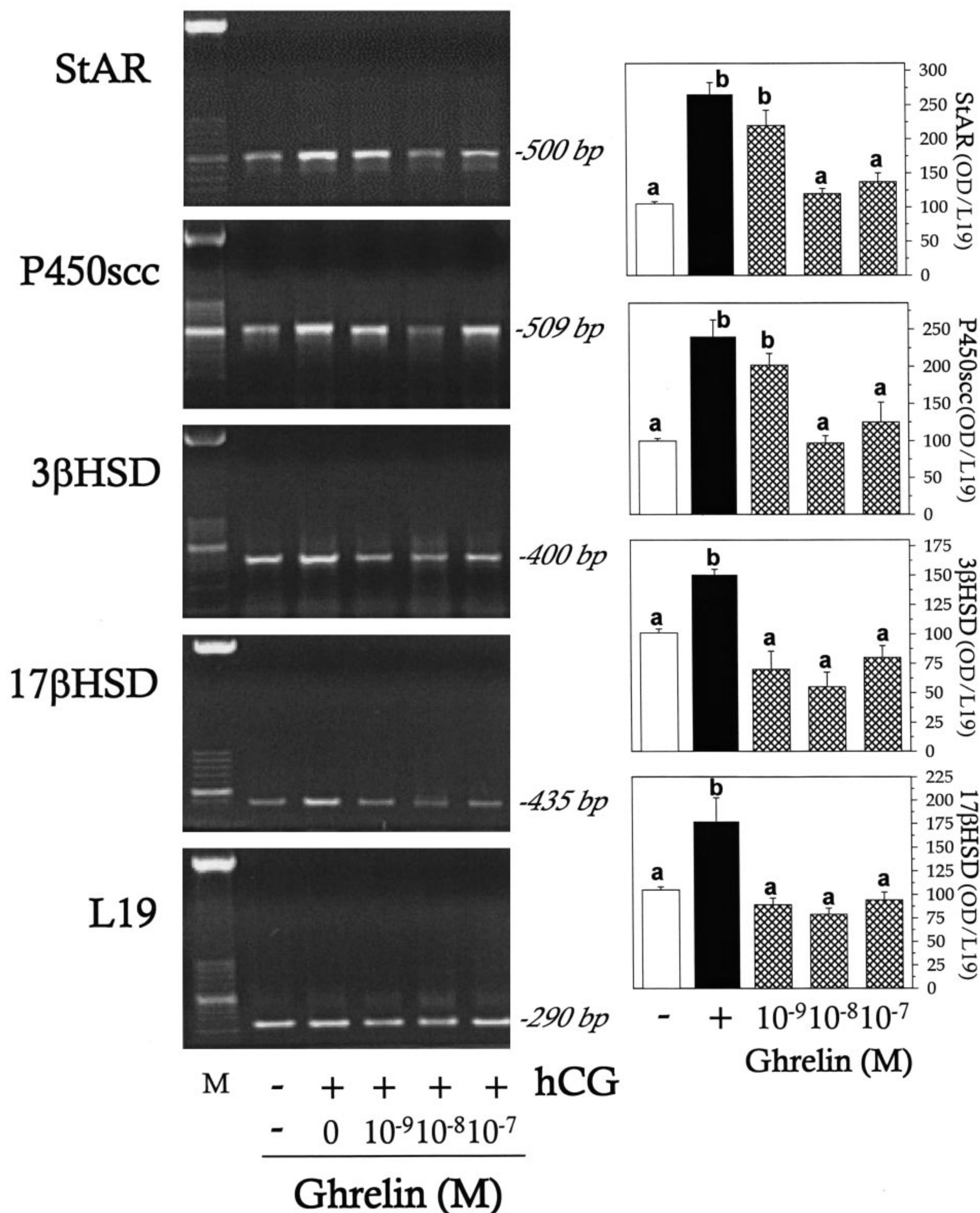

FIG. 8. Effects of ghrelin upon hCG-stimulated mRNA expression levels of StAR, P450scc, 3β-HSD, and testis-specific 17β-HSD type III in rat testis. *Left*, Representative semiquantitative RT-PCR assays of expression levels of the targets in testicular samples incubated in the presence of medium, 10 IU/ml hCG alone, or hCG plus increasing doses of ghrelin (10^{-7} to 10^{-9} M). *Right*, Compilation of semiquantitative data on the steady-state levels of StAR, P450scc, 3β-HSD, and 17β-HSD mRNAs in hCG-stimulated testicular samples challenged with increasing concentrations of ghrelin. Relative expression levels were obtained in each sample by normalization of absolute ODs of the specific target to that of L19 signal. Values are the mean ± SEM of at least three independent determinations. *Groups with different letters above them* are statistically different (ANOVA followed by Tukey's test).

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remained unaffected after exposure to increasing concentrations of ghrelin. However, ghrelin, in a dose-dependent manner, was able to significantly inhibit both hCG- and cAMP-stimulated T release *in vitro*. The mechanisms and cell types involved in such an inhibitory response are presently under investigation. The fact that ghrelin equally decreased hCG- and cAMP-induced T secretion indicates that this inhibitory action must take place in a step beyond cAMP formation. Concerning cell types involved, our results *in vitro* are compatible either with a direct inhibitory effect of ghrelin upon the steroidogenic Leydig cells, or indirect actions mediated through other testicular cell type(s). In favor of the latter, invariant levels of GHS-R mRNA were detected after selective Leydig cell destruction (see Fig. 5). Moreover, our preliminary functional analyses, including assessment of expression and hormonal regulation of GHS-R gene in testicular cell lines and tissue, as well as evaluation of the ability of ghrelin to modulate gene expression of several non-Leydig cell products, strongly suggest that Leydig cells are not the primary testicular target of ghrelin (Tena-Sempere, M., and M. L. Barreiro, manuscript in preparation). Direct assessment of the cellular location of GHS-R and biological actions of ghrelin in purified testicular cell preparations (*e.g.* Sertoli and Leydig cells) will help to identify the targets of this molecule within the rat testis.

The inhibitory effect of ghrelin upon T secretion was associated with a significant decrease in hCG-stimulated expression levels of the mRNAs encoding several key factors in the steroidogenic route: StAR and enzymes P450scc, 3β-HSD, and testis-specific 17β-HSD type III. It must be stressed, however, that causative relationship between these phenomena is yet to be proven. Nevertheless, our data showing that ghrelin was able to consistently inhibit stimulated T secretion, both after coincubation with hCG or Bu_2-cAMP, and to decrease mRNA expression levels of several key steroidogenic factors strongly suggest that functional ghrelin receptors are expressed in rat testis and that ghrelin signaling negatively regulates testicular steroidogenic function. In our laboratory, we are currently assessing the effects of blockade of endogenous ghrelin upon testicular T secretion *in vivo* to evaluate the physiological relevance of such a phenomenon.

Interestingly, the pattern of response to ghrelin in terms of StAR and P450scc mRNA expression closely mirrored that observed in terms of T release: a lack of inhibitory effect of 10^{-9} M ghrelin was followed by significant decreases after challenge with 10^{-8} to 10^{-7} M ghrelin. In this sense, cholesterol translocation to the inner mitochondrial membrane (StAR-mediated event) and its subsequent conversion to pregnenolone (P450scc-mediated event) are the first and rate-limiting steps in steroid biosynthesis (21, 22). Moreover, it is well documented that regulation of steroidogenesis by various hormonal signals is tightly correlated with concomitant changes in StAR and P450scc gene expression in different experimental settings (21, 22, 30, 31). However, the possibility that ghrelin-induced decrease in StAR and P450scc expression levels may directly contribute to the inhibition of stimulated T secretion after exposure to ghrelin *in vitro* must be substantiated by additional experimental work, including analysis of protein expression and/or activity of the above steroidogenic factors. From a general standpoint, the facts that the three major steroidogenic tissues, namely adrenal, testis, and ovary, possess high amounts of GHS binding sites in humans (10) and that, besides the testis, GHS-R gene is expressed in rat adrenal and ovary (our unpublished observation) make it worthy to evaluate the potential effects and mechanism(s) of action of ghrelin upon the steroidogenic function in different systems.

Notably, a similar direct inhibitory action on testicular T secretion was recently documented for leptin, the adipocyte-derived plasma hormone (16, 32). Both leptin and ghrelin appear as regulatory signals in growth and body weight homeostasis (1, 2, 7, 8, 33). Moreover, the involvement of leptin in the control of the reproductive axis has been well established (33). In this context, our current data on the expression of ghrelin and its functional receptor in rat testis open up the possibility that ghrelin may represent an additional regulatory signal linking growth, food intake, and reproductive function.

The testis is a complex endocrine organ in which different cell types cooperate to ensure adequate male fertility. Besides pituitary gonadotropins, an ever-growing group of extragonadal and intragonadal hormones and growth factors have been implicated in recent years in the control of testicular function (13). Overall, the results presented herein strongly suggest that ghrelin participates in such a regulatory network, thus providing evidence for an unexpected reproductive facet of this newly discovered molecule.

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