BIOLOGY OF REPRODUCTION **67,** 1768–1776 (2002)
Published online before print 04 October 2002.
DOI 10.1095/biolreprod.102.006965

Cellular Location and Hormonal Regulation of Ghrelin Expression in Rat Testis[1]

M.L. Barreiro,[3] F. Gaytán,[3] J.E. Caminos,[4] L. Pinilla,[3] F.F. Casanueva,[5] E. Aguilar,[3] C. Diéguez,[4] and M. Tena-Sempere[2,3]

Department of Cell Biology, Physiology, and Immunology,[3] University of Córdoba, 14004 Córdoba, Spain
Departments of Physiology[4] and Medicine,[5] University of Santiago de Compostela, 15705 Santiago de Compostela, Spain

ABSTRACT

Ghrelin, the endogenous ligand for the growth hormone-secretagogue receptor, is a recently cloned 28-amino acid peptide, expressed primarily in the stomach and hypothalamus, with the ability to stimulate growth hormone (GH) release and food intake. However, the possibility of additional, as yet unknown biological actions of ghrelin has been suggested. As a continuation of our recent findings on the expression and functional role of ghrelin in rat testis, we report here the pattern of cellular expression of ghrelin peptide in rat testis during postnatal development and after selective Leydig cell elimination, and we assess hormonal regulation of testicular ghrelin expression, at the mRNA and/or protein levels, in different experimental models. Immunohistochemical analyses along postnatal development demonstrated selective location of ghrelin peptide within rat testis in mature fetal- and adult-type Leydig cells. In good agreement, ghrelin protein appeared undetectable in testicular interstitium after selective Leydig cell withdrawal. In terms of hormonal regulation, testicular ghrelin mRNA and protein expression decreased to negligible levels after long-term hypophysectomy, whereas replacement with human chorionic gonadotropin (CG) (as superagonist of LH) partially restored ghrelin mRNA and peptide expression. Furthermore, acute administration of human CG (25 IU) to intact rats resulted in a transient increase in testicular ghrelin mRNA levels, with peak values 4 h after injection, an effect that was not mimicked by FSH (12.5 IU/rat). In contrast, testicular expression of ghrelin mRNA remained unaltered in GH-deficient rats, under hyper- and hypothyroidism conditions, as well as in adrenalectomized animals. In conclusion, our results demonstrate that mature Leydig cells are the source of ghrelin expression in rat testis, the protein being expressed in both fetal- and adult-type Leydig cells. In addition, our data indicate that testicular expression of ghrelin is hormonally regulated and is at least partially dependent on pituitary LH.

gene regulation, Leydig cells, luteinizing hormone, testis

INTRODUCTION

Ghrelin is a 28-amino acid peptide with an essential *n*-octanoyl modification at Ser3, recently identified as the endogenous ligand for the growth hormone (GH) secretagogue (GHS) receptor [1, 2]. This peptide is primarily expressed in the stomach and hypothalamus [1, 2], and, as anticipated for the endogenous counterpart of GHSs, it is able to elicit GH secretion from anterior pituitary cells in culture and in a variety of species in vivo [1, 3–5], thus suggesting the involvement of this novel molecule in the physiological control of the growth axis. Moreover, in addition to its hallmark effects on GH release, ghrelin has been proven to induce a number of biological responses acting at central neuroendocrine levels, including stimulation of food intake and adiposity [4, 6, 7] as well as modulation of corticotropic and gonadotropic axes [3–5, 8, 9].

Besides its central biological effects, growing evidence strongly suggests that additional, as yet poorly characterized, peripheral actions of ghrelin are likely to take place. In this sense, novel expression of ghrelin in noncentral tissues, such as placenta and kidney, has been reported very recently [10, 11]. Furthermore, a wide range of endocrine and nonendocrine human tissues has been shown to possess GHS/ghrelin binding sites [12]. However, the physiological relevance of ghrelin signaling in such peripheral systems remains to be fully established.

In keeping with data pointing to peripheral actions of ghrelin, we have recently provided evidence for the expression of ghrelin and its functional GHS receptor in rat testis [13], thus underscoring an unexpected, potential role of this molecule in the direct control of gonadal function. Notably, the testis is a complex endocrine organ where different cell types interplay to ensure male fertility, under the control of an array of extragonadal and intragonadal hormones and growth factors [14, 15]. Our initial data suggest that ghrelin may participate in such a regulatory network [13]. Thus, to expand our knowledge on the role of ghrelin in testicular function, in the present study, we evaluated the pattern of cellular expression of ghrelin peptide in rat testis during postnatal development and after selective Leydig cell elimination. In addition, hormonal regulation of testicular ghrelin expression, at the mRNA and/or protein levels, was assessed using different experimental paradigms, including the hypophysectomized rat (with or without human chorionic gonadotropin [CG] replacement treatment), the intact rat after acute administration of human CG or recombinant FSH, the GH-deficient rat, and the adrenalectomized rat, as well as models of hyper- and hypothyroidism. On this point, the relevant role of gonadotropins and other endocrine signals, as GH, glucocorticoids, and thyroid hormones, in the control of testicular function has been firmly established previously [16–19]. Overall, the proposed analysis would enable us to characterize in detail the developmental and hormonal regulation of ghrelin expression in rat testis, thus paving the way for a better understanding of the potential role of this newly discovered molecule in testicular physiology.

[1]This work was supported by grant PM-98-0163 from DGESIC (Ministerio de Educación y Cultura, Spain) and project 1FD97-0696-02 (FEDER).
[2]Correspondence: Manuel Tena-Sempere, Physiology Section, Department of Cell Biology, Physiology, and Immunology, Faculty of Medicine, University of Córdoba, Avda. Menéndez Pidal s/n, 14004 Córdoba, Spain. FAX: 34 957 218288; e-mail: fi1tesem@lucano.uco.es

Received: 1 May 2002.
First decision: 20 May 2002.
Accepted: 25 June 2002.

ISSN: 0006-3363. http://www.biolreprod.org

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 Day 1 of age. The animals were maintained under constant conditions of light (14 h of light beginning at 0700 h) and temperature (22°C) and were weaned at Day 21 of age in groups of five rats with free access to pelleted food and tap water. In addition, groups of adult (75-day-old) hypophysectomized Wistar male rats were purchased from Criffa (Barcelona, Spain) and male normal Lewis and Lewis-derived dwarf (*dw*) rats were obtained from the vivarium of the University of Santiago de Compostela (Spain). 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. Highly purified human CG (Profasi) was purchased from Serono (Madrid, Spain). Human recombinant FSH was kindly provided by Prof. J.A.F. Tresguerres (Gonal-F, Serono). Ethylene dimethane sulfonate (EDS) was synthesized as previously described [20] and dissolved in dimethylsulfoxide: water (1:3, vol/vol). Amino-triazole and L-thyroxine were obtained from Sigma (St. Louis, MO).

Experimental Designs

In a first set of experiments, the pattern of expression and cellular location of ghrelin peptide within rat testicular tissue was assessed throughout postnatal development and after selective Leydig cell elimination. Thus, based on previous reports on the timing of sex development of the rat [21], samples of testicular tissue from 1-, 5-, 15-, 20-, 30-, 45-, and 75-day-old rats, i.e., corresponding to neonatal (1-day), infantile (5-day), prepubertal (15- and 20-day), pubertal-early adult (30- and 45-day), and adult (75-day) periods, were subjected to immunohistochemical analysis using an anti-ghrelin polyclonal antibody (see below). In addition, expression of ghrelin peptide was studied at early stages (i.e., 5 and 15 days) after selective Leydig cell elimination by systemic administration of the cytotoxic drug EDS (in a single dose of 75 mg/kg i.p.). In this model, mature Leydig cells are completely and selectively eliminated from the testicular interstitium within 24–48 h of administration of the toxicant in vivo [22], thus providing an optimal experimental background in which to test Leydig cell-specific expression of testis-derived factors (e.g., [23, 24]).

In a second set of experiments, hormonal regulation of ghrelin expression in rat testis was evaluated at the mRNA and/or protein levels by means of semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. To this end, testicular ghrelin mRNA and peptide expression was comparatively analyzed in control and long-term hypophysectomized (HPX) rats, i.e., 4 wk after pituitary removal. Moreover, expression of ghrelin mRNA and peptide was studied in testes from long-term HPX rats injected i.p. with human CG (10 IU/rat/24 h; hCG as superagonist of LH) for 7 days before sampling. In addition, expression levels of ghrelin mRNA were assayed in testes from adult intact rats injected i.p. with hCG (25 IU/rat) or human recombinant FSH (12.5 IU/rat) and sampled 2, 4, 8, and 24 h after administration. Similarly, to evaluate regulation of testicular ghrelin gene expression by hormonal signals other than gonadotropins, testicular ghrelin mRNA levels were determined by semiquantitative RT-PCR in animal models of GH deficiency and hyper- and hypothyroidism as well as in adrenalectomized rats. As a model for GH deficiency, a dwarf rat strain derived from the Lewis rat, which is selectively deficient in GH synthesis with the remaining hormonal parameters within the normal ranges [25], was used. Hypothyroidism was induced as previously described [26] by administration of 0.1% amino-triazole in drinking water for a period of 3 wk. Hyperthyroidism was induced by chronic subcutaneous administration of L-thyroxine (100 μg/day, dissolved in 200 μl of saline). As an index of treatment efficiency, amino-triazole administration significantly increased plasma thyroid stimulating hormone (TSH) levels (control rats: 3.17 ± 0.38 ng/ml; amino-triazole treated rats: 27.17 ± 1.71 ng/ml; $P < 0.05$), whereas administration of L-thyroxine significantly decreased plasma TSH levels (0.42 ± 0.09 ng/ml versus 3.17 ± 0.38 ng/ml in control animals; $P < 0.05$). Bilateral adrenalectomy was performed by the dorsal approach under ketamine/xilazine (5 mg/kg) anesthesia. Adrenalectomized and control sham-operated rats were maintained on 0.9% NaCl and 0.6% glucose in drinking water. An additional control sham-group with tap drinking water was included. Sampling of testicular tissue was carried out 7 days after adrenalectomy.

Ghrelin Immunohistochemistry

Detection of ghrelin protein was carried out in 4% paraformaldehyde-fixed sections of rat testes sampled at different age points of postnatal development, 5 days after EDS administration and from long-term HPX rats, with or without human CG replacement treatment (see *Experimental Designs*). For analysis, a rabbit anti-ghrelin polyclonal antiserum, kindly provided by Drs. Kojima and Kangawa (Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan), was used as the primary antibody. This antibody was generated as described in detail elsewhere [27] using [Cys0]-rat ghrelin (13–28) as antigen. High specificity of the antiserum is demonstrated by absence of significant cross-reactivity with other peptides, as reported earlier [28]. Ghrelin immunostaining was conducted in 5-μm-thick paraffin sections. After dewaxing and rehydration of the samples, endogenous peroxidase was inhibited by incubation in 2% hydrogen peroxide for 30 min. After washing in PBS, sections were blocked with normal rabbit serum and incubated overnight with the primary antibody (dilution 1:400). The sections were then processed according to the avidin-biotin-peroxidase complex method. Briefly, sections were treated sequentially with the secondary antibody (anti-rabbit IgG-biotin conjugate; Sigma) at a 1:200 dilution for 1 h and the avidin-peroxidase complex (Vector Labs, Burlingame, CA). Tissue-bound peroxidase was visualized by incubation in 0.03% diamino benzidine-tetrahydrochloride (type IV; Sigma) and 0.01% hydrogen peroxide in 0.1 M Tris buffer (pH 7.6) for 1 min. Sections were counterstained with hematoxylin. In all reactions, negative controls were run in parallel by omitting the primary anti-ghrelin antibody (substituted either by PBS or preimmune serum) with absence of specific immunoreactivity.

RNA Analysis by Semiquantitative RT-PCR

In the experimental groups, testicular expression of ghrelin mRNA was assessed by RT-PCR optimized for semiquantitative detection. Total RNA was isolated from testis samples from different experimental settings using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method [29]. For amplification of ghrelin signals, the following primer pair was used: ghrelin-sense (nt 205–224; 5′-CCA GAG GAC AGA GGA CAA GC-3′) and ghrelin-as (nt 451–430; 5′-AGT TGC AGA GGA GGC AGA AGC T-3′), synthesized according to the published cDNA sequence of rat ghrelin [1]. In addition, to provide an appropriate internal control, parallel amplification of a 290-base pair (bp) fragment of L19 ribosomal protein mRNA or a 603-bp fragment of β-actin mRNA was carried out in each sample using, respectively, the primer pairs L19-sense (nt 91–110; 5′-GAA ATC GCC AAT GCC AAC-3′) and L19-as (nt 380–361; 5′-ACC TTC AGG TAC AGG CTG TG-3′); β-actin-sense (nt 45–63; 5′-TAC AAC TCC TTG CAG CTC C-3′) and β-actin-as (nt 648–628; 5′-ATC TTC ATG AGG TAG TCA GTC-3′), as described in detail elsewhere [11, 13].

For amplification of the target gene, RT and PCR were run in two separate steps. Furthermore, to enable appropriate amplification in the exponential phase, PCR amplification of specific signal and internal control (L19 ribosomal protein or β-actin) transcripts was carried out in separate reactions with different numbers of cycles (see below) but using similar amounts of the corresponding cDNA templates generated in single RT reactions, as previously described [13, 30]. Briefly, equal amounts of total testicular 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), 20 U ribonuclease inhibitor RNasin (Promega), 200 nM deoxy-NTP mixture, and 1 nM specific and internal control antisense primers in a final volume of 30 μl of 1× AMV-RT buffer. 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 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), 200 nM deoxy-NTP mixture, and the appropriate primer pairs (1 nM of each primer). 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 at 63°C for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 15 min was included. Different numbers of cycles were tested to optimize amplification in the exponential phase of PCR for specific (ghrelin) and internal control (L19 ribosomal protein and β-actin) signals (not shown), and 33 and 22 cycles, respectively, were chosen for further analysis.

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 using a fluorescent

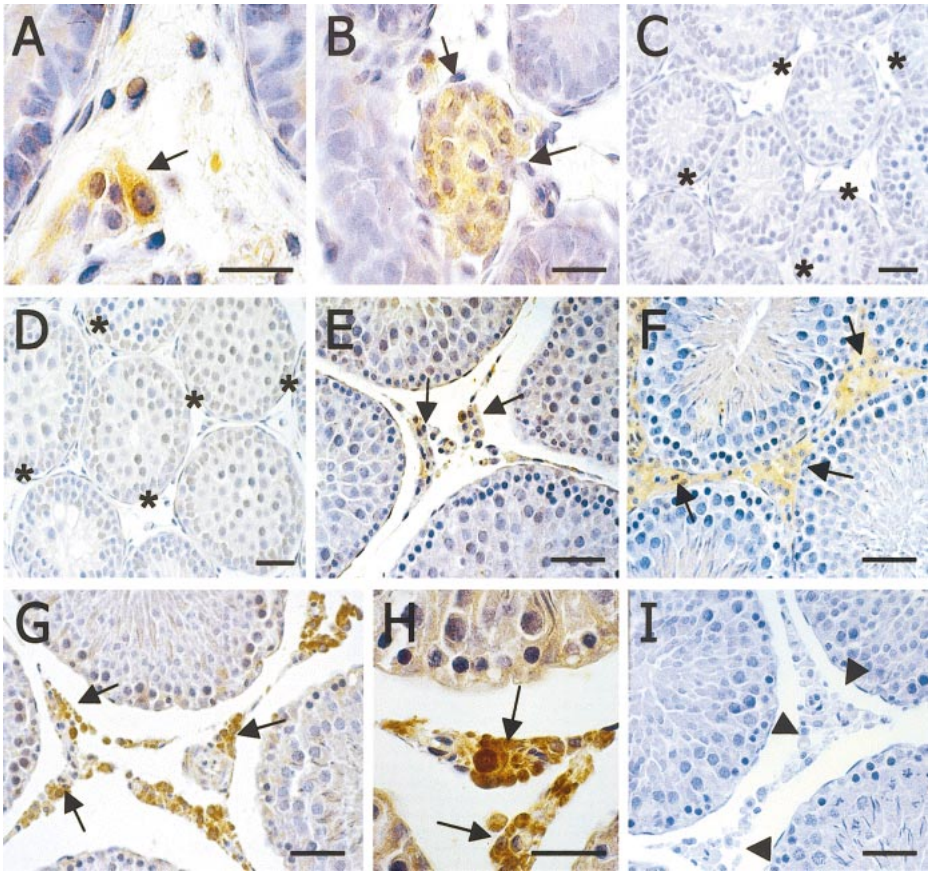

FIG. 1. Sections of testicular tissue obtained from rats at different age points of postnatal development immunostained with a rabbit anti-ghrelin polyclonal antibody and counterstained with hematoxylin. In detail, samples from 1- (**A**), 5- (**B**), 15- (**C**), 20- (**D**), 30- (**E**), 45- (**F**), and 75-day-old rats (**G**, **H**) were analyzed. At the latter age point, two different magnifications are presented. During the study period, strong immunostaining was detected in morphologically discernible mature Leydig cells within testicular interstitium (arrows). Lack of ghrelin immunoreactivity in clusters of immature adult-type Leydig cell progenitors (**C**, **D**) is denoted by asterisks. As control for the specificity of immunohistochemical labeling, substitution of the primary antibody by preimmune serum resulted in absence of staining in testicular preparations from adult (75-day-old) rats (**I**), where clusters of interstitial Leydig cells are indicated by arrowheads. Bar = 50 μm.

dye termination reaction and an automated sequencer (NewBiotechnic Ltd., Sevilla, Spain). In all assays, liquid controls and reactions without RT were included, yielding negative amplification. 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 target (ghrelin) being normalized to those of internal controls to indicate arbitrary units of relative expression.

Presentation of Data and Statistics

Immunohistochemical and RT-PCR analyses were carried out at least in quadruplicate using independent tissue and RNA samples, respectively. When relevant, semiquantitative RNA data are presented as mean ± SEM. Quantitative results were analyzed for statistically significant differences using ANOVA, followed by the Tukey test. $P \leq 0.05$ was considered significant.

RESULTS

Pattern of Cellular Expression of Ghrelin in Rat Testis Throughout Postnatal Development and after Selective Leydig Cell Elimination

Evaluation of the pattern of cellular expression of ghrelin protein in rat testis was conducted by immunohistochemistry using a rabbit anti-ghrelin polyclonal antibody. Analysis throughout postnatal development demonstrated that ghrelin peptide is selectively located in interstitial Leydig cells, both of the fetal and adult type, at advanced stages of differentiation (Fig. 1). Thus, strong ghrelin immunostaining was observed in fetal-type Leydig cells in testis tissue from 1- and 5-day-old rats (Fig. 1, A and B). Similarly, intense ghrelin immunoreactivity was detected in adult-type Leydig cells from puberty (30-day-old) onward, with increasing intensity of the signal in adulthood (Fig. 1, E–H). In contrast, weak to negligible ghrelin immunostaining was observed in undifferentiated Leydig cell progenitors, present in testicular interstitium of prepubertal (15- and 20-day-old) rats (Fig. 1, C and D) despite immunohistochemical reactions run in parallel to those in neonatal (Fig. 1, A and B), pubertal (Fig. 1, E and F), and adult (Fig. 1, G and H) preparations that yielded strong staining of interstitial mature Leydig cells. It is noteworthy that, in all positive cells, ghrelin immunoreactivity showed specific cytoplasmic location. Negligible immunostaining in the seminiferous tubules was impossible to differentiate from the background and was considered negative. As a control for specificity of detection, omission of primary anti-ghrelin antibody and its substitution either by PBS (data not shown) or preimmune serum (Fig. 1I) resulted in negative staining of interstitial cells.

Leydig cell-specific expression of ghrelin peptide within rat testis was confirmed by immunohistochemical analyses after selective elimination of adult-type Leydig cells by administration of the toxicant EDS. Ghrelin immunostaining in testicular interstitium became undetectable 5 days after EDS treatment, a time point when testis tissue is completely devoid of mature Leydig cells (Fig. 2). Indeed, no ghrelin signal was detected in proliferating Leydig cell precursors or in other interstitial cell types, as macrophages, that accumulate at such an early stage after administration of the cytotoxic compound [31]. Lack of ghrelin immunostaining was persistently observed in the interstitial space of testicular tissue 15 days after EDS treatment (data not shown).

Ghrelin Expression in Rat Testes from Hypophysectomized Rats With or Without Human CG Replacement

Assessment of hormonal regulation of testicular ghrelin expression at the gene and protein level was first evaluated

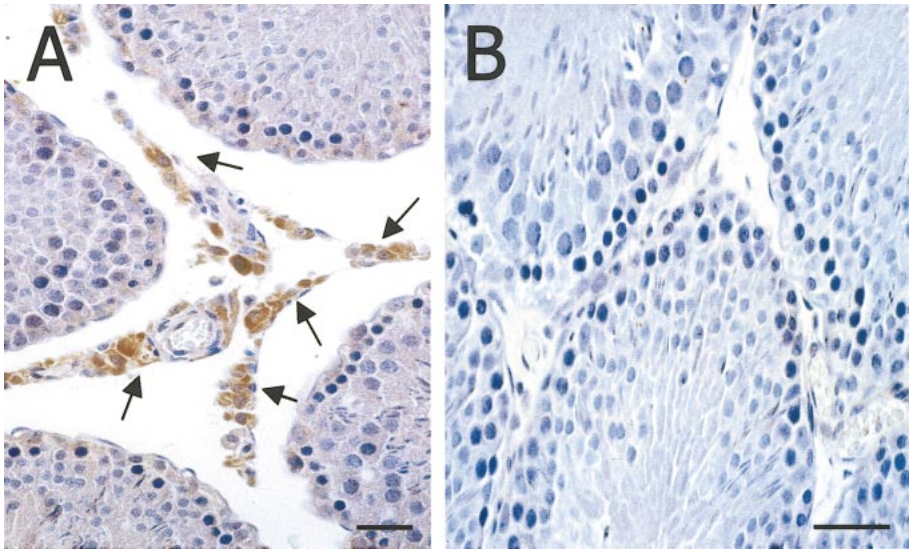

FIG. 2. Ghrelin immunostaining in sections of testicular tissue obtained from 75-day-old rats before (**A**) and 5 days after (**B**) ethylene dimethane sulfonate (EDS) administration. Efficiency of Leydig cell elimination by EDS is demonstrated by the absence of differentiated Leydig cells in testicular interstitium following administration of the cytotoxic compound. Whereas clear immunostaining was observed in mature Leydig cells in control sections (**A**, arrows), ghrelin peptide was absent in testicular interstitium from EDS-treated rats (**B**). Bar = 50 μm.

using the HPX rat as the experimental model. Long-term (4-wk) hypophysectomy resulted in the atrophy of all testicular compartments. In detail, atrophic Leydig cells in the interstitial space showed highly reduced cytoplasm, whereas regression of seminiferous epithelium with arrest of spermatogenesis at early meiosis was observed within the tubules, with some round spermatids being still detected due to the limited 4-wk elapsed period. Immunohistochemical analyses demonstrated that long-term hypophysectomy induced a decrease in ghrelin peptide expression in rat testis to virtually negligible levels (Fig. 3, A and B), a response that was prevented by replacement with human CG (10 IU/rat/24 h) for 7 days before sampling (Fig. 3C). In keeping with protein data, semiquantitative RT-PCR analyses demonstrated that relative expression levels of ghrelin mRNA were significantly reduced in testes from HPX rats (~20% of control values). Replacement of HXP rats with human CG partially restored testicular ghrelin mRNA expression, which reached values of ~75% of control levels (Fig. 4).

Regulation of Testicular Ghrelin mRNA Expression by Acute Human CG Administration

Hormonal regulation of ghrelin gene expression in rat testis was further assessed in a number of experimental models using semiquantitative RT-PCR. Acute administration of human CG to intact, adult rats (25 IU/rat) induced a transient, significant increase in testicular ghrelin mRNA levels that peaked at 4 h and declined thereafter (Fig. 5). In contrast, exposure to a single bolus of recombinant FSH (12.5 IU/rat) in vivo failed to modify ghrelin mRNA levels in rat testis at any time point (2–24 h) studied (data not shown). In addition, to ascertain whether endocrine signals other than gonadotropins are involved in the regulation of

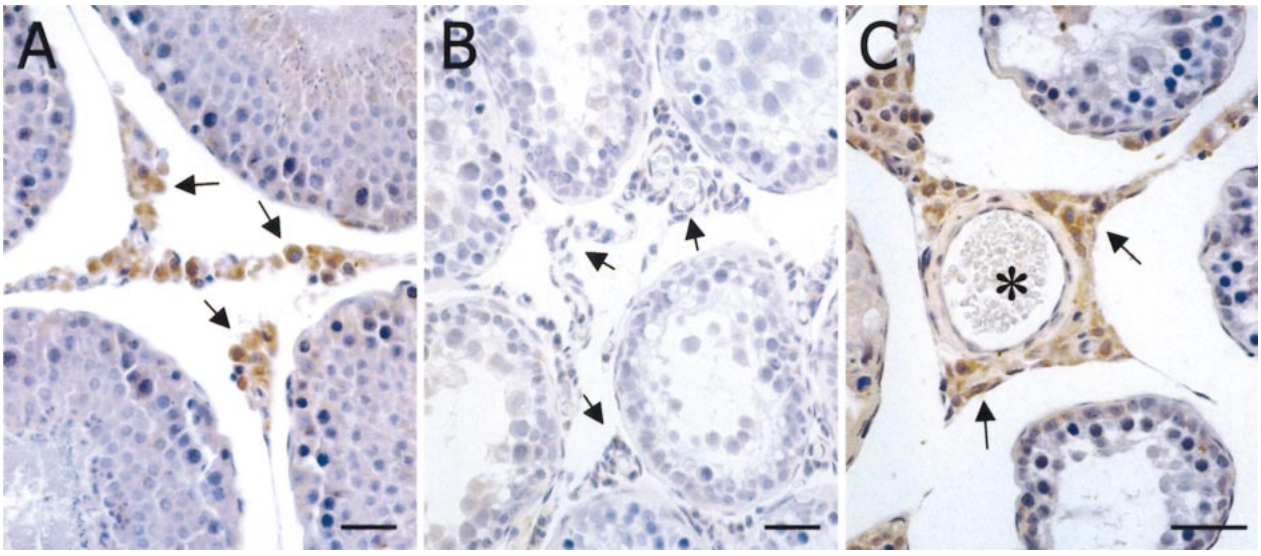

FIG. 3. Immunohistochemical analysis of ghrelin protein expression in rat testes from long-term (4-wk) hypophysectomyzed (HPX) rats with or without human CG replacement treatment. Sections of testicular tissue obtained from control (**A**), HPX (**B**), and HPX+hCG-treated rats (**C**) were immunostained with a rabbit anti-ghrelin polyclonal antibody and counterstained with hematoxylin. Whereas clear immunostaining was observed in mature Leydig cells in control sections (**A**, arrows), negligible ghrelin signal was detected in testicular sections from HPX rats (**B**). In contrast, human CG replacement for 7 days (10 IU/rat/24 h) restored ghrelin immunostaining in testicular interstitium to apparent Leydig cells (**C,** arrows). Bar = 50 μm. Asterisk denotes blood vessels.

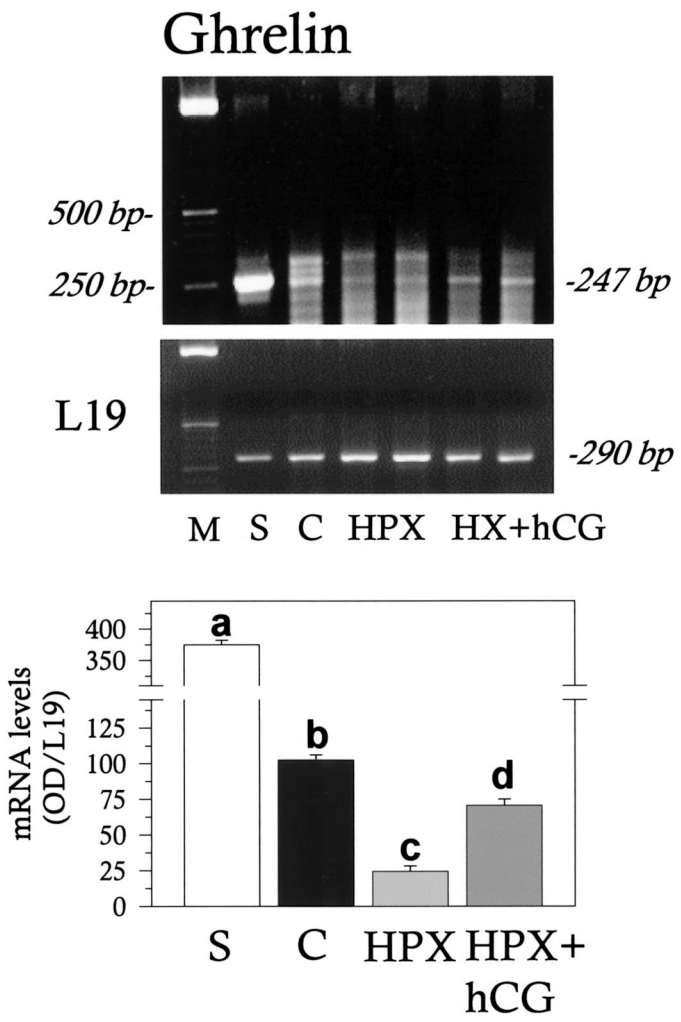

FIG. 4. Expression of ghrelin mRNA in testes from long-term (4-wk) hypophysectomyzed (HPX) rats with or without human CG replacement treatment (10 IU/rat/24 h for 7 days). In the upper panel, a representative RT-PCR assay of expression levels of ghrelin mRNA in testicular samples from control (C), HPX, and HPX+hCG-treated rats is shown; HPX and HPX+hCG groups are presented in duplicate. In addition, amplification of ghrelin signal from stomach (S) sample was used as a positive control, whereas amplification of L19 ribosomal protein mRNA served as an internal control. A 50-bp molecular weight marker (M) was used. In the lower panel, semiquantitative data on the expression levels of ghrelin mRNA in the different experimental groups are shown. Relative expression levels were obtained in each sample by normalization of absolute optical densities (OD) of the specific target to that of the L19 signal. For presentation of data, expression levels in the control (C) group were taken as 100% and the others were normalized accordingly, thus allowing semiquantitative comparison. Values are the mean ± SEM of at least four independent determinations. Groups with different superscript letters are statistically different (ANOVA followed by the Tukey test).

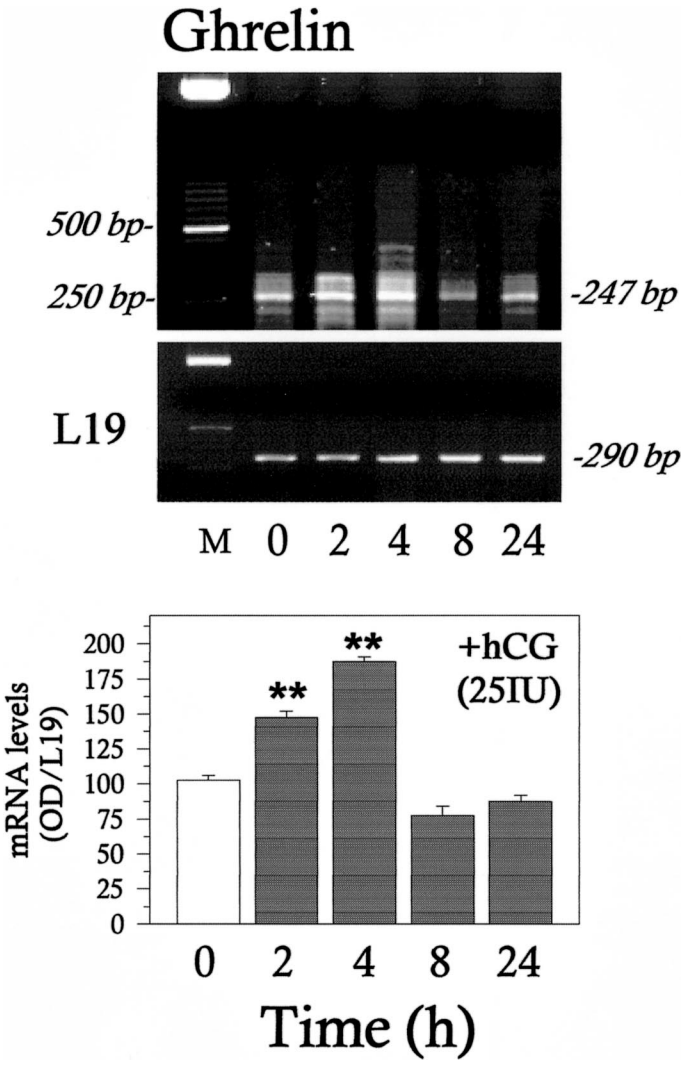

FIG. 5. Expression of ghrelin mRNA in rat testes after acute administration of a bolus of human CG. In the upper panel, a representative RT-PCR assay of expression levels of ghrelin mRNA in testicular samples from adult rats before (0) and 2, 4, 8, and 24 h after injection of a single dose of human CG (25 IU/rat) is presented. A 50-bp molecular weight marker (M) was used. Amplification of L19 ribosomal protein mRNA served as an internal control. In the lower panel, semiquantitative data on the expression levels of ghrelin mRNA in the experimental groups are shown. For presentation of data, expression levels in the control basal (0) group were taken as 100% and the others were normalized accordingly, thus allowing semiquantitative comparison. Values are the mean ± SEM of at least four independent determinations. $^{**}P \leq 0.01$ versus control (0) values (ANOVA followed by the Tukey test).

testicular expression of the ghrelin gene, relative expression levels of ghrelin mRNA were monitored in testes from GH-deficient rats, hyper- and hypothyroid animals, and adrenalectomized rats. Neither severe GH deficiency, as that observed in the Lewis-derived dwarf strain [25], nor alterations of the thyroid status induced significant changes in testicular ghrelin mRNA levels (Fig. 6). Similarly, invariant expression levels of ghrelin transcript were detected in testes from adult rats 7 days after adrenalectomy (Fig. 7).

DISCUSSION

Extensive research efforts following identification of ghrelin, as the endogenous ligand for the GHS receptor [1, 2], have pointed out the involvement of this newly discovered molecule not only in the physiological regulation of GH secretion but also in a variety of additional biological functions, including feeding and neuroendocrine control [4, 6, 8, 9]. In this context, an unexpected reproductive facet of ghrelin has recently emerged. Thus, intracerebroventricular administration of ghrelin has been shown to rapidly suppress pulsatile LH secretion [9]. In addition, our group has provided evidence for the expression of ghrelin and its functional receptor in rat testis [13]. Moreover, a specific ghrelin gene-derived transcript has been recently identified in mouse testis [32]. Our present results extend those previous observations and unambiguously demonstrate that testicular expression of ghrelin is selectively located in ma-

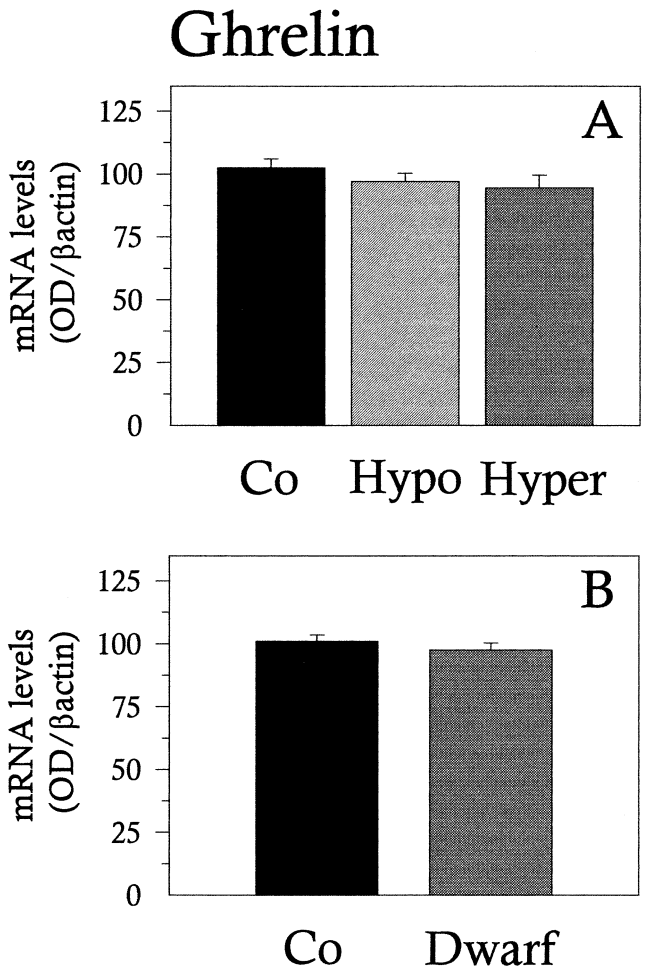

FIG. 6. Expression levels of ghrelin mRNA in rat testes from experimental models of hypo- and hyperthyroidism as well as GH deficiency. In the upper panel, semiquantitative data are presented on testicular levels of ghrelin mRNA in control euthyroid (Co) as well as in hypo- and hyperthyroid rats, induced as described in *Materials and Methods*. In the lower panel, a compilation of semiquantitative data on relative expression levels of ghrelin mRNA in testes from control Lewis (Co) and Lewis-derived dwarf rats is shown. The Lewis-derived dwarf rat is characterized by a profound GH deficiency, with the remaining hormonal parameters within the normal range. For presentation of data, expression levels in the control (Co) groups were taken as 100% and the others were normalized accordingly, thus allowing semiquantitative comparison. Values are the mean ± SEM of seven independent determinations. No statistically significant differences between groups were detected (ANOVA followed by the Tukey test).

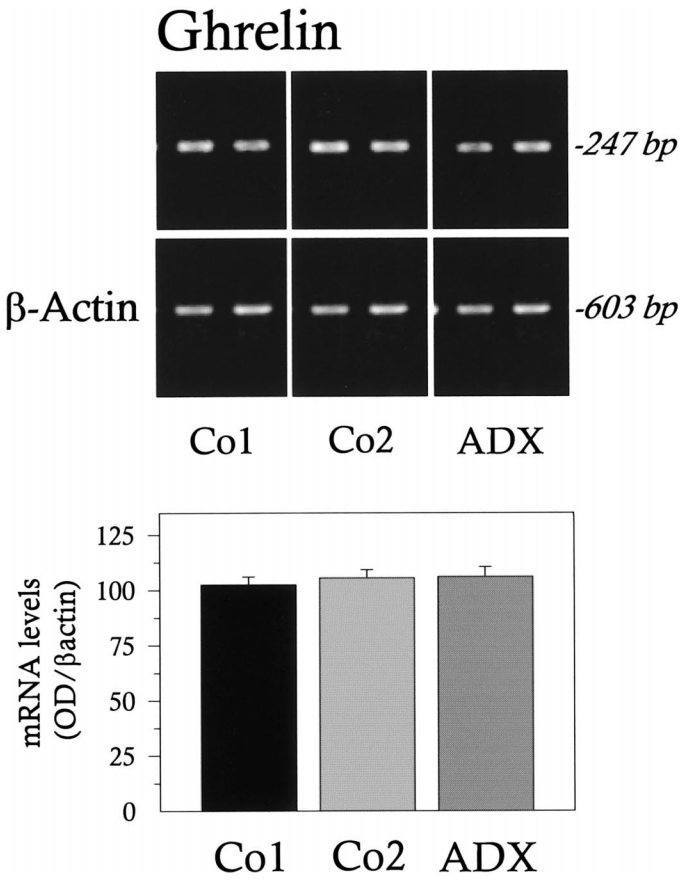

FIG. 7. Expression of ghrelin mRNA in rat testes after adrenalectomy. In the upper panel, a representative RT-PCR assay is presented showing expression levels of ghrelin mRNA in testicular samples from control (Co) and bilaterally adrenalectomized (ADX) adult rats. ADX and control (Co1) sham-operated rats were maintained on 0.9% NaCl and 0.6% glucose in drinking water. An additional control sham group (Co2) with tap drinking water was included. Amplification of β-actin mRNA served as an internal control. In the lower panel, semiquantitative data on the expression levels of ghrelin mRNA in the experimental groups are presented. For presentation of data, expression levels in the control (Co1) group were taken as 100% and the others were normalized accordingly, thus allowing semiquantitative comparison. Values are the mean ± SEM of seven independent determinations. No statistically significant differences between groups were detected (ANOVA followed by the Tukey test).

ture Leydig cells throughout postnatal development and is under hormonal regulation of pituitary LH.

Leydig cell-specific expression of testicular ghrelin was confirmed by immunohistochemical analyses at different developmental stages, from the neonatal period up to adulthood, and after selective elimination of mature Leydig cells by administration of the cytotoxic compound EDS. Worthy to note is that two different populations of Leydig cells, namely fetal-type and adult-type Leydig cells, can be identified in testis during development. In the rat, fetal-type Leydig cells appear in utero and predominate during the first 2 wk postnatally, although decreasing in number after birth [33]. In contrast, the adult-type Leydig cell population emerges during pubertal sexual development through a complex process of proliferation and differentiation of preexisting precursors into fully mature Leydig cells, responsible for androgen secretion by the pubertal-adult testis [33]. Our data indicate that testicular ghrelin peptide is selectively detected in Leydig cells at advanced stages of maturation regardless of their fetal or adult origin. Thus, intense ghrelin immunostaining was observed in mature fetal-type Leydig cells (Days 1 and 5 postpartum) as well as in adult-type Leydig cells from puberty (Day 30) onward, with increasing intensity of the signal in adult age. In contrast, undifferentiated Leydig cell progenitors, abundantly present in rat testicular interstitium during the prepubertal period [34], showed weak to negligible expression of ghrelin protein. These results are in keeping with our previous data on ghrelin gene expression in rat testis along pubertal maturation [13] and are in good agreement with our current findings on the pattern of testicular expression of ghrelin peptide after EDS treatment. Indeed, selective elimination of mature adult-type Leydig cells by EDS activates a subsequent wave of proliferation and further differentiation of preexisting undifferentiated Leydig cell precursors that mimics the normal developmental events of adult-type progenitors during puberty [31, 35]. Thus, the lack of ghrelin immunostaining in testis tissue 5 days after EDS not only

confirms Leydig cell specificity of testicular expression of this peptide but also supports the hypothesis that only Leydig cells at advanced stages of differentiation do express ghrelin, a contention further confirmed by the absence of a ghrelin signal in testicular interstitial cells 15 days after EDS treatment (data not shown). Regarding the latter, it has to be noted that, in our rat strain, no discernible mature Leydig cells are identified at this time point following EDS, yet some strain differences in the precise rate of Leydig cell repopulation seem to exists (e.g., [36]). Interestingly, the pattern of cellular expression of ghrelin in rat testis, both along postnatal development and after Leydig cell elimination, closely resembles that of other novel Leydig cell-specific products, as relaxin-like factor (RLF). In mouse and rat testes, RLF has been proven to be mostly expressed in mature Leydig cells, although its expression has been demonstrated also, albeit at lower levels, in developing Leydig cell progenitors. Thus, RLF has been proposed as a useful index of the differentiation status of this cell type [24, 37]. Similarly, ghrelin may function also as a differentiation marker of Leydig cells.

It is noteworthy that previous evidence indicates that, as is the case for ghrelin, GH-releasing hormone (GHRH), a hypothalamic key factor in the control of GH secretion, is expressed in rat Leydig cells under the positive control of LH [38]. However, the effects of these signals on Leydig cell endocrine function appear to be opposite because GHRH enhanced LH-induced cAMP production and steroidogenesis in Leydig cell cultures [38] whereas ghrelin significantly inhibited human CG-stimulated testosterone secretion in vitro [13]. Additional differences include a distinct pattern of cellular expression within the testis, as GHRH was also detected in germ cells [39]. Interestingly, testicular GHRH has been proven to be a positive paracrine regulator of Sertoli cell function [38, 39]. Whether ghrelin has a similar role remains to be evaluated. The lack of effect of acute administration of FSH on ghrelin mRNA levels suggests that Sertoli cell factors may not be primarily involved in the regulation of testicular ghrelin expression. However, our initial evidence on the expression of the gene encoding the cognate ghrelin receptor in rat seminiferous tubules (unpublished results) is highly suggestive of a direct action of ghrelin on rat seminiferous epithelium. Overall, different signals with pivotal roles in the neuroendocrine control of the growth axis, as ghrelin and GHRH, are likely involved in the direct control of testicular function, yet their effects and mechanisms of actions are partially different.

Recently, a ghrelin gene-derived transcript (GGDT) was identified in mouse testis. This species contains a unique 68-bp sequence at the 5′-end and an additional 252-bp sequence identical to that of exons 4 and 5 of the mouse ghrelin gene [32]. Notably, the developmental profile of expression of GGDT in mouse testis was grossly similar to that of ghrelin mRNA reported by our group in rat testis [13]. However, the possibility that GGDT may account for testicular ghrelin expression, as shown in our studies [13 and present results], can be ruled out as different sets of primer pairs, complementary to areas of ghrelin cDNA outside exons 4 and 5, were used for RT-PCR amplification of ghrelin transcript in testicular samples. In detail, the primer pair used in this report was designed to span over the intron between exons 3 and 4 of the ghrelin gene, thus making it impossible to accidentally amplify a potential rat counterpart of mouse GGDT. In addition, the polyclonal antibody used for our immunohistochemical analyses was raised against the mature ghrelin peptide that is not encoded by exons 4 and 5 of the ghrelin gene. Indeed, no evidence available demonstrates that GGDT is actually translated into a functional protein. Taken together, our results strongly indicate that, in rat testis, expression of the ghrelin gene results in the generation of a full-length mRNA transcript encoding the mature ghrelin peptide. Whether a species similar to testis-specific mouse GGDT is expressed in rat testis is presently under evaluation.

Besides constitutive expression along postnatal development, our combined analysis, using different experimental models, allowed us to delineate major hormonal regulators of ghrelin expression in rat testis. Deprivation of pituitary hormones after long-term hypophysectomy lead to a clear-cut decrease in testicular ghrelin mRNA and protein expression. Noteworthy, replacement with human CG (as superagonist of LH) partially restored ghrelin mRNA and peptide expression. In this sense, testicular LH/CG receptors are selectively expressed in Leydig cells, LH being the major tropic input for development and maintenance of Leydig cell-differentiated functions [16]. Thus, the biological effects of this gonadotropin on Leydig cells involve induction of proliferation and differentiation of progenitors into fully functional, mature Leydig cells, which proliferate also under LH/CG stimulation as well as activational acute regulatory actions. Assumedly, constitutive expression of ghrelin in LH-driven, fully differentiated Leydig cells may account, at least partially, for the findings reported herein. However, our current data on the effects of acute administration of human CG to intact rats, which resulted in a transient increase in testicular ghrelin mRNA levels with peak values 4 h after injection, is suggestive of a genuine regulatory action of LH on testicular ghrelin expression. The specificity of such an effect is supported by the fact that FSH was not able to mimic the acute response to human CG in terms of testicular ghrelin mRNA expression. Overall, our present results suggest that ghrelin expression is, at least partially, under hormonal regulation of pituitary LH. Whether this action is carried out directly or is mediated by LH-driven locally produced factors, such as testosterone, deserves further investigation.

Of interest, the ability of ghrelin to inhibit human CG-stimulated testosterone secretion in vitro, as reported previously by our group [13], might be in apparent contrast with the stimulatory role of pituitary LH on testicular ghrelin expression presented herein, as this gonadotropin is the major elicitor of testosterone production [16]. A tempting explanation, however, is that ghrelin may operate as a local regulator in the fine tuning of the steroidogenic actions of LH. In this model, ghrelin would participate in the autolimitation of the testicular testosterone response to gonadotropic stimulation, a well characterized phenomenon that involves several regulatory mechanisms, such as ligand-induced receptor desensitization [16]. Furthermore, as stated above, LH-induced ghrelin may serve additional biological actions within the testis, which opens up the possibility that this novel molecule may function as a mediator of testicular actions of LH other than its steroidogenic effects.

In addition to pituitary gonadotropins, testicular function is under the control of a plethora of endocrine signals. Among them, relevant biological actions of GH, glucocorticoids, and thyroid hormones have been demonstrated in rodent testis [16–19]. Moreover, compelling evidence indicates that Leydig cells are direct targets for those signals (e.g., [18, 19, 40]). On this basis, we evaluated testicular expression of ghrelin mRNA in several experimental models, including GH deficiency and hyper- and hypothyroid-

ism conditions as well as in adrenalectomized rats. Notably, the effects of hypophysectomy on testicular ghrelin expression, presented herein, might partially derive from disturbance of the above-mentioned neuroendocrine axes. Our semiquantitative RT-PCR analyses, however, demonstrated invariant expression levels of ghrelin mRNA in all the paradigms tested. These results cast doubt on the potential contribution of endocrine signals other than pituitary LH in the regulation of ghrelin gene expression in rat testis. In this sense, unaltered levels of ghrelin mRNA in testes from GH-deficient rats suggest that, as for systemic GH, circulating IGF-I may not participate in the direct control of testicular ghrelin expression. Nevertheless, the modulatory role, if any, of additional pituitary hormones with proven direct testicular actions, such as prolactin ([41] and references therein), remains to be elucidated.

In conclusion, our current results unambiguously demonstrate that, regardless of their fetal or adult origin, differentiated Leydig cells express ghrelin, a phenomenon that takes place in a LH/CG-dependent manner. Given the major physiological role of LH in the functional regulation of testicular Leydig cells throughout development [16], it is tempting to hypothesize that ghrelin may operate as a novel mediator and/or regulator of different actions of pituitary LH in rat testis. Further analysis of the mechanisms of action and novel end points for the testicular effects of ghrelin will help to fully elucidate the role of this recently identified signal in the direct control of male gonadal function.

ACKNOWLEDGMENTS

The authors are indebted to Rocío Campón for her excellent technical assistance. Rabbit anti-ghrelin polyclonal antibody was kindly donated by Drs. M. Kojima and K. Kangawa (Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan). M.T.-S. is indebted to Dr. Jorma Toppari (Department of Physiology, University of Turku, Finland) for helpful comments during preparation of the manuscript.

REFERENCES

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone acylated peptide from stomach. Nature 1999; 402:656–666.
2. Kojima M, Hosoda H, Matsuo H, Kangawa K. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab 2001; 12:118–122.
3. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 2000; 85:4908–4911.
4. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 2000; 141:4325–4328.
5. Arvat E, Maccario M, di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. J Clin Endocrinol Metab 2001; 86:1169–1174.
6. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 2000; 141:4797–4800.
7. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000; 407:908–913.
8. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Fujimiya M, Katsuura G, Makino S, Fujino MA, Kasuga M. A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology 2001; 74:143–147.
9. Furuta M, Funabashi T, Kimura F. Intracerebroventricular administration of ghrelin rapidly suppresses pulsatile luteinizing hormone secretion in ovariectomized rats. Biochem Biophys Res Commun 2001; 288:780–785.
10. Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K. Kidney produces a novel acylated peptide, ghrelin. FEBS Lett 2000; 486:213–216.
11. Gualillo O, Caminos JE, Blanco M, García-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva F. Ghrelin, a novel placental-derived hormone. Endocrinology 2001; 142:788–794.
12. Papotti M, Ghé C, Cassoni P, Catapano F, Deghenghi R, Ghigo E, Muccioli G. Growth hormone secretagogue binding sites in peripheral human tissues. J Clin Endocrinol Metab 2000; 85:3803–3807.
13. Tena-Sempere M, Barreiro ML, Gonzalez LC, Gaytan F, Zhang FP, Caminos JP, Pinilla L, Casanueva FF, Dieguez C, Aguilar E. Novel expression and functional role of ghrelin in rat testis. Endocrinology 2002; 143:717–725.
14. Skinner MK. Cell-cell interactions in the testis. Endocr Rev 1991; 12: 45–77.
15. Saez JM. Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev 1994; 15:574–626.
16. Tena-Sempere M, Huhtaniemi I. Gonadotropin receptors. In: Fauser BCJM, Rutherford AJ, Strauss JF, van Steirteghem A (eds.), Molecular Biology in Reproductive Medicine. New York: Parthenon Publishing; 1999: 165–200.
17. Jannini EA, Ulisse S, D'Armiento M. Thyroid hormone and male gonadal function. Endocr Rev 1995; 16:443–459.
18. Ge R-S, Hardy DO, Catterall JF, Hardy MP. Developmental changes in glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells. Endocrinology 1997; 138:5089–5095.
19. Kanzaki M, Morris PL. Growth hormone regulates steroidogenic acute regulatory protein expression and steroidogenesis in Leydig cell progenitors. Endocrinology 1999; 140:1681–1686.
20. Jackson CM, Jackson H. Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethane sulphonate. J Reprod Fertil 1984; 71:393–401.
21. Ojeda SR, Urbanski HF. Puberty in the rat. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994: 363–410.
22. Molenaar R, de Rooij DG, Rommerts FFG, van der Molen HJ. Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with ethylene dimethane sulfonate is dependent on luteinizing hormone and not follicle stimulating hormone. Endocrinology 1986; 118:2546–2554.
23. Koga M, Tanaka H, Yomogida K, Tsuchida J, Uchida K, Kitamura M, Sakoda S, Matsumiya K, Okuyama A, Nishimune Y. Expression of selenoprotein-P messenger ribonucleic acid in the rat testis. Biol Reprod 1998; 58:261–265.
24. Spiess AN, Balvers M, Tena-Sempere M, Huhtaniemi I, Parry L, Ivell R. Structure and expression of the rat relaxin-like factor (RLF) gene. Mol Reprod Dev 1999; 54:319–325.
25. Charlton HM, Clark RG, Robinson ICAF, Porter-Gof AE, Cox BS, Bugnon C, Bloch BS. Growth hormone-deficient dwarfism: a new mutation. J Endocrinol 1988; 119:51–58.
26. Coya R, Carro E, Mallo F, Dieguez C. Retinoic acid inhibits in vivo thyroid-stimulating hormone secretion. Life Sci 1997; 60:247–250.
27. Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000; 279:909–913.
28. Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, Hayashi Y, Kangawa K. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol 2001; 280:R1483–R1487.
29. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
30. Tena-Sempere M, Pinilla L, Zhang F-P, Gonzalez LC, Huhtaniemi I, Casanueva FF, Dieguez C, Aguilar E. Developmental and hormonal regulation of leptin receptor (Ob-R) messenger ribonucleic acid in rat testis. Biol Reprod 2001; 64:634–643.
31. Teerds KJ, Veldhuizen-Tsoerkan MB, Rommerts FFG, de Rooij DG, Dorrington H. Proliferation and differentiation of testicular interstitial cells: aspects of Leydig cell development in the (pre)pubertal and adult testis. In: Verhoeven G, Habenicht U-F (eds.), Molecular and

Cellular Endocrinology of the Testis. Berlin: Springer-Velag; 1994: 37–65.
32. Tanaka M, Hayashida Y, Nakao N, Nakai N, Nakashima K. Testis-specific and developmentally induced expression of a ghrelin gene-derived transcript that encodes a novel polypeptide in the mouse. Biochim Biophys Acta 2001; 1522:62–65.
33. Huhtaniemi IT. The fetal testis—a very special endocrine organ. Eur J Endocrinol 1994; 130:25–31.
34. Benton L, Shan L-X, Hardy MP. Differentiation of adult Leydig cells. J Steroid Biochem Mol Biol 1995; 53:61–68.
35. Tena-Sempere M, Rannikko A, Kero J, Zhang F-P, Huhtaniemi IT. Molecular mechanisms of reappearance of luteinizing hormone receptor expression and function in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. Endocrinology 1997; 138:3340–3348.
36. Teerds KJ, de Boer-Brouwer M, Dorrington JH, Balvers M, Ivell R. Identification of markers for precursor and Leydig cell differentiation in the adult rat testis following ethane dimethyl sulphonate administration. Biol Reprod 1999; 60:1437–1445.
37. Balvers M, Spiess A-N, Domagalski R, Hunt N, Kilic E, Mukhopadhyay AK, Hanks E, Charlton HM, Ivell R. Relaxin-like factor expression as marker of differentiation in the mouse testis and ovary. Endocrinology 1998; 139:2960–2970.
38. Ciampani T, Fabbri A, Isidori A, Dufau ML. Growth hormone-releasing hormone is produced by rat Leydig cells in culture and acts as a positive regulator of Leydig cell function. Endocrinology 1992; 131: 2785–2792.
39. Fabbri A, Ciocca DR, Ciampani T, Wang J, Dufau ML. Growth hormone-releasing hormone in testicular interstitial and germ cells: potential paracrine modulation of follicle-stimulating hormone action on Sertoli cell function. Endocrinology 1995; 136:2303–2308.
40. Manna PR, Tena-Sempere M, Huhtaniemi IT. Molecular mechanisms of thyroid hormone-stimulated steroidogenesis in mouse Leydig tumor cells. Involvement of the steroidogenic acute regulatory (StAR) protein. J Biol Chem 1999; 274:5909–5918.
41. Manna PR, El-Hefnawy T, Kero J, Huhtaniemi IT. Biphasic action of prolactin in the regulation of murine Leydig tumor cell functions. Endocrinology 2001; 142:308–318.