Ghrelin Inhibits Steroid Biosynthesis by Cultured Granulosa-Lutein Cells

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Context: Growing evidence indicates that ghrelin may participate in the regulation of different aspects of reproductive function. The genes encoding for this peptide and its receptor are expressed in the human ovary, but their functional role is still unknown.

Objective: The aim of our study was to assess whether ghrelin has any effect on steroid synthesis by human granulosa-lutein cells and to identify the receptor isoform through which this potential effect is exerted.

Design, Patients, and Methods: Thirty-five women with spontaneous ovulatory cycles undergoing *in vitro* fertilization for infertility due to uni- or bilateral tubal impatency or male factor were studied. Granulosa-lutein cells obtained from follicular fluid were incubated with increasing amounts of human acylated ghrelin (10^{-11} to 10^{-7} mol/liter) either alone or together with a 1:500 concentration of a specific anti-ghrelin receptor antibody [GH secretagogue receptor 1a (GHS-R1a)]. Culture media were tested for estradiol (E_2) and progesterone (P_4). The expression of GHS-R1a and GHS-R1b in human granulosa-lutein cells was also studied by real-time quantitative PCR.

Results: E_2 and P_4 concentrations in the culture media were significantly reduced by ghrelin in a dose-dependent fashion. The maximal decrease in E_2 (25%) and P_4 (20%) media concentrations was obtained with the 10^{-7} and 10^{-8} mol/liter ghrelin concentrations, respectively. The inhibitory effect of all ghrelin concentrations used was antagonized by the specific anti-ghrelin receptor-1a antibody added to the culture media and not by the specific anti-ghrelin receptor-1b antibody. Both 1a and 1b isoforms of the GHS-R were expressed in human granulosa-lutein cells, with the latter exceeding the former's expression (GHS-R1b/GHS-R1a ratio, 143.23 $\pm$ 28.15).

Conclusions: Ghrelin exerts an inhibitory effect on granulosa-lutein cells steroidogenesis by acting through its functional GHS-R1a. This suggests that ghrelin may serve an autocrine-paracrine role in the control of gonadal function and be part of a network of molecular signals responsible for the coordinated control of energy homeostasis and reproduction. (***J Clin Endocrinol Metab* 93: 1476–1481, 2008**)

Ghrelin, a 28-amino-acid peptide primarily secreted by the stomach, was first identified as the endogenous ligand of the GH secretagogue receptor (GHS-R) (1, 2). Since its discovery in 1999, the evidence for potential involvement of this molecule in the regulation of numerous endocrine and nonendocrine functions is constantly growing. Ghrelin, indeed, not only displays strong GH-releasing activity but also regulates food intake and energy balance, and it is involved peripherally in the control of gastric motility and acid secretion, cardiovascular system, glucose metabolism, pancreatic insulin secretion, and cell proliferation (3–5).

Although ghrelin is predominantly secreted by the human gastric mucosa, substantially lower amounts of ghrelin were detected in many other organs, such as the bowel, pancreas,

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Abbreviations: E_2, Estradiol; GHS-R, GH secretagogue receptor; P_4, progesterone.

kidney, placenta, lymphatic tissues, gonads, thyroid, adrenal, lung, pituitary, and hypothalamus and in different human neoplastic tissues and related cancer cell lines such as gastric and intestinal carcinoids, lymphomas, and thyroid, breast, liver, lung, and prostate carcinomas (6–8). Although peripheral actions of ghrelin cannot be ruled out, the functional roles, if any, of the hormone in peripheral systems remain unexplored.

Ghrelin is secreted in a pulsatile fashion (9) and acts through the interaction with the GHS-R, which belongs to the large family of the G protein-coupled seven-transmembrane receptors. Two major subtypes, generated by the alternative splicing of a single gene located at chromosome 3q26.2, were described: the full-length GHS-R type 1a and the truncated GHS-R type 1b (10, 11). The GHS-R1a is the functionally active, signal-transducing form of the receptor. In contrast, the GHS-R1b lacks transmembrane domains 6 and 7, and it is apparently devoid of high-affinity ligand-binding and signal transduction capacity. Thus, its functional role, if any, remains to be clarified.

Ghrelin, as well as GHS-R1a and GHS-R1b, are widely distributed. The selective tissue-specific expression of the human GHS-R1a is regulated by the promoter region and transcriptional control of the GHS-R gene. Although GHS-R1a is mainly expressed at central neuroendocrine tissues, a widespread pattern of expression of the gene encoding GHS-R1a was demonstrated in a variety of peripheral tissues, including the thyroid gland, pancreas, spleen, myocardium, adrenal gland, testes, ovaries, stomach, and the neuronal cells of the gut (12). With regard to GHS-R1b expression, also this variant is widespread, showing different levels of expression in a large variety of tissues and organs.

Based on its well-recognized action as modulator of feeding behavior and energy metabolism, and the close relationship between the latter, nutritional state, and reproductive physiology, a possible role, either systemic or local, of ghrelin in the control of gonadal axis was recently hypothesized. Ghrelin gene expression was demonstrated in human and rodent placenta, and ghrelin was reported to inhibit early embryo development (13). Ghrelin was shown to suppress LH secretion *in vivo* (14, 15) and to decrease LH responsiveness to LHRH *in vitro* (15). In addition, a role of the hormone in the control of prolactin secretion in rats and in adult humans was also described (16, 17). Direct gonadal effects of ghrelin were studied in rat testis where the hormone was able to inhibit testosterone synthesis (18), whereas no effect upon adrenal steroidogenesis was reported (19).

Recently, evidence for the expression of ghrelin and its functional receptor in the ovary was reported (20, 21), highlighting the possibility for a role of ghrelin in the direct control of ovarian physiology. To investigate further the ghrelin signaling system in human ovary, the expression of GHS-R1a and GHS-R1b in human granulosa-lutein cells and the effects of ghrelin on estradiol (E_2) and progesterone (P_4) secretion by this cell type were evaluated in the present study.

Subjects and Methods

Subjects

Thirty-five women aged between 26 and 45 yr with spontaneous ovulatory cycles and infertility due to uni- or bilateral tubal impatency or male factor were studied. This study was approved by the Ethical Research Committee of the Department of Pediatrics at the University of Parma (Parma, Italy), and informed consent was obtained from all subjects. All patients received a GnRH analog (Leuproreline, 1.9 mg im) 1 month before FSH therapy was started. Subsequently, superovulation for *in vitro* fertilization/embryo transfer was induced with recombinant human FSH (225 IU Gonal F 75; Serono, Rome, Italy) from d 1–3 and individualized thereafter, depending on daily plasma E_2 levels and measurements of follicular growth by transvaginal ultrasound examination. Thirty-six hours before harvest, human chorionic gonadotropin (10,000 IU Profasi; Serono) was administered to increase the number of follicles with enhanced steroidogenic activity (22) and, in turn, E_2 and P_4 secretion by granulosa-lutein cells. This results in maximal steroid secretion allowing amplification of the steroid response to ghrelin administration. Follicular aspiration for oocyte retrieval was performed by laparoscopy under general anesthesia 34–38 h after human chorionic gonadotropin administration.

Granulosa-lutein cell cultures

After oocyte recovery, granulosa-lutein cells were obtained from follicular fluid by centrifugation at 800 rpm for 15 min; cells were resuspended with a 1:1 mixture of medium 199 and Hank's salt solution, and pools were prepared from each individual patient. The suspension (10 ml) was layered in 10 ml lymphocyte separation medium (Ficoll; ICN Biomedicals, Irvine, CA) and centrifuged at 1800 rpm at room temperature for 30 min to separate the red cells. Granulosa-lutein cells were recovered from the Ficoll interface, washed three times with Hank's solution, and resuspended with 3 ml medium 199. An aliquot of collected cells was frozen at −20 C for RNA extraction after adding RNase inhibitors.

Aliquots of the cell suspension were counted with a hemocytometer to determine the number of cells and placed into multiwell dishes (5×10^5 cells per well) in 1 ml medium 199 with Earle's salt supplemented with 2% L-glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin-amphotericin, and 10% fetal bovine serum. Cultures were maintained in humidified 95% air/5% CO_2 at 37 C. After 24 h, culture medium was replaced with 1 ml serum-free medium, and cells were cultured for 24 h with the following treatments: medium alone (control) and medium supplemented with increasing concentrations of human acylated ghrelin (NeoMPS, Strasburg, France) (10^{-11} to 10^{-7} mol/liter). The serum-free medium contained androstenedione (10^{-7} mol/liter) as the substrate for E_2 production. Culture media were gently aspirated after 24 h and centrifuged, and the supernatants were stored at −20 C until assayed for E_2 and P_4 content.

When the number of granulosa-lutein cells obtained from the same subject was adequate, a concentration of 1:500 of a specific anti-GHS-R1a antibody (Phoenix Pharmaceuticals, Belmont, CA) was added to the cell culture media supplemented with the different human ghrelin doses. After 24 h, the supernatants were collected and stored at −20 C until assayed for E_2 and P_4 content. A similar set of experiments was performed using a specific anti-GHS-R1b antibody (Phoenix Pharmaceuticals). Both the GHS-R1a and -1b antibodies used were raised against the C-terminal end of the ghrelin receptor isoforms. The two antibodies differ at the carboxyl-terminal region, and in particular, the 1b isoform lacks the last 77 amino acids. Because the anti-GHS-R1a antibody was raised against the final 36 amino acids of the peptide, it is highly unlikely that it might detect the GHS-R1b receptor.

Quantitation of GHS-R1a and GHS-R1b mRNA: real-time PCR

Isolation of total RNA from untreated granulosa-lutein cells (10^7 cells) was performed by the single-step liquid-phase separation (TRI

reagent kit; Molecular Research Center, Cincinnati, OH) as recommended by the manufacturer. cDNA synthesis was accomplished with the random primer using the RT-PCR kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (23).

Quantitation of human ghrelin receptors (GHS-R1a and GHS-R1b) mRNA was performed using the real-time quantitative PCR (TaqMan PCR). An identical fluorogenic probe and two specific primers for type 1a and 1b receptors were used (Table 1) (4). DNA contamination was evaluated by replacing the RNA sample with diethylpyrocarbonate-treated water. The TaqMan rRNA control primers and probe were used to detect the 18S rRNA levels, which provide an endogenous control for PCR quantitative studies.

Following the TaqMan protocol, the reactions were prepared in triplicate using 80 ng of each cDNA and adding the following reagents at the following concentrations: 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM primers, 100 nM probes, and 50 nM rRNA primers and probes. Reactions were started at 95 C for 10 min to activate AmpliTaqGold polymerase (Promega, Madison, WI) and were run for 50 cycles at 95 C for 15 sec and 60 C for 1 min.

PCR amplification was performed simultaneously for GHS-R1a and GHS-R1b cDNA using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Results were normalized by dividing the amount of mRNA by the amount of 18S RNA present in each sample. Each experiment was repeated at least three times.

Hormone assay

E_2 and P_4 concentrations were measured in the media by commercially available RIA kits (Ultra-sensitive estradiol RIA from Diagnostic Systems Laboratories Inc., Webster, TX; Coat-A-Count progesterone RIA from Diagnostic Products Corp., Los Angeles, CA). The mean intra- and interassay coefficients of variation were 7.4 and 9.3% for E_2 and 4.6 and 5.9% for P_4, respectively. Assay sensitivity was 8.1 pmol/liter and 0.06 nmol/liter for E_2 and P_4, respectively.

Statistical analysis

Results are expressed as mean ± SEM. The results were analyzed by one-way or two-way repeated-measures ANOVA, as appropriate, followed by Scheffé test. Statistical significance was set at $P < 0.05$.

Results

GHS-R1a and GHS-R1b mRNA expression

GHS-R1a and GHS-R1b were both expressed in human granulosa-lutein cells, with the latter exceeding the former's expression (GHS-R1b/GHS-R1a ratio, 143.23 ± 28.15).

Effects of human ghrelin on E_2 production by granulosa-lutein cells

Baseline E_2 levels produced by granulosa-lutein cells were 59.08 ± 2.07 nmol/liter. Exposure of these cells to increasing concentrations of human ghrelin resulted in significant reduction of E_2 concentrations in the media (Fig. 1A). E_2 production was inhibited by all ghrelin concentrations used between 10^{-11} and 10^{-7} mol/liter (10–25%) (one-way repeated-measures ANOVA, $P < 0.001$). The maximal inhibitory effect was observed at the concentration of 10^{-7} mol/liter, which caused a 25% decrease in E_2 production.

The concentration of 1:500 of a specific anti-ghrelin receptor-1a polyclonal antibody antagonized the suppressive effect on E_2 secretion by all ghrelin doses used but had no effect when added alone to the culture media (two-way repeated-measures ANOVA, $P < 0.05$) (Fig. 1B). On the contrary, the same effect was not observed with the addition of a specific anti-ghrelin receptor-1b polyclonal antibody (data not shown).

Effects of human ghrelin on P_4 production by granulosa-lutein cells

Baseline P_4 levels produced by granulosa-lutein cells were 5.89 ± 0.9 mmol/liter. Exposure of these cells to increasing concentrations of human ghrelin induced a significant inhibition of the produced P_4 similar to that observed when E_2 was measured (Fig. 2A) (one-way repeated-measures ANOVA, $P < 0.001$), with a maximal inhibitory effect at ghrelin concentration of 10^{-8} mol/liter (20%). This inhibitory effect was antagonized by the specific anti-ghrelin receptor-1a polyclonal antibody (1:500) added to the culture media (two-way repeated measures ANOVA, $P < 0.05$) (Fig. 2B).

Discussion

Assessment of the biological actions of ghrelin in the ovary by an *in vitro* model showed that ghrelin is able to significantly inhibit, in a dose-dependent manner, E_2 and P_4 production by granulosa-lutein cells. In addition, we were able to show that both 1a and 1b isoforms of the GHS-R gene are expressed in ovarian granulosa-lutein cells, with a prevalence of the 1b isoform.

In a previous study, systematic screening of GHS-R mRNA expression in a wide variety of human tissues, using real-time quantitative PCR (24), demonstrated the presence of type 1b GHS-R mRNA in ovarian tissue obtained at surgery but failed to detect the 1a transcript. In contrast, immunohistochemical analyses of the presence and cellular location of GHS-R1a protein

TABLE 1. Primers and probes

	Sequence
GHS-R1a and GHS-R1b primers and probe	
GHS-R1a/b forward primer	5′-TCG TGG GTG CCT CGC T-3′
GHS-R 1a reverse primer	5′-CAC CAC TAC AGC CAG CAT TTT C-3′
GHS-R 1b reverse primer	5′-GCT GAG ACC CAC CCA GCA-3′
GHS-R1a/b probe	5′-FAM- AGG GAC CAG AAC CAC AAG CAA ACC G -TAMRA-3′
18S rRNA primers and probe	
Forward primer	5′-CGG CTA CCA CAT CCA AGG AA-3′
Reverse primer	5′-GCT GGA ATT ACC GCG GCT-3′
Probe	VIC- TGC TGG CAC CAG

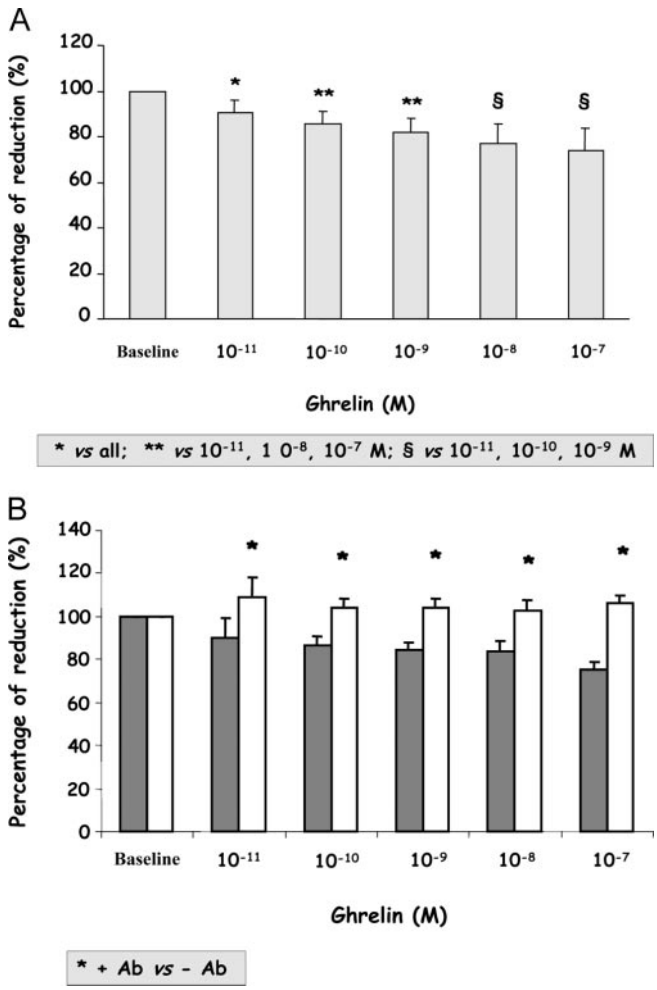

FIG. 1. A, Effects of increasing concentrations of human ghrelin (10^{-11} to 10^{-7} mol/liter) on E_2 production by granulosa-lutein cells. Data are expressed as the percentage of control values ± SEM for a total of 35 experiments performed in triplicate. Control hormone levels were measured in granulosa-lutein cell culture media cultured with serum-free media not containing ghrelin. *, $P < 0.05$ *vs.* all; **, $P < 0.05$ *vs.* 10^{-11}, 10^{-8}, and 10^{-7} mol/liter; §, $P < 0.01$ *vs.* 10^{-11}, 10^{-10}, and 10^{-9} mol/liter. B, Effects of a specific anti-GHS-R1a polyclonal antibody on granulosa-lutein cell E_2 production after stimulation with increasing doses of ghrelin. Data are expressed as the percentage of control values ± SEM for a total of 10 experiments performed in triplicate. Control hormone levels were measured in granulosa-lutein cell culture media cultured with serum-free media not containing ghrelin. *, $P < 0.001$, group treated with the antibody *vs.* untreated group. *Gray bars*, no antibody (− Ab); *white bars*, antibody (+ Ab).

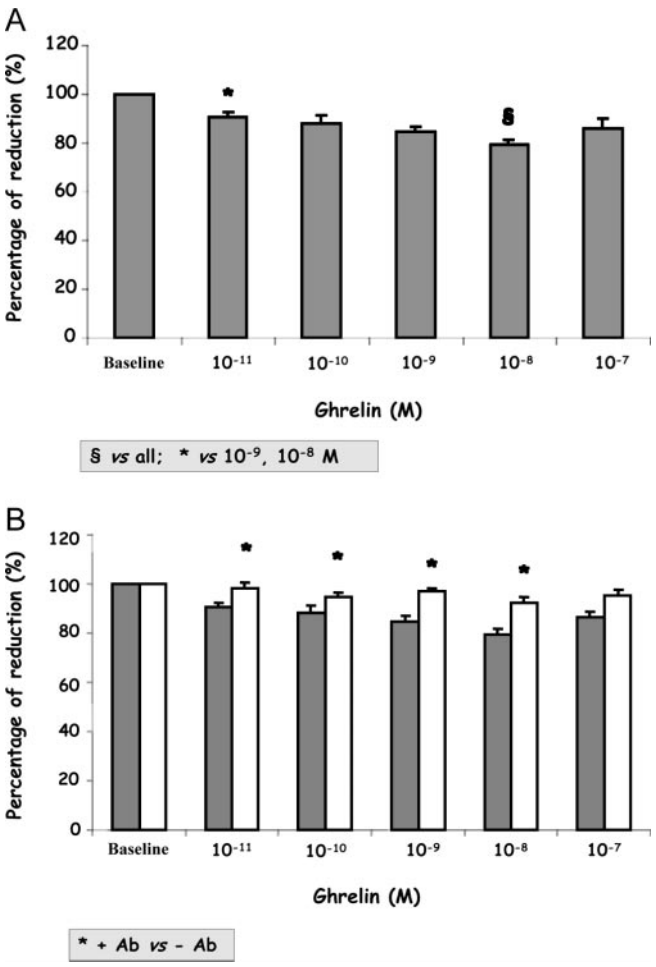

FIG. 2. A, Effects of increasing concentrations of ghrelin (10^{-11} to 10^{-7} mol/liter) on P_4 production by granulosa-lutein cells. Data are expressed as the percentage of control values ± SEM for a total of 35 experiments performed in triplicate. Control hormone levels were measured in granulosa-lutein cell culture media cultured with serum-free media not containing ghrelin. §, $P < 0.05$ *vs.* all; *, $P < 0.05$ *vs.* 10^{-9} and 10^{-8} mol/liter. B, Effects of a specific anti-GHS-R1a polyclonal antibody on granulosa-lutein cell P_4 production after stimulation with increasing doses of ghrelin. Data are expressed as the percentage of control values ± SEM for a total of 10 experiments performed in triplicate. Control hormone levels were measured in granulosa-lutein cell culture media cultured with serum-free media not containing ghrelin. *, $P < 0.05$, group treated with the antibody *vs.* untreated group. *Gray bars*, no antibody (− Ab); *white bars*, antibody (+ Ab).

within the cyclic ovary indicated a wide pattern of distribution of the 1a isoform, with detectable specific signals in oocytes as well somatic follicular cells, luteal cells from young and mature corpora lutea, and interstitial cells (21). The reasons for the discrepancies among different studies may be accounted for by the striking changes that the ovary undergoes during development and within the cycle, making it possible that the net expression levels of a gene may vary dramatically. Moreover, changes in the balance between 1a and 1b isoforms of GHS-R gene may occur in the human ovary under certain conditions, as shown in rat testis in which changes in the alternative splicing of the gene are observed throughout postnatal development (25). Specifically, during pubertal development, a shift in the pattern of splicing of the GHS-R gene takes place in rat testis, favoring expression of the biological active type 1a form of the receptor and indicating that the balance between receptor subtypes may represent a novel mechanism for the regulation of ghrelin sensitivity in gonads.

Although GHS-R1a is the fully functional form of the receptor, the GHS-R1b isoform is apparently devoid of high-affinity ligand-binding domain and signal transduction capacity, and its functional role is yet to be elucidated. Recently, it was shown that GHS-R1b behaves as a dominant-negative mutant of the full-length receptor in human embryonic kidney 293 cells, suggesting that conditions that up-regulate the expression of GHS-R1b may have pathophysiological consequences caused by the attenuation in GHS-R constitutive sig-

nal (26). Because estrogen has been shown to be involved in the regulation of ghrelin expression (27), it can be speculated that the high estrogen concentrations induced in women undergoing *in vitro* fertilization may result in a shift of the GHS-R gene splicing, leading to an excess of GHS-R1b in granulosa-lutein cells such as those analyzed in the present study. In view of the inhibitory effects of ghrelin on ovarian steroidogenesis described in the present study, the excess of GHS-R1b would counteract the negative effects of ghrelin on estrogen and be beneficial in a condition in which high estrogen concentrations are desirable. The GHS-R1b excess in granulosa-lutein cells would also explain the quantitatively limited inhibitory effect of ghrelin on steroid biosynthesis.

In the present study, we provide compelling evidence for a biological action of ghrelin in the ovary. Ghrelin, in a dose-dependent manner, was able to significantly inhibit E_2 and P_4 secretion by granulosa-lutein cells by binding to its functional receptor. The inhibitory effect was in fact counteracted by the addition to culture media of an antibody specific for the full-length isoform of GHS-R. Similar inhibitory effects of ghrelin on steroidogenesis were reported in the rat testis where, as in the ovary, the simultaneous expression of ghrelin and its cognate receptor is compatible with a potential action of locally produced ghrelin in the auto-paracrine regulation of gonadal function (18). Such a mechanism could contribute to the suppression of the reproductive axis in situations of negative energy balance, such as starvation. The latter is characterized by low blood gonadotropins and E_2 levels and high ghrelin concentrations. Ghrelin was shown to significantly decrease hypothalamic LHRH *in vitro* and LH pulse frequency *in vivo*, both in animals and humans (28). This inhibitory effect was suggested to be mediated by hypothalamic neuropeptide Y and agouti-related peptide, because both peptides were shown to inhibit pulsatile LH release, and their synthesis was shown to be decreased by ghrelin. In malnutrition states, the high circulating gut-derived ghrelin levels acting at the hypothalamus may interact with the ghrelin locally produced in the ovary to inhibit hypothalamic-pituitary-gonadal axis function. Such a mechanism would have adaptive value because it would divert limited resources toward important physiological processes and away from energy-consuming processes that are not so essential for immediate survival, such as reproductive function.

In conclusion, provided that *in vitro* studies may not necessarily reflect normal physiology *in vivo*, growing evidence indicates that ghrelin may participate in the regulation of different aspects of the reproductive function. These actions likely involve both the systemic gut-derived hormone, with potential actions at different levels of the hypothalamic-pituitary-gonadal axis, and the locally produced ghrelin, which may serve additional autocrine-paracrine roles in the control of gonadal function. Thus, ghrelin may be, together with leptin and other neuroendocrine integrators, part of a network of molecular signals responsible for the coordinated control of energy homeostasis and reproduction.

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