LH down-regulates gonadotropin-releasing hormone (GnRH) receptor, but not GnRH, mRNA levels in the rat testis

M-C Botté¹, Y Lerrant^{1,2}, A Lozach¹, A Bérault¹, R Counis¹ and M-L Kottler^{1,3}

¹Endocrinologie Cellulaire et Moléculaire de la Reproduction, Université P&M Curie, CNRS ESA 7080, 75005 Paris, France

²Université Française du Pacifique, Tahiti, France

³Service de Biochimie Médicale, CHU Pitié-Salpêtrière, 75013 Paris, France

(Requests for offprints should be addressed to M-C Botté, Endocrinologie Cellulaire et Moléculaire de la Reproduction, Université P&M Curie, CNRS ESA 7080, case 244, 4 place Jussieu, 75252 Paris cedex 05, France)

Abstract

The demonstration of an inhibitory effect of gonadotropin-releasing hormone (GnRH) agonists upon steroidogenesis in hypophysectomized rats and the presence of mRNA coding for GnRH and GnRH receptors (GnRH-R) in rat gonads suggests that GnRH can act locally in the gonads. To assess this hypothesis, we investigated the effects of GnRH analogs, gonadotropins and testosterone on the levels of both GnRH and GnRH-R mRNA in the rat testis. Using dot blot hybridization, we measured the mRNA levels 2 to 120 h after the administration of the GnRH agonist, triptorelin. We observed an acute reduction of both GnRH and GnRH-R mRNAs 24 h after the injection (about 38% of control). However, the kinetics for testis GnRH-R mRNA were different from those previously found for pituitary GnRH-R mRNA under the same conditions. Initially, the concentrations of serum LH and FSH peaked, then declined, probably due to the desensitization of the gonadotrope cells. In contrast, the GnRH antagonist, antarelix, after 8 h induced a 2.5-fold increase in GnRH-R mRNA, but not in GnRH mRNA, while gonadotropins levels were reduced. Human recombinant FSH had no significant effect on either GnRH or

GnRH-R mRNA levels. Inversely, GnRH-R mRNA levels markedly decreased by 21% of that of control 24 h after hCG injection. Finally, 24 h after testosterone injection, a significant increase in GnRH-R mRNA levels (2.3 fold vs control) was found, but a reduction in the concentration of serum LH, probably by negative feedback on the pituitary, was observed. In contrast, GnRH mRNA levels were not significantly altered following testosterone treatment.

Since LH receptors, GnRH-R and testosterone synthesis are colocalized in Leydig cells, our data suggest that LH could inhibit the GnRH-R gene expression or decrease the GnRH-R mRNA stability in the testis. However, this does not exclude the possibility that GnRH analogs could also affect the GnRH-R mRNA levels via direct binding to testicular GnRH-R. In contrast, the regulation of GnRH mRNA levels appeared to be independent of gonadotropins. Taken together, our results suggest a regulation of GnRH and GnRH-R mRNA specific for the testis.

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Introduction

In addition to the pituitary gonadotropin-releasing hormone (GnRH) receptors (GnRH-R) which are known to mediate GnRH effects on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion, GnRH-binding sites have also been described on Leydig cells in rat testes (Clayton *et al.* 1980, Lefebvre *et al.* 1980, Sharpe & Fraser 1980a), and we have shown that their nucleotide sequence is identical to that of the pituitary GnRH-R (Moumni *et al.* 1994). Moreover, an endogenous GnRH-like peptide has been found in Sertoli cells (Sharpe & Fraser 1980b, Paull *et al.* 1981, Bhasin *et al.* 1983) and, recently, we have demonstrated the presence of a GnRH

mRNA in the rat testis, with a sequence identical to that of the hypothalamic GnRH (Botté *et al.* 1998). Taken together, these studies suggest the presence of authentic GnRH and GnRH-R in the testis, but their physiological role in the endogenous regulation of testis functions remains unknown.

In male hypophysectomized rats, gonadal functions are no longer stimulated, and testosterone secretion can be further reduced by the administration of GnRH agonists at high doses or over long periods (Arimura *et al.* 1979, Hsueh & Erickson 1979). This strongly suggests, first, that GnRH agonists have a direct effect on steroidogenesis, possibly via gonadal GnRH-R, and secondly, that this action is independent of gonadotropins.

By contrast, in intact rats, it is well known that a treatment with GnRH agonists induces first a peak called 'flare-up', then a depletion of circulating gonadotropins, and later a decrease in steroid secretion. Thus, due to the regulation by the pituitary, the potential interactions of the agonists with the testicular GnRH-R are difficult to evaluate.

Elucidation of the physiological role of gonadal GnRH in the control of testicular functions requires analysis of the regulation of testicular GnRH and GnRH-R gene expression. In the pituitary, GnRH-R expression is regulated by both GnRH and steroids (Clayton & Catt 1981, Counis *et al.* 1993, Kaiser *et al.* 1993, Stojilkovic & Catt 1995) and, as we have previously shown, by GnRH agonists, the administration of which causes an acute depression of pituitary GnRH-R mRNA levels (Lerrant *et al.* 1995). The aim of this study was to investigate the hormonal regulation of both GnRH and GnRH-R mRNA levels in testes. We first assessed the effects of GnRH agonist and antagonist treatments. To complete the study, we investigated the effects of gonadotropins and finally that of testosterone. In all cases, GnRH and GnRH-R mRNA levels were quantified in the adult rat testis using dot blot hybridization, and serum gonadotropins and testosterone concentrations were measured in parallel.

Materials and Methods

Animals and treatments

Adult (230–250 g) male Wistar rats (Centre d'Élevage Janvier, Le Genest, France), were housed under controlled photoperiods (10 h light:14 h darkness), with water and food available *ad libitum*.

They were randomly assigned to the following groups: (1) animals injected i.m. with 300 µg/kg body weight (BW) of the GnRH agonist triptorelin ([D-Trp⁶]GnRH, decapeptyl long-acting formulation; Ipsen Biotech, Paris, France), (2) injected i.m. with 200 µg/kg BW of the GnRH antagonist antarelix (Reissmann *et al.* 1995) (Europeptides, Argenteuil, France), (3) injected s.c. with 300 IU/kg BW human chorionic gonadotropin (hCG 'Endo'; Organon, Eragny-sur-Epte, France), (4) injected s.c. with 10 or 100 IU/kg BW human recombinant FSH (h-rec FSH) (Puregon; Organon), (5) injected s.c. with 50 mg/kg BW testosterone enanthate (Androtardyl; Schering, Lys-Lez-Lannoy, France). In each group, control rats were sham-injected with vehicle.

Rats were killed by decapitation 2–120 h after injection. Blood was collected and serum was stored at –20 °C until analysis. Testes were dissected and immediately frozen in liquid nitrogen before being stored at –80 °C until use.

Hormone measurements

Serum LH and FSH levels were measured by RIA using kits generously provided by Dr A F Parlow (National Hormone and Pituitary Program, NIDDK). The post-injection hCG level was measured in serum, by a micro-particular enzymologic immunoassay using the AxSYM β-hCG kit (Abbott France, Rungis, France). Serum testosterone was measured by RIA using the DSL-4000 active testosterone coated-tube RIA kit (Chiron Diagnostics, Eragny sur Oise, France).

DNA probes

The 612 bp GnRH-R cDNA probe was described by Moumni *et al.* (1994) and the 344 bp GnRH cDNA probe by Botté *et al.* (1998). The cyclophilin cDNA probe was a gift from Dr J Douglass (Danielson *et al.* 1988). Probes were labeled by random priming with [α -³²P]dCTP (specific activity 3000 Ci/mmol) (Amersham, Arlington Heights, IL, USA) using an Amersham Megaprime labeling kit.

RNA extraction and dot blot hybridization

Total RNA was extracted using the Tri-Insta-Pure procedure (Eurogentec, Seraing, Belgium) (Chomczynski & Sacchi 1987), and was quantified by optical density at 260 nm.

Total RNA was formaldehyde denatured and diluted aliquots were spotted on Protran filters (Schleicher and Schuell, Ecqueville, France) using a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA, USA). At least three parallel determinations, using total RNA extracted from testes of distinct animals, were performed. Filter 1 (aliquots of 15 µg, 10 µg and 5 µg total RNA) was hybridized with the ³²P-labeled GnRH cDNA. Filter 2 (aliquots of 10 µg, 5 µg and 2.5 µg total RNA) was hybridized with the ³²P-labeled GnRH-R cDNA. Then both filters were stripped and rehybridized with the ³²P-labeled cyclophilin probe. Hybridization and washes were performed using standard methods, previously described (Garrel *et al.* 1993). Radioactivity on the filters was revealed by autoradiography (Kodak X-Omat AR) and quantified by densitometry of the entire surface of the spot, using a Biocom (Biocom S.A., Les Ulis, France) or an Autorad 210 (IMSTAR, Paris, France) device system.

Data analysis

The densitometric data for GnRH and GnRH-R were corrected for variability in loading by expressing the values as a ratio to cyclophilin. Results are presented as the percentage of the value obtained for the untreated control. All results, dot blot data and hormone assays are presented as the means ± S.E.M. of *n* determinations. Differences

Figure 1 Effects of triptorelin on GnRH and GnRH-R mRNA levels in the testis. Adult male rats received a single i.m. injection of 300 µg/kg BW triptorelin, and were killed at the times indicated. (A) Total RNA from testes were analyzed for their GnRH and GnRH-R mRNA content, by dot blot hybridization with 32 P-labeled GnRH and GnRH-R cDNA probes, followed by densitometry. Densitometric values were standardized to cyclophilin mRNA and expressed relative to untreated rats, as the means $\pm$ S.E.M. of n independent determinations ($n=4$ for GnRH, $n=6$ for GnRH-R). (B) Serum concentrations of LH and FSH were determined by RIA, and are represented as the means $\pm$ S.E.M. of six independent determinations. In all cases difference between means was assessed by ANOVA followed by Dunnett's t -test: * $P \leq 0.05$, ** $P \leq 0.01$ (vs control).

between means were assessed by ANOVA followed by Dunnett's t -test. A value of $P \leq 0.05$ was considered significant.

Results

Regulation of testicular GnRH and GnRH-R mRNA levels by GnRH analogs

Effects of a GnRH agonist The effects of a single injection of the long-acting GnRH agonist triptorelin on GnRH and GnRH-R mRNA levels in testes are represented in Fig. 1A, as percentage of the control value. GnRH-R mRNA levels started to decrease after a lag-time of 8 h; however, this decrease was significant ($P < 0.05$) only after 24 h, with a minimum of

Figure 2 Effects of a single i.m. injection of 200 µg/kg BW GnRH antagonist, antarelix, on GnRH and GnRH-R mRNA levels in the rat testis. (A) GnRH and GnRH-R mRNA levels were determined by dot blot hybridization and results are expressed as described in Fig. 1. Values are the means $\pm$ S.E.M. of three independent determinations. (B) Serum concentrations of LH and FSH were determined by RIA, and are represented as the means $\pm$ S.E.M. of three independent determinations. * $P \leq 0.05$, ** $P \leq 0.01$ (vs control).

40.7 $\pm$ 13.6%. Levels returned to control values within 4 days. GnRH mRNA levels followed a similar pattern, except that the decrease was significant ($P < 0.05$) as early as 2 h after the injection.

As shown in Fig. 1B, within minutes following the injection of the GnRH agonist both LH and FSH increased markedly ($P < 0.01$) in serum, reaching, 2 h after the injection, a maximum of 21 and 7 times greater than the control levels respectively. Both hormones then returned progressively to control levels within 24 h, and remained constant.

Effects of a GnRH antagonist Treatment with the GnRH antagonist, antarelix, induced a significant increase in GnRH-R mRNA levels (Fig. 2A) 5 h after injection, which peaked at 8 h showing a 2.5-fold increase vs control levels ($P < 0.01$). Levels returned to near control values after 24 h. In contrast, no significant variation of GnRH mRNA levels was seen. In the same experiment, the concentration of serum LH and FSH decreased significantly ($P < 0.05$ and $P < 0.01$ respectively), and remained

Figure 3 GnRH and GnRH-R mRNA levels in the rat testis, after a single s.c. injection of 300 IU/kg BW hCG. (A) GnRH and GnRH-R mRNA levels were determined by dot blot hybridization and results are expressed as in Fig. 1. Values are the means $\pm$ S.E.M. of three independent determinations. (B) hCG was assayed in serum by a microparticle enzymologic immunoassay using the AxSYM β -hCG kit, and testosterone was measured by RIA using the DSL-4000 active testosterone coated-tube RIA kit. Values are the means $\pm$ S.E.M. of three independent determinations. ** $P \leq 0.01$ (vs control).

about 50% that of the control value (47 and 58% respectively) from 2 to 8 h after injection (Fig. 2B). LH then returned to the control level, whereas the concentration of FSH remained low.

Regulation of testicular GnRH and GnRH-R mRNA levels by gonadotropins

Effects of hCG Figure 3A represents the variations in GnRH and GnRH-R mRNA levels after a single injection of hCG. GnRH-R mRNA levels began to decrease slightly after 2 h, then maintained a plateau at about 75% of the control levels. After 24 h, an acute decrease ($P < 0.01$) in GnRH-R mRNA levels to $21.4 \pm 4.0\%$ of the control value was observed. GnRH-R mRNA levels then increased progressively and returned to control levels after day 5. In contrast, GnRH mRNA levels were not significantly affected by the hCG treatment, the slight increase observed 48 h after the injection was not significant.

Figure 4 Effects of a single s.c. injection of 50 mg/kg BW testosterone enanthate on GnRH and GnRH-R mRNA levels in the rat testis. (A) GnRH and GnRH-R mRNA levels were determined by dot blot hybridization and results are expressed as in Fig. 1. Values are the means $\pm$ S.E.M. of three independent determinations. (B) The plasmatic concentration of LH was determined by RIA, and is presented as the means $\pm$ S.E.M. of three independent determinations. * $P \leq 0.05$, ** $P \leq 0.01$ (vs control).

Figure 3B shows hCG concentrations in blood. The level increased rapidly ($P < 0.01$ at 2 h), reaching a maximum value of 377 mU/ml at 8 h, then decreased and became undetectable after 5 days. During the same experiments, the concentration of testosterone in the serum exhibited a biphasic profile, with a peak ($P < 0.01$) at 2 and 24 h after the injection (19.2 ng/ml and 13.5 ng/ml respectively). On day 5, the level was near normal values.

Effects of h-rec FSH Neither GnRH nor GnRH-R mRNA levels were affected by the treatment, whatever the dose administered (data not shown).

Regulation of testicular GnRH and GnRH-R mRNA levels by testosterone

As shown in Fig. 4A, GnRH-R mRNA levels increased significantly ($P < 0.05$) 8 h after the injection of testosterone enanthate, and reached a maximum of $226 \pm 16.4\%$ ($P < 0.01$) at 24 h. GnRH-R mRNA levels returned to

control values in the 3 days that followed. By contrast, GnRH mRNA levels were not significantly affected.

Over the same time, LH decreased as expected, reaching a minimum 48% of the control value 24 h after the injection ($P < 0.05$) (Fig. 4B).

Discussion

In the present study we have shown that treatment with a long-acting GnRH agonist such as triptorelin induces an acute decrease in GnRH and GnRH-R mRNA levels in testes, 24 h after the injection. Studies performed previously in hypophysectomized rats have suggested that GnRH agonists could act directly on the testis to regulate testosterone secretion (Arimura *et al.* 1979, Hsueh & Erickson 1979), possibly via binding to GnRH-R present on Leydig cells. However, the effects of a long-acting agonist are complex. In fact, the injection of a GnRH agonist into intact rats is followed by a rapid and transient release of the pituitary content of gonadotropins, an effect called 'flare-up', followed immediately by a transient stimulation of steroid secretion. Then the desensitization of the gonadotrope cells leads to a fall in circulating concentrations of gonadotropins, and consequently to a reduction in androgen secretion. Thus, in intact rats, it is difficult to determine whether the inhibition of GnRH and GnRH-R mRNA levels we observed after triptorelin administration is due to a direct effect of the agonist on the testis, and/or to an indirect effect via the variations in gonadotropin or testosterone levels. We thus studied the consequences of each of these hormonal variations in an attempt to elucidate the factor responsible for the decrease in the GnRH and GnRH-R mRNA levels in the testis.

First, we injected a GnRH antagonist to avoid the initial flare-up and to inhibit gonadotropin secretion. We observed that GnRH-R mRNA levels increased when LH and FSH concentration were low, and then returned to control values when LH concentration returned to normal. However, the FSH level remained low. Such a differential effect between GnRH agonist and GnRH antagonist on GnRH-R mRNA levels in the testis may be related either to their different mechanisms of action, or to their distinct effects on gonadotropin secretion.

In the pituitary, the expression of GnRH-R is under the control of GnRH secretion by the hypothalamus (Clayton & Catt 1981). The acute administration of a potent antagonist *in vivo* induces a diminution of pituitary GnRH-R mRNA levels (Pinski *et al.* 1996), as do the GnRH agonists under desensitizing conditions (Lerrant *et al.* 1995). In the murine cell line α T3-1, it has been shown that high concentrations of GnRH agonists could inhibit and low concentrations stimulate the translation of GnRH-R mRNA without altering the mRNA levels (Tsutsumi *et al.* 1993, 1995). Taken together these data indicate that high concentrations of GnRH analogs induce a decrease in GnRH-R expression in the pituitary.

GnRH analogs could act directly on the testis, by binding to GnRH-R present on Leydig cells, as previously shown in hypophysectomized rats. In the present study, the GnRH antagonist induced an increase in the GnRH-R mRNA levels; however, in contrast, the GnRH agonist exerted a negative effect. This suggests that the antagonist would prevent the binding of an inhibiting factor, the endogenous GnRH secreted by Sertoli cells being a good candidate.

However, compared with the antagonist, the negative effect of the agonist was delayed (24 h vs 8 h). Indeed, when GnRH agonist was administered, the gonadotropin secretion underwent an initial flare-up, then an inhibition (only after 8 h), whereas it decreased quickly (as soon as 2 h) after the antagonist administration. To examine the role of each gonadotropin, we injected separately either hCG or h-rec FSH. Neither GnRH nor GnRH-R mRNA levels were affected by the h-rec FSH treatment, at doses previously shown to restore the testis weight after hypophysectomy (Tapanainen *et al.* 1993). Only hCG, which is known to bind to the LH receptor, affected the GnRH-R mRNA profile. It induced a biphasic decrease of the mRNA levels, the second diminution occurring 24 h after the injection, this pattern being quite similar to that observed after the agonist administration.

hCG induces an acute release of testosterone followed by a delayed stimulation of its secretion (Tremblay & Bélanger 1985). After testosterone administration, we observed an increase in GnRH-R mRNA levels, as found under antagonist treatment but with a delay (24 h vs 8 h) that could correspond to the negative feedback of testosterone on LH release.

Taken together these data suggest that LH decreases the GnRH-R mRNA levels. This hypothesis is strengthened by the demonstration that hCG also induces a significant decline of GnRH-R mRNA in other non-pituitary target cells containing LH/CG receptors, such as GT1-7 neurons (Li *et al.* 1996), and human granulosa-luteal cells (Peng *et al.* 1994). In our study, when LH was further increased, for example by treatment with GnRH agonist or hCG, GnRH-R mRNA levels decreased, whereas when LH decreased, for example by GnRH antagonist or testosterone administration, GnRH-R mRNA increased. However, these alterations in the GnRH-R mRNA levels could result from regulation of gene expression, as well as from modification of mRNA stability.

Consistent with a regulation of GnRH-R mRNA levels by LH within Leydig cells, LH receptors, GnRH-R and testosterone secretion have been found together in Leydig cells. Inversely, the absence of FSH receptors on Leydig cells could explain the lack of effect of FSH on GnRH-R mRNA.

The present study also points out that the regulation of GnRH-R mRNA levels in the testis is different from that in the pituitary. For example, identical GnRH agonist treatment induces an earlier inhibition (8 h after the

injection vs 24 h) and a more prolonged effect on GnRH-R mRNA levels in the pituitary (Lerrant *et al.* 1995) than in the testis. In addition, GnRH antagonists decrease GnRH-R mRNA levels in the pituitary whereas they are increased in the testis. Finally, in the pituitary, the GnRH-R mRNA levels are either increased or decreased by steroids, and this regulation is, at least in part, mediated by changes in the pattern of hypothalamic GnRH secretion (Kaiser *et al.* 1993). In contrast, in the testis they are increased by testosterone, and this stimulation appears to be mediated by a decrease in LH secretion.

Although GnRH mRNA levels were decreased by the GnRH agonist, neither GnRH antagonist, hCG nor h-rec FSH had an effect, suggesting that the regulation of the GnRH mRNA levels in the testis is independent of the gonadotropins. As previously demonstrated in cultured testicular cells, GnRH agonists exert an inhibitory effect on the steroidogenesis in Leydig cells (Hsueh *et al.* 1983); however, we have found that testosterone had no effect on the GnRH mRNA levels. Thus the decrease in these levels under GnRH agonist treatment may involve other paracrine factors which remain to be elucidated.

In summary, we have shown opposing effects of GnRH agonist and GnRH antagonist on testicular GnRH-R mRNA levels, and we have found that this difference may depend either on LH secretion pattern or on distinct direct effects on the testis. We have shown that LH is a regulator of the GnRH-R mRNA levels in the testis, whereas in the pituitary they are essentially regulated by steroids and GnRH. In contrast, the testicular GnRH mRNA levels appear to be independent of gonadotropins, but are reduced under GnRH agonist treatment. All these data suggest that the regulation of the GnRH and GnRH-R mRNA levels demonstrated in this study may be specific to the testis.

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