Expression of GnRH receptor in mouse and rat testicular germ cells*

P.Bull¹, P.Morales², C.Huyser³, T.Socías¹ and E.A.Castellón⁴

¹Departamento de Genética Molecular y Microbiología, P Universidad Católica de Chile, ²Unit of Reproductive Biology, Faculty of Health Sciences, University of Antofagasta, ³Reproductive Biology Laboratory, Department of Obstetrics and Gynaecology, University of Pretoria, South Africa and ⁴ICBM, Universidad de Chile, Chile

5To whom correspondence should be addressed at: Unit of Reproductive Biology, Faculty of Health Sciences, University of Antofagasta, PO Box 170, Antofagasta, Chile. E-mail: pmorales@uantof.cl

The expression of gonadotrophin-releasing hormone receptor (GnRH-R) in germinal cells of mouse testis, whole testes, pituitary glands, and mouse ovaries was determined by means of Northern hybridization using a mouse GnRH-R [³²P]-labelled cDNA probe. Also, the expression of GnRH-R in rat germinal cells, testis and pituitary gland was determined by Northern blot analysis using the same mouse-specific probe. Three receptor transcripts were detected in all cases. In mouse pituitary, ovary and testis, we found two GnRH-R transcripts in the proximity of 4.6–4.7 and 3.4 kb, as well as a 1.6 kb transcript in the pituitary and a 2.0–2.1 kb transcript in both the ovary and testis. Mouse germ cells also exhibited three GnRH-R transcripts of 4.7, 3.5 and 2.2 kb. Two distinct GnRH-R transcripts were also detected in the rat pituitary (4.6 and 2.1 kb), testis (4.7 and 3.5 kb) and germ cells (4.5 and 3.5 kb). In addition, a third transcript was detected in rat pituitary (1.9 kb) and in rat testis and germinal cells (2.1 kb). The present study demonstrates that GnRH-R mRNA is expressed in rat and mouse testicular germ cells. We suggest that GnRH-R present in these cells may interact with GnRH or GnRH-like peptides produced in the testis and may be part of a paracrine system. The presence of multiple GnRH-R encoding transcripts is also of interest and warrants further studies to evaluate their regulation and function.

Key words: GnRH/GnRH receptors/spermatozoa/spermatogenic cells/testis

Introduction

Gonadotrophin-releasing hormone (GnRH) is a hypothalamic decapetide, which regulates mammalian gonadotrophin secretions by binding to specific, high-affinity receptors (GnRH-R) on the pituitary (Naor, 1990; Stojilkovic and Catt, 1992; Catt et al., 1993; Hawes and Conn, 1993; Stojilkovic et al., 1994). These receptors have been localized in the anterior pituitary gland, brain, steroid-dependent tumours and reproductive organs, and they belong to the family of heptahelical G protein-coupled receptor proteins (Catt et al., 1993; Sealfon and Millar, 1995; Anderson, 1996; Anderson et al., 1996). In addition to the orchestration of gonad activity through the pulsatile release of LH and FSH, GnRH may play an important role in the regulation of extrapituitary physiological functions (Hsueh and Jones, 1981; Stojilkovic et al., 1994).

GnRH and GnRH-like material has been observed in human seminal plasma (Izumi et al., 1985; Sokol et al., 1985) and follicular fluid (Ying et al., 1981; Li et al., 1993). Moreover, the presence of GnRH or GnRH-like material has been demonstrated in the ovary (Birnbaumer et al., 1985; Aten et al., 1987; Oikawa et al., 1990; Li et al., 1993), testis (Sharpe and Fraser, 1980; Bhasin et al., 1983; Sharpe and Cooper, 1987), and prostate gland (Azad et al., 1993) of several mammalian species. GnRH-R has also been demonstrated in these organs. In the testis, GnRH-R are expressed in Leydig but not Sertoli cells (Belisle et al., 1984; Dufau et al., 1984; Eidne et al., 1985a,b, 1987; Iwashita and Catt, 1985; Butzow et al., 1987; Kadar et al., 1988; Marchetti et al., 1989). Based on this evidence, it has been suggested that GnRH or GnRH-like material have an endocrine/paracrine function in the gonads of several mammalian species, including humans. Using in-situ hybridization, the expression of ovarian GnRH-R mRNA was demonstrated in granulosa cells of atretic rat follicles (Kogo et al., 1995). These authors suggested that the receptor might be involved in the control of various ovarian functions. Moreover, by using in-situ hybridization and enzymatic receptor localization, Bahk et al. identified (i) GnRH mRNA in Sertoli and spermatogenic cells of mature rat testis; (ii) stage-specific expression of GnRH in mRNA of human adult testis; and (iii) GnRH-R in the interstitial tissue of the testis, including Leydig cells (Bahk et al., 1995). It was postulated that GnRH is produced in Sertoli cells and reacts with GnRH-R in interstitial cells as a paracrine hormone (Bahk et al., 1995). Other authors (Azad et al., 1993) reasoned that GnRH-like material in seminal plasma might originate from the prostate, since pro-GnRH and its mRNA have been identified in the gland.

*This work was presented in part at the 14th Annual Meeting of the European Society of Human Reproduction and Embryology, Göteborg, June 21–24, 1998.

© European Society of Human Reproduction and Embryology
Expression of GnRH/GnRH-R in reproductive tissues such as the ovary and testis may modulate various processes or mechanisms during the fertilization process. Recent reports have indicated that while GnRH increased sperm–zona pellucida binding in humans (Morales, 1998), several GnRH antagonists inhibited this process (Morales et al., 1999). It was suggested that GnRH might bind to specific sites on the sperm plasma membrane, whereby the affinity of zona ligands on the spermatozoa could change or previously masked zona ligands could be exposed (Morales, 1998). Given the importance of GnRH and GnRH-R in the treatment of various reproductive disorders, as well as an information surge which indicates interaction between spermatozoa and GnRH or GnRH-like molecules, the expression of GnRH-R by Northern blot analysis in mouse and rat testicular germ cells using a mouse specific probe was investigated in the present study.

Materials and methods

Reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Germ cell isolation

Immature mice (C57Bl-MDX, n = 6–8 per experiment) and rats (Sprague–Dawley, n = 4–6 per experiment) were killed at 22 and 30 days of age respectively. Individual germ cell preparations were obtained by means of enzymatic procedures (collagenase/trypsin treatment), as described (Janecki et al., 1988; Castellón, 1998). Briefly, detumescated testes were injected with 0.3–0.4 ml CaCl\_2, MgCl\_2 free Hanks’ balanced salt solution (HBSS) medium containing 0.2% bovine serum albumin (BSA), 0.25% collagenase, 0.1% hyaluronic acid, 0.01% DNase and 20 mmol/l HEPES (pH 7.4). The testes were then incubated in the latter mentioned solution at 34°C in a shaking waterbath until no individual tubules were visualized (c. 20 min). The supernatant containing Leydig cells was discarded after washing and the tubules were re-incubated in HBSS supplemented with 0.2% trypsin, 0.01% DNase, 0.2% BSA and 20 mmol/l HEPES (pH 7.4) at 34°C in a shaking waterbath until no individual tubules were visualized. The tubule fragments were gently washed and sedimented at unit gravity for 20 min at room temperature. The supernatant containing germ cells was centrifuged at 150 g for 5 min. Then, the germ cell pellet was gently washed and resuspended in a small volume of HBSS without BSA. Cell samples were microscopically evaluated (20 individual fields of two slides) by means of Harris haematoxylin and eosin and 3β-hydroxysteroid-dehydrogenase cytochemical staining (Contreras and Ronco, 1994).

RNA isolation and Northern hybridization

Total RNA was extracted from individual germ cell preparations, as well as from whole testes, pituitary glands, and mice ovaries with the TRIzol reagent (Gibco BRL Products, Gaithersburg, MD, USA), as directed by the manufacturer. The RNAs were processed separately, quantified by UV spectroscopy and any degradation was assessed by ethidium bromide staining intensity of 28S and 18S ribosomal RNA after formaldehyde agarose gel electrophoresis. The purity of the RNA was assessed by the ratio of absorbance at 260 and 280 nm. Electrophoresis of RNA (usually 50 µg/loading), transfer onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA), Northern hybridization, and visualization by autoradiography were performed using conditions previously described (Sambrook et al., 1989). Hybridization probes were labelled with [\textsuperscript{32}P]-dCTP DNA labelling system (Random primer labelling, Gibco BRL Products). Filters were hybridized by using the mouse GnRH-R cDNA EcoR1/XhoI fragment (Tsutsumi et al., 1992) or [\textsuperscript{32}P]-radiolabelled β-actin cDNA as a probe (Ng et al., 1985). Membranes were washed at high stringency, 0.1× 150 mmol/l NaCl, 15 mmol/l sodium citrate pH 7 (SSC), 0.5% sodium dodecyl sulphate (SDS) at 65°C, for homologous probe, or low stringency (1× SSC, 0.1% SDS, 42°C) for heterologous probe as indicated.

Results

To assess residual contamination of other cell types in the germinal cell fractions, cell smears were fixed and stained with haematoxylin and eosin. Light microscope evaluation of samples showed 80 ± 2% and 82 ± 2% mid- and late pachytene spermatocytes (Figure 1) in the mouse and rat isolated germ cell preparations respectively. Most of the spermatocytes were in the process of meiotic cell division. The samples contained <2% Leydig cells and 7 ± 1% interstitial cells (macrophages and mast cells), 8–10% Sertoli cells and 3–5% other cell types.

To compare and verify the expression of GnRH-R mRNA in mouse and rat germ cell samples, other tissue samples were analysed concurrently. As shown in Table I and Figure 2, mRNA from isolated germ cells, testis and pituitary tissues from mouse and rat, as well as mouse ovary tissue were hybridized with a mouse receptor cDNA probe. Three receptor transcripts were detected in all cases. Under high stringency conditions of hybridization, the mouse pituitary and testis, ovary and germinal cells revealed two receptor transcripts in the proximity of 4.6–4.7 and 3.4–3.5 kb (Figure 2A). A third transcript of 1.6 kb was found in the mouse pituitary. Similarly, a third transcript of 2.1 kb in the ovary, a 2.0 kb transcript in the testis and a 2.2 kb transcript in germ cells of the mouse were observed. Using low stringency conditions of hybridization, mRNAs encoding GnRH-R were also detected in rat tissues (Figure 2B). Likewise, the mouse receptor cDNA probe hybridized to three distinct receptor transcripts, sized 4.6, 2.1 and 1.9 kb in the rat pituitary. The rat testis and germinal cells revealed both two transcripts of 3.5 and 2.1 kb, with an additional 4.5 kb transcript in the germinal cells and a 4.7 kb transcript in the testis. The precise identity of the additional bands present at low abundance remains to be elucidated. A comparison of GnRH-R mRNA values in whole testes from immature (aged 22 days) and mature (aged 45 days) mice was inconclusive (data not shown). Ethidium bromide staining intensity of 28S and 18S ribosomal RNA after formaldehyde agarose gel electrophoresis as well as signal intensity of β-actin indicated RNA overloading.

Discussion

It has been shown that GnRH or GnRH-like material is produced in the testis of several mammalian species. Different authors have demonstrated that Sertoli cells (Sharpe and Fraser, 1980; Bhasin et al., 1983; Verhoeven and Cailleau, 1985; Sharpe and Cooper, 1987; Saint et al., 1988) can produce GnRH and that Leydig cells express GnRH-R (Hsueh and
Table I. Gonadotrophin-releasing hormone receptor (GnRH-R) transcripts in mouse and rat tissues, using a mouse GnRH-R [32P]-labelled cDNA probe. mRNA from mouse and rat tissues was extracted and then Northern blotted with a [32P]-labelled cDNA probe against the mouse GnRH receptor. Molecular weights were estimated from the 18S and 28S rRNA migration.

<table>
<thead>
<tr>
<th>Mouse Tissue</th>
<th>Transcript 1 (kb)</th>
<th>Transcript 2 (kb)</th>
<th>Transcript 3 (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>4.7</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Ovary</td>
<td>4.6</td>
<td>3.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Testis</td>
<td>4.7</td>
<td>3.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Germ cells</td>
<td>4.7</td>
<td>3.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rat tissue</th>
<th>Transcript 1 (kb)</th>
<th>Transcript 2 (kb)</th>
<th>Transcript 3 (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>4.6</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Testis</td>
<td>4.7</td>
<td>3.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Germ cells</td>
<td>4.5</td>
<td>3.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Schaeffer, 1985; Iwashita and Catt, 1985; Jegou et al., 1985; Nikula and Huhtaniemi, 1988; Petersson et al., 1989; Kakar et al., 1992; Reinhart et al., 1992). In the present study, we have found for the first time that GnRH-R mRNA is expressed in both mouse and rat testicular germ cells, analysed by Northern blotting. Although this is not conclusive evidence for the presence of GnRH-R in mature sperm cells, certainly these findings are consistent with our previous work that indicated that GnRH regulates sperm–zona pellucida binding. We have shown that GnRH increases sperm binding to the human zona pellucida by a direct effect (Morales, 1998) that is mediated by calcium influx into the sperm cells (Morales et al., 1998). In addition, we showed that GnRH antagonists blocked the stimulatory effect of GnRH and that, at higher concentrations, they had a clear inhibitory effect on sperm–zona binding (Morales et al., 1999). These observations are in agreement with the action of GnRH upon stimulation of LH and FSH secretion in pituitary gonadotrophs. GnRH exerts its action on pituitary cells by binding to specific receptors located on the plasma membrane. The activation of GnRH-R leads to a rapid increase in inositol 1,4,5-trisphosphate (InsP$_3$) and diacylglycerol, with the consequent onset of Ca$^{2+}$ signal- and translocation of protein kinase C to the plasma membrane (Stojilkovic and Catt, 1992; Catt et al., 1993; Hawes and Conn, 1993). Through the binding to the same specific receptors, agonists and antagonists of GnRH inhibit the release of gonadotrophins and, therefore, gonadal function. Thus, this work provides the connection between the biological action and binding site for GnRH on the sperm cells.

In all the tissues examined, we found three GnRH-R transcripts of different sizes. Other investigators have also found multiplicity of sizes in GnRH-R transcripts. One study (Kaiser et al., 1992) reported a large transcript of 5.0–5.5 kb, another of 4.5 kb and a small one of 1.8 kb in rat pituitary, ovary and testis. Reinhart et al. found two transcripts of 3.5 kb and 1.6 kb in mouse pituitary gland, and a single receptor mRNA of 4.6 kb in rat pituitary, ovary, and Leydig cells (Reinhart et al., 1992), while other authors (Illing et al., 1993) described four transcripts of 5.4, 3.6, 2.3 and 1.3 kb in sheep pituitary, and demonstrated up-regulation of these transcripts by castration. In bovine pituitary four transcripts of 5, 3.5, 2 and 1.5 kb, were found (Kakar et al., 1993), of which the 5.0 kb was the most abundant. Finally, three transcripts were reported (Quiñones-Jenab et al., 1996) in female rat pituitary of 5, 4.5 and 1.4 kb, and that the transcripts were up-regulated after oestrogen treatment. In all cases examined, there were transcripts ~5, 3.5, 2.5 and 1.5 kb, were found (Kakar et al., 1993), of which the 5.0 kb was the most abundant. Finally, three transcripts were reported (Quiñones-Jenab et al., 1996) in female rat pituitary of 5, 4.5 and 1.4 kb, and that the transcripts were up-regulated after oestrogen treatment. In all cases examined, there were transcripts ~5, 3.5, 2 and 1.5 kb, with more or less variability due to the broad bands obtained. Sometimes the smallest fragment is not seen due to their lesser abundance. Multiple initiation sites and multiple polyadenylation signals could be responsible for the multiplicity of sizes, as demonstrated for the human receptor gene (Fan et al., 1995). More recently, fetal expression of GnRH and GnRH-R genes was reported (Botté et al., 1998) in rat testis and ovary. Since their demonstration was based upon polymerase chain reaction (PCR), they did not report size differences. Lastly, Chen et al. found, using PCR, expression of GnRH-R in human peripheral blood mononuclear cells (Chen et al., 1999). It would be very important to confirm the presence of
GnRH-R protein in mature sperm cells, by using antibodies against the protein; however, this has not yet been possible. The presence of functional GnRH receptor protein in sperm cells should open a whole new field of investigation.

In conclusion, GnRH-R mRNA is expressed in rat and mouse germ cells. We detected at least three transcripts, ~4.7, ~3.4, and 2 kb. The function of each of them is currently being studied.

Acknowledgements

We wish to thank R.P. Millar (UCT Medical School, South Africa) for the gift of the mouse GnRH-R cDNA. This project was supported by FONDECYT 197/1243, CONRAD MSG 9617 and RF 94025#15 and by financial support from the UNDP UNFPA/WHO/World Bank Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organisation. Project no. 98184.

References


Marchetti, B., Guarcello, V., Morales, M.C. et al. (1989) Luteinizing hormone-releasing hormone (LHRH) agonist restoration of age-associated decline of thymus weight, thymic LHRH receptors, and thymocyte proliferative capacity. Endocrinology, 125, 1037–1045.


Received on January 28, 2000; accepted on April 17, 2000