Expression of type I GNRH receptor and in vivo and in vitro GNRH-I effects in corpora lutea of pseudopregnant rabbits

Massimo Zerani, Francesco Parillo, Gabriele Brecchia¹, Gabriella Guelfi¹, Cecilia Dall’Aglio², Lorena Lilli¹, Margherita Maranesi¹, Anna Gobbetti³ and Cristiano Boiti¹

¹Sezione di Fisiologia veterinaria and ²Sezione di Anatomia veterinaria, Laboratorio di Biotecnologie fisiologiche, Dipartimento di Scienze biopatologiche ed Igiene delle produzioni animali e alimentari, Università di Perugia, 06100 Perugia, Italy
³Scuola di Bioscienze e biotecnologie, Università di Camerino, 62032 Camerino, Italy

(Correspondence should be addressed to M Zerani; Email: massimo.zerani@unicam.it)

Abstract

The expression of type I GNRH receptor (GNRHR-I) and the direct role of GNRH-I on corpora lutea (CL) function were studied in the pseudopregnant rabbit model. Immunohistochemistry evidenced GNRHR-I and GNRH-I in luteal cells at early (day 4 pseudopregnancy)-, mid (day 9)-, and late (day 13)-luteal stages. Real-time RT-PCR and western blotting revealed GNRHR-I mRNA and protein at the three luteal stages. Buserelin in vivo treatment at days 9 and 13 decreased plasma progesterone levels for 48 and 24 h respectively. In in vitro cultured CL, buserelin reduced progesterone secretion, increased prostaglandin F2α (PGF2α) secretion and cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) activities at days 9 and 13, and decreased PGE2 at day 13. Co-incubation with antagonists for GNRH-I (antide), inositol 1,4,5-trisphosphate (IP3, 2-amino-ethoxydiphenylborate), and diacylglycerol (DAG, 1-hexadecyl-2-acetyl glycerol) or inhibitors for phospholipase C (PLC, compound 48/80), and protein kinase C (PKC, staurosporine) counteracted the buserelin effects. Buserelin co-incubated with COX inhibitor (acetylsalicylic acid) increased progesterone and decreased PGF2α and NOS activity at days 9 and 13, whereas co-incubation with NOS inhibitor (N-nitro-L-arginine methyl ester) increased progesterone at the same luteal stages. These results suggest that GNRHR-I is constitutively expressed in rabbit CL independently of luteal stage, whereas GNRH-I down-regulates directly CL progesterone production via PGF2α at mid- and late-luteal stages of pseudopregnancy, utilizing its cognate type I receptor with a post-receptorial mechanism that involves PLC, IP3, DAG, PKC, COX-2, and NOS.

Journal of Endocrinology (2010) 207, 289–300

Introduction

GNRH is a hypothalamic neuronal secretory decapeptide that plays a central regulatory role in mammalian reproductive physiology. GNRH influences reproductive processes mainly by regulating pituitary gonadotropin synthesis and release, which in turn modulate steroidogenesis and gametogenesis (Conn & Crowley 1994). Even if the hypothalamus and pituitary are, respectively, the principal source of and target site for GNRH, several studies have reported an extra-hypothalamic origin of GNRH, as well as an extrapituitary presence of GNRH receptor (GNRHR) in numerous peripheral tissues including reproductive organs, such as testis, prostate, ovary, oviduct, placenta, and mammary gland (Ramakrishnappa et al. 2005).

There are currently 14 identified naturally occurring GNRH structural variants across the vertebrate species (Somoza et al. 2002). However, it is evident that at least three forms of GNRH are present in the majority of the vertebrate species studied: hypothalamic GNRH (GNRH-I), mid-brain GNRH (GNRH-II), and telencephalic GNRH (GNRH-III) (Gorbman & Sower 2003). In extrapituitary reproductive tissues, GNRH is considered to act in an autocrine or a paracrine manner and to regulate ovarian steroidogenesis by exerting a stimulatory as well as an inhibitory effect on the production of steroid hormones and apoptosis in ovarian follicles and corpora lutea (CL; Dubois et al. 2002, Ramakrishnappa et al. 2005). Although three types of GNRHR have been identified thus far, mammals have only two types of receptors: GNRHR-I and -II (Millar 2005). All known GNRHRs are transmembrane G protein-coupled receptors that produce their effects by activating phospholipase A2 (PLA2), phospholipase C (PLC), PLD, or adenylate cyclase (AC) cell signaling pathways (Millar 2005). Phospholipase activation may generate arachidonic acid (AA) that is converted into prostaglandins (PGs) by the action of the cyclo-oxygenase-1 and -2 (COX-1 and -2) as well as by that of other PG synthase enzyme types (Naor 2009).

Various studies have shown that GNRH exerts both inhibitory and stimulatory effects in the gonads, with either...
down- or up-regulation of the ovarian cellular steroid production (Ramakrishnappa et al. 2005). Few studies have addressed the direct effects of GNRH on rabbit CL, with conflicting results: earlier works pointed to CL regression with serum progesterone decline (Hilliard et al. 1976, Rippel et al. 1976), while a later study indicated that there was no effect on steroid production by ovary cultured in vitro (Eisenberg et al. 1984). Therefore, the main objectives of the present study were to examine the in vivo and in vitro modulatory effects of GNRH on rabbit CL during early-, mid-, and late-luteal stages of pseudopregnancy. With this end in view, experiments were devised to clarify the presence of GNRHR-I and GNRH-I and their possible post-receptorial mechanisms, as well as the effects of GNRH-I on the production of progesterone, PGE₂, and PGF₂α and on the activities of COX-1 and -2 and nitric oxide synthase (NOS), the enzymes involved in the regulation of rabbit CL life span (Boiti et al. 2000, 2005, Zerani et al. 2007).

Materials and Methods

Reagents

Reagent for isolation of total RNA (TRIzol) was purchased from Invitrogen. iSCRIPT cDNA and IQ SYBR Green SuperMix were purchased from Bio-Rad. QIAquick PCR Purification Kit for sequencing PCR product was from Qiagen. Random hexamer primers, DNase I Amplification Grade, real-time PCR primers for GNRHR-I and 18S were supplied by Invitrogen.

Tritiated progesterone, PGE₂, PGF₂α, and AA were purchased from Amersham Biosciences Ltd, while non-radioactive hormones and specific antisera were from Sigma–Aldrich. The NOS detect TM assay kit was purchased from Biomol Research Laboratories (South San Francisco, CA, USA). The avidin–biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3’-diaminobenzidine tetrachloride (DAB) were purchased from Vector Laboratories (Burlingame, CA, USA). The mouse monoclonal anti-β-tubulin antibody was from Sigma–Aldrich. PageRuler Ladder for western blot (WB) analysis was obtained from Fermentas (Burlington, Ontario, Canada). The enhanced chemiluminescence detection system for WB (Immobilon Western Chemiluminescent HRP Substrate) was purchased from Millipore (Billerica, MA, USA). The HRP-conjugated rabbit anti-goat IgG antibody as well as the Restore Western Blot Stripping Buffer was obtained from Thermo Fisher Scientific (Rockford, IL, USA). The Protran nitrocellulose membranes were from Whatman (Dassel, Germany). Biomax film for chemiluminescent blot imaging was from Kodak Laboratories. The bands were quantified using Quantity One software (Bio–Rad Laboratories).

The kit for the protein assay was purchased from Bio–Rad Laboratories. Medium 199 and Earle’s Balanced Salt Solution were from Gibco. HEPES, BSA, buserelin, antide, eCG, 4-bromophenacyl bromide, compound 48/80, propranolol, 2-O-methyladenosine, acetylcholine (ASA), and N-nitro-l-arginine methyl were purchased from Sigma, 2-amino-ethoxydiphenylborate was from Tocris Cookson Ltd (Northpoint Fourth Way Avonmouth, UK), and 1-hexadecyl-2-acetyl glycerol was from Biomol Research Laboratories (Plymouth Meeting, PA, USA), whereas all other pure grade chemicals and reagents were obtained locally.

Animals and hormonal regimen

Sexually mature New Zealand White female rabbits (3.5–4 kg body weight and 5 months of age) raised in premises owned by the University of Perugia were used for all experiments. The rabbits were housed individually in wire mesh cages under controlled light (14 h light:10 h darkness; lights off at 2100 h) and temperature (18–24 °C) conditions. Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU eCG followed by 0.8 μg buserelin 3 days later (Stradaioli et al. 1997). The day of buserelin injection was designated as day 0. The animals (n = 5 for each luteal stage) were killed by cervical dislocation in accordance with the guidelines and principles for the care and use of research animals. The protocols involving the use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

Tissue collection

Upon killing, reproductive tracts were promptly removed and thoroughly washed with saline. Within a few minutes, the CL were excised from ovaries and, after careful dissection of non-luteal tissue by fine forceps under stereoscopic magnification, immediately processed for in vitro experiments or frozen at −80 °C, after rinsing with RNase-free PBS, for later evaluation of gene and protein expression. For the immunohistochemical detection of GNRHR-I and GNRH-I, the ovaries of two additional animals for each luteal stage were fixed by immersion in 4% (w/v) formaldehyde in PBS (pH 7.4) for 24 h at room temperature, and subsequently processed for embedding in paraffin following routine tissue preparation procedures. The pituitary gland of a control rabbit was taken and processed as described above.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated through graded ethanol, and finally rinsed in distilled water; then they were submitted to antigen retrieval by
microwaving in citrate buffer solution (10 mmol/l, pH 6) at 700 W for 10 min. The slides were cooled at room temperature. After rinsing with Tris-buffered saline (TBS), the specimens were dipped in 3% H2O2 (v/v) in methanol for 1 h to quench the endogenous peroxidase activity and rinsed in TBS. Background labeling was prevented by incubating the sections with normal rabbit serum (for GNRHR-I) and normal goat serum (for GNRH-I), diluted 1:10, for 30 min at room temperature. Subsequently, the sections were incubated for 1 h at 37 °C and then overnight at 4 °C in a moist chamber with goat polyclonal anti-GNRHR-I primary antibody, raised against 18 C-terminal residues, diluted 1:200 in TBS containing 0.1% BSA, whereas the ovary sections were treated also with mouse monoclonal anti-GNRH primary antibody diluted as described above. The next day, the slides were rinsed three times in TBS for 5 min each, treated again with normal serum and then incubated for 30 min at room temperature with biotin rabbit anti-goat (for GNRHR-I) or biotin goat anti-rabbit (for GNRH-I) secondary antibodies diluted 1:200 in TBS. After TBS washes, the slides were exposed to ABC for 30 min and rinsed again with TBS. The peroxidase activity sites were visualized using DAB kit as chromogen; then the specimens were rinsed twice with distilled water for 5 min each, counterstained with hematoxylin, washed in running tap water and, finally, dehydrated passing through graded ethanol (70, 95, and 100% v/v), cleared in xylene and water, and, finally, dehydrated passing through graded ethanol (70, 95, and 100% v/v), cleared in xylene and mounted with medium for light microscopy. Tissue sections in which the primary antibody was omitted or substituted by goat or mouse IgG were used as negative controls of non-specific staining. Pituatory tissue sections from a control rabbit were included as positive controls for the GNRH-I reaction. The intensity of GNRHR-I and GNRH-I immunostaining in follicles and CL was assessed by goat or mouse IgG were used as negative controls of non-specific staining. Pituatory tissue sections from a control rabbit were included as positive controls for the GNRH-I reaction. The intensity of GNRHR-I and GNRH-I immunostaining in follicles and CL was assessed by

**Table 1** Primers used for gene quantification by real-time PCR

| Gene       | Accession number       | bp     | Primers                                                                 
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GNRHR-I</td>
<td>AV781779 Orcytalus cuniculus</td>
<td>191</td>
<td>TATCCACCTCTACAAATGGA ATGAAAGGAACCGGTGTCAGAG GAGGGATCCATGGAGTACCTG GGAAGGTTGGAGAAACACATCTG</td>
</tr>
<tr>
<td>FSH</td>
<td>AW429104.1 Homo sapiens</td>
<td>150</td>
<td>GAGGGATCCATGGAGTACCTG GGAAGGTTGGAGAAACACATCTG CTGGAAGAAGATCCAAATG CGATTAGCCCTCTGAGGACCTG</td>
</tr>
<tr>
<td>LHR</td>
<td>S57793 Homo sapiens</td>
<td>118</td>
<td>CTTTCTTGAGTACCTGAGCCTTTC</td>
</tr>
<tr>
<td>18S</td>
<td>X03205.1 Homo sapiens</td>
<td>148</td>
<td>TTTCTTTAAGTTCAGCGCTTTC</td>
</tr>
</tbody>
</table>

**GNRH-I, FSHR, and LHR real-time RT-PCR**

Total RNA was extracted from CL of three rabbits for each luteal stage as described previously (Boiti et al. 2005). According to the protocol provided by the manufacturer, 5 μg total RNA were reverse transcribed in 20 μl of iSCRIPT cDNA using random hexamer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. Serial experiments were performed to optimize the quantitative reaction, efficiency, and threshold cycle (Ct) values. The optimal 25 μl PCR volume contained 12.5 μl SYBR Green SuperMix, 1 μl forward and reverse primers (stock concentration 10 μmol/l), and 25 μl water. The primers used are listed in **Table 1**. All reagents were mixed as a master mix and distributed into a 96-well PCR plate before adding 2 μl cDNA (diluted 10-fold with water). For every PCR run were included reaction controls without template, as negative controls, and control without reverse transcriptase in RT, to assess whether the RNA was free by genomic DNA contamination. Samples’ amplification fidelity was also verified by agarose gel electrophoresis. PCR was performed on iCycler iQ (Bio-Rad) with an initial incubation at 95 °C for 1.5 min, followed by 40 cycles at 95 °C for 15 s, 53 °C for 30 s, during which fluorescence data were collected. The Ct value of each trace was automatically computed. PCR products were purified and sequenced by Qiagen PCR Purification Kit according to the manufacturer’s protocol. The 18S Ct housekeeping gene, was determined to normalize sample variations in the amount of starting cDNA. Standard curves generated by plotting the Ct value against the log cDNA standard dilution (1/10 dilution) in nuclease-free water were used to compare the relative amount of target genes. The slope of this graph was used to determine the reaction efficiency. Quantification of mRNA samples was performed by iCycler system software. The melting curve analysis, performed immediately after the PCR end cycle, was used to determine the specificity of each primer set. A melt-curve protocol was performed by repeating 80 °C heating for 10 s, from 55 °C with 0.5Cc increments, during which fluorescence data were collected. The melting temperatures of the products were determined graphically with fluorescence change rate (Δd(RFU)/dT) versus temperature.
Western blotting

Total luteal proteins were extracted from a pool of ten CL of three rabbits for each luteal stage. The CL were homogenized in 1 ml ice-cold RIPA buffer (PBS containing 1% Igepal CA-630, 0·5% sodium deoxycholate, 0·1% SDS v/v) as described previously (Zerani et al. 2004). After incubation at 4°C for 20 min, the homogenates were centrifuged at 12 000 g for 60 min at 4°C. The supernatants were collected, and their protein concentrations were measured using the protein assay kit with BSA as a standard. Equal amounts of proteins (20 μg) were separated by discontinuous 10% SDS-PAGE (w/v) with 4% stacking gel (w/v) for 40 min at 200 V and 500 mA, after which proteins were transferred onto a nitrocellulose membrane for 1 h at 100 V and 350 mA. After the transfer, non-specific binding of antiseraum was prevented by incubation for 1 h at 100 V and 350 mA. The supernatants were collected, and their protein concentrations were measured using the protein assay kit with BSA as a standard. All antibody incubations were performed in TBS containing 5% BSA (w/v) and 0·05% Tween-20 (v/v). Blocked membranes were then probed with antibody against GNRHR (1:500) overnight at 4°C. After washing with TBS, the membranes were incubated with HRP-labeled rabbit anti-goat IgG secondary antibody (1:20 000) at room temperature for 1 h. All antibody incubations were performed in TBS containing 5% BSA (w/v) and 0·05% Tween-20 (v/v). After washing with TBS, the immunocomplexes were visualized by using an enhanced chemiluminescence detection kit according to the manufacturer’s protocol and exposed to X-ray film. Blot images were acquired, and the intensities of the bands were quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories). Membranes were then rebotted with mouse anti-β-tubulin monoclonal antibody (0·05 μg/ml) and processed as described above to verify equal loading of proteins. Data were normalized by reference to β-tubulin after membrane stripping for 15 min with stripping solution. Values were expressed as arbitrary units of relative abundance of the specific protein normalized with that of β-tubulin used as a loading control.

In vivo experiments

At days 4, 9, and 13 of pseudopregnancy, two groups of rabbits (n=6/group per day) were treated i.m. with either saline (0·2 ml) or buserelin (0·8 μg). From each rabbit, blood samples were collected by venipuncture of the marginal ear vein 0, 24, and 48 h after injection. The samples, collected in EDTA vacutainers, were centrifuged at 3000 g for 15 min, and the frozen plasma was stored until assayed for progesterone concentrations to assess the functional status of the ovarian CL. For the purposes of this work, functional luteolysis was defined as a 50% decrease in plasma progesterone from pre-treatment values, while complete luteolysis as the failure of CL to secrete progesterone so that blood levels fall below 1·0 ng/ml, which are found in estrous rabbits (Browning et al. 1980).

In vitro experiments

A method reported previously was used for the in vitro study (Boiti et al. 2001). Day 4, 9, or 13 CL were randomly distributed (one CL/well) into incubation wells (Becton Dickinson Co., Clifton, N J, USA) in 1 ml culture medium 199 with Earle’s Balanced Salt Solution containing 2·2 mg/ml sodium bicarbonate, 2·3 mg/ml HEPES, and 3% BSA (w/v), referred to here as M199. Before treatment, the CL were quartered inside each well using fine forceps. Each set of incubation wells was divided into 12 experimental groups, each were formed of five wells as follows: I) medium alone as control; II) GNRH-I agonist (buserelin, 200 nmol/l); III) buserelin + GNRH-I antagonist (antide, 100 nmol/l); IV) buserelin + PLA2 inhibitor (4-bromophenacyl bromide, 2 μmol/l); V) buserelin + PLC inhibitor (compound 48/80, 2 μmol/l); VI) buserelin + PLD inhibitor (propranolol, 10 μmol/l); VII) buserelin + AC inhibitor (2-O-methyladenosine, 2 μmol/l); VIII) buserelin + inositol 1,4,5-trisphosphate (IP3) antagonist (2-aminoethyl diphenylborinate, 100 μmol/l); IX) buserelin + diacylglycerol (DAG) antagonist (1-hexadecyl-2-acetyl glycerol, 100 μmol/l); X) buserelin + protein kinase C (PKC) inhibitor (staurosporine 20 μmol/l); XI) buserelin + COX inhibitor (ASA, 100 μmol/l); XII) buserelin + NOS inhibitor (N-nitro-L-arginine methyl ester, 100 μmol/l). The culture plates were incubated at 37°C in air with 5% CO2. The media of each well were collected after 4 h of incubation and stored immediately at −20°C for later determination of progesterone, PGF2α, and PGE2. Preliminary evidence led us to choose the incubation conditions and the minimum effective dose for buserelin used in the present in vitro study (Fig. 1).

Hormone determination

Progesterone, PGF2α, and PGE2 were determined following the RIA protocols reported previously (Boiti et al. 2001). Intra- and inter-assay coefficients of variation and minimum
Figure 2  Immunostaining for GNRHR-I of rabbit ovaries during the mid stage of pseudopregnancy. (a) The immunopositivity is evident in the cytoplasm of luteal cells (L) and endothelial cells (arrows), (insert: control section in the absence of primary antibody); (b) immunosignals are detected in the ooplasm of oocytes (O) and cytoplasm of follicular (F), thecal cells (T), and endocrine interstitial cells (I) present in antral follicles, (insert: control section); (c) the immunolabeling is localized in the ooplasm of oocytes (O) and cytoplasm of follicular (F) and thecal cells (T) present in pre-antral follicles; (d) immunosignals are evident in the ooplasm of oocytes and cytoplasm of follicular cells present in primordial follicles (arrow) and in ovarian epithelium (arrowhead), (insert: control section); (e) rabbit pituitary gland: cell groups show an intense immunoreactivity for GNRHR-I. Scale bar = 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-10-0109.
detectable dose were progesterone: 5 and 9%, 10 pg; PGF2α: 8 and 12%, 19 pg; PGE2: 7 and 13%, 18 pg.

**Enzyme activity determination**

Luteal COX-1 and -2 activities were determined by measuring the disappearance of the radiolabeled substrate [3H]AA using a method reported previously (Zerani et al. 2007). Selective COX-2 inhibitor (NS-398 1 μmol/l) and non-selective COX inhibitor (ASA, 1 mmol/l) were used. For each sample, COX-1 activity was determined by calculating the rate of loss of [3H]AA incubated with selective COX-2 inhibitor. Conversely, the COX-2 activity of each corresponding sample was determined by calculating the rate of loss of [3H]AA incubated without selective COX-2 inhibitor and subtracting from this value that of COX-1. The values for COX-1 and -2 were corrected by subtracting the [3H]AA disappearance values due to other enzymatic activities and to non-enzymatic reactions.

NOS activity was determined by monitoring the conversion of [3H]-arginine to [3H]-citrulline with a commercial NOS assay kit, according to the experimental protocol described by Boiti et al. (2000).

**Statistical analysis**

Data on gene and protein expressions, hormone levels, and enzyme activities were examined by ANOVA followed by the Student–Newman–Keuls t-test. Differences were considered significant at \( P<0.01 \).

**Results**

**GNRHR-I and GNRH-I immunolocalization**

**GNRH-I** Immunopositivity for GNRH-I was evidenced in the cytoplasm of luteal cells and endothelial cells (Fig. 2, photo a); additionally, it was detected in the ooplasm of oocytes and cytoplasm of follicular and thecal cells present both in antral and pre-antral follicles (Fig. 2, photos b and c). Immunosignals were also evident in the ooplasm of oocytes and cytoplasm of follicular cells present in primordial follicles (Fig. 2, photo d), ovarian epithelium (Fig. 2, photo d), and endocrine interstitial cells (Fig. 2, photo a). The intervening fibroblast cells within the CL were always unreactive. Differences in the presence and intensity of immunostaining were not observed during the three luteal stages. The control staining procedure failed to disclose appreciable reactivity at any of the sites described for GNRHR (inserts of Fig. 2, photos a, b and d). The cytoplasm of pituitary cells exhibited intense immunostaining (Fig. 2, photo a). The intensity of GNRH-I immunostaining did not show any difference between CL and follicles at any luteal stage considered (data not shown).

**GNRH-I** Positive immunosignals for GNRH-I were evidenced in the cytoplasm of luteal cells at all stages of pseudopregnancy (Fig. 3, photo a). The cytoplasm of follicular and thecal cells and the ooplasm within antral follicles were immunopositive (Fig. 3, photo b) as well as the follicular cells and the ooplasm within pre-antral and primordial follicles (Fig. 3, photo c). The ovarian epithelium (Fig. 3, photo b), endothelial cells (Fig. 3, photo a), and interstitial cells displayed an intense immunoreactivity. The connective tissue within the CL was not immunostained, whereas that of the ovarian cortex immunoreacted to GNRH-I (Fig. 3, photo c). The control sections were not stained at any sites examined for GNRH-I (insert of Fig. 3, photo b). The intensity of GNRH-I immunostaining did not show any difference between CL and follicles at any luteal stage considered (data not shown).
Expression of mRNA and protein for luteal GNRHR-I, FSHR, and LHR

**GNRHR-I** transcript was expressed in CL at different stages of pseudopregnancy with no changes in its relative abundance (Fig. 4, panels A and B). The protein abundance of GNRHR-I did not differ between days 4, 9, and 13 of pseudopregnancy (Fig. 4, panels C and D). The expressions of FSHR and LHR transcripts were higher \((P<0.01)\) in day 13 CL than in day 4 and 9 CL (Fig. 5).

**In vivo experiments**

Buserelin administration at days 9 and 13 of pseudopregnancy decreased \((P<0.01)\) the peripheral plasma progesterone concentrations for 48 and 24 h respectively (Fig. 6), whereas buserelin injection at day 4 did not affect progesterone levels (Fig. 6, panel B).

**In vitro experiments**

**Hormone releases** Basal *in vitro* progesterone secretion was higher in day 9 CL \((P<0.01)\) than in day 4 and 13 CL, secretion in day 13 CL was higher \((P<0.01)\) than in day 4 CL (Fig. 7, top panel). Buserelin reduced \((P<0.01)\) progesterone *in vitro* secretion by both day 9 and 13 CL, but had no effect on day 4 CL (Fig. 7, top panel). The co-incubation of buserelin with antid, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted \((P<0.01)\) the effects of buserelin alone on day 9 and 13 CL (Fig. 7, top panel). The co-treatments with ASA or N-nitro-L-arginine methyl ester inhibitor increased \((P<0.01)\) progesterone secretion by both day 9 and 13 CL (Fig. 7, top panel). The co-treatment with 4-bromophenacyl bromide, propranolol, or 2-O-methyladenosine did not affect the buserelin-decreased progesterone secretion on day 9 and 13 CL (data not shown).

Basal *in vitro* PGE\(_{2x}\) secretion was higher in day 13 CL \((P<0.01)\) than in day 4 and 9 CL, and secretion in day 9 CL was higher \((P<0.01)\) than in day 4 CL (Fig. 7, bottom panel). Buserelin increased \((P<0.01)\) PGE\(_{2x}\) *in vitro* secretion by both day 9 and 13 CL (Fig. 7, bottom panel). The co-incubation of buserelin with antid, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted \((P<0.01)\) the effects of buserelin alone on day 9 and 13 CL (Fig. 7, bottom panel). The co-treatment with ASA decreased \((P<0.01)\) PGE\(_{2x}\) secretion in all CL types, while the co-treatment with N-nitro-L-arginine methyl ester did not affect the buserelin-induced PGE\(_{2x}\) secretion (Fig. 7, bottom panel).

Basal *in vitro* PGF\(_{2\alpha}\) secretion was higher in day 4 CL \((P<0.01)\) than in day 9 and 13 CL (Fig. 7, middle panel). Buserelin reduced \((P<0.01)\) PGF\(_{2\alpha}\) *in vitro* secretion by day 13 CL (Fig. 7, middle panel). The co-incubation of buserelin with antid, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted \((P<0.01)\) the effects of buserelin alone on day 13 CL (Fig. 7, middle panel). The co-treatment with ASA decreased \((P<0.01)\) PGF\(_{2\alpha}\) secretion in all CL types, while the co-treatment with N-nitro-L-arginine methyl ester did not affect the buserelin-induced PGF\(_{2\alpha}\) secretion (Fig. 7, bottom panel).

![Image](image-url)
all CL types, while the co-treatment with N-nitro-L-arginine methyl ester did not affect the buserelin-induced PGE2 secretion (Fig. 7, middle panel).

**Enzyme activities** Basal COX-1 activity was higher in day 13 CL \((P<0.01)\) than in day 4 and 9 CL (Fig. 8, top panel). All treatments did not affect COX-1 activity in all CL types, except for ASA that decreased \((P<0.01)\) the enzymatic activity (Fig. 8, top panel).

Basal COX-2 activity of day 13 CL was higher \((P<0.01)\) than that of day 4 and 9 CL; COX-2 activity of day 9 CL was higher \((P<0.01)\) than that of day 4 CL (Fig. 8, middle panel). Buserelin increased \((P<0.01)\) COX-2 activity in CL of all stages (Fig. 8, middle panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted \((P<0.01)\) the effects of buserelin alone (Fig. 8, middle panel). The co-treatments with ASA decreased \((P<0.01)\) COX-2 activity in all CL types, while the co-treatments with N-nitro-L-arginine methyl ester did not affect the buserelin-induced COX-2 activity increase (Fig. 8, middle panel).

Basal NOS activity of day 4 CL was higher \((P<0.01)\) than that of day 9 CL (Fig. 8, bottom panel). Buserelin increased \((P<0.01)\) NOS activity in both day 9 and 13 CL (Fig. 8, bottom panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted \((P<0.01)\) the effects of buserelin alone at day 9 and 13 (Fig. 8, bottom panel). The co-treatments with N-nitro-L-arginine methyl ester decreased \((P<0.01)\) NOS activity in all stages, while that with ASA in both day 9 and 13 CL (Fig. 8, bottom panel).

All hormone release and enzyme activity data are summarized in the synoptic Table 2.
The first evidence for the presence of ovarian GNRHR, through ligand-specific binding sites, was observed in rat granulosa and luteal cells (Reeves et al. 1980, Pieper et al. 1981, Olofsson et al. 1995). Subsequently, the presence of GNRHR mRNA was also identified in human granulosa luteal cells (Kang et al. 2000) and in both follicles and CL of bovine ovary (Ramakrishnappa et al. 2001). Among the different stages of CL, GNRHR transcripts were clearly detectable in stage III luteal tissues, whereas the expression levels were slightly lower in stage II, and barely or no detectable signal was evident from stages I and IV luteal tissues (Milvae et al. 1984).

Our study showed the presence of GNRHR-I at both transcript and protein levels in CL of pseudopregnant rabbits. Apparently, both mRNA and protein abundances did not change at different luteal stages, suggesting that in the rabbit this receptor is not dynamically regulated during the CL lifespan. In both early- and mid-luteal stages, luteal GNRHR-I, FSHR, and LHR transcript levels were expressed at approximately the same relative abundance. By converse, the GNRHR-I to FSHR or to LHR mRNAs ratio decreased in CL of late-luteal stage as a consequence of the two- to three-fold up-regulation of these two ovarian markers. Positive staining for GNRHR-I was clearly evidenced in the cytoplasm of luteal cells during all stages of pseudopregnancy.

The presence of GNRH-I has been revealed in the ovary of rats (Aten et al. 1986, Schirman-Hildelsheim et al. 2005, Sengupta et al. 2008), monkeys (Chakrabarti et al. 2008), and humans (Aten et al. 1987). The present investigation confirms that the ovary of rabbits expresses GNRH-I in the cytoplasm of different cell types, including the luteal cells, independently of the luteal stage examined.

The presence of GNRHR-I and GNRHR-I, similarly to what found in other species, supports a direct role of GNRH in the regulation of luteal functions in rabbits. GNRHR-I immunoreactivity was also observed in other rabbit ovary cells as oocytes, follicular and thecal cells, so stressing the potential paracrine and/or autocrine role of the GNRH/GNRHR system in modulating ovary function in mammals (Ramakrishnappa et al. 2005). Interestingly, in the early-luteal stage, the microdensitometry results revealed the presence of larger GNRHR-I amounts in luteal than in follicle cells suggesting that GNRH-I is involved also in the luteotropic processes.

GNRH has been shown to exert a mixture of both inhibitory and stimulatory effects in the gonads, causing either inhibitory or stimulatory effects on ovarian cellular steroid output (Ramakrishnappa et al. 2005). In bovine, two GNRH antagonists, buserelin and deslorelin, showed in vivo inhibitory effects on progesterone release (Milvae et al. 1984, D’Occhio & Aspden 1999). Conversely, GNRH stimulatory in vitro effects on progesterone production were demonstrated in monkey and human granulosa cells (Casper et al. 1984, Liu et al. 1991, Bussenot et al. 1993). Finally, other authors documented that GNRH has no effect on progesterone production in human granulosa–lutein cells (Casper et al. 1984). In rabbits, GNRH agonist directly affected the

---

**Discussion**

The present study, for the first time, indicates the presence of a GNRH/GNRHR system that modulates the steroidogenic luteal function of pseudopregnant rabbits.
Table 2  In vitro effects of GNRH-I agonist (buserelin, GNRH-I) alone or co-incubated with antagonists for GNRH-I (antide, GNRH-la), IP3 (2-aminoethyl diphenylborinate, IP3-a), and DAG (1-hexadecyl-2-acetyl glycerol, DAGa), or inhibitors for PLC (compound 48/80, PLCi), PKC (staurosporine, PKCi), COX (acetylsalicylic acid, COXi), and NOS (N-nitro-l-arginine methyl ester, NOSi) on progesterone, PGF2a, and COX-1, COX-2, and NOS activities by rabbit CL at days 4, 9, and 13 of pseudopregnancy. Values are means ± S.D. of five replicates.

<table>
<thead>
<tr>
<th></th>
<th>Progesterone</th>
<th>PGF2a</th>
<th>PGE2</th>
<th>COX-1</th>
<th>COX-2</th>
<th>NOS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1997 ± 273</td>
<td>707± 225</td>
<td>796 ± 200</td>
<td>7859 ± 1135</td>
<td>3145 ± 825</td>
<td>19 202 ± 1702</td>
</tr>
<tr>
<td>GNRH-I</td>
<td>1755 ± 152</td>
<td>640 ± 167</td>
<td>860 ± 105</td>
<td>6728 ± 1178</td>
<td>9705 ± 2101</td>
<td>18 910 ± 1613</td>
</tr>
<tr>
<td>GNRH-I + GNRH-la</td>
<td>1671 ± 336</td>
<td>702 ± 172</td>
<td>899 ± 358</td>
<td>6264 ± 2667</td>
<td>3478 ± 492</td>
<td>17 096 ± 4393</td>
</tr>
<tr>
<td>GNRH-I + PLCi</td>
<td>2099 ± 248</td>
<td>597 ± 112</td>
<td>699 ± 138</td>
<td>7030 ± 1912</td>
<td>3044 ± 508</td>
<td>16 475 ± 1788</td>
</tr>
<tr>
<td>GNRH-I + IP3a</td>
<td>2280 ± 332</td>
<td>715 ± 183</td>
<td>899 ± 140</td>
<td>8152 ± 1881</td>
<td>2587 ± 437</td>
<td>20 172 ± 3336</td>
</tr>
<tr>
<td>GNRH-I + DAGa</td>
<td>1808 ± 389</td>
<td>627 ± 114</td>
<td>785 ± 187</td>
<td>7961 ± 803</td>
<td>3219 ± 567</td>
<td>17 438 ± 2313</td>
</tr>
<tr>
<td>GNRH-I + PKCi</td>
<td>2001 ± 305</td>
<td>617 ± 152</td>
<td>725 ± 247</td>
<td>7526 ± 1640</td>
<td>2476 ± 305</td>
<td>18 762 ± 3655</td>
</tr>
<tr>
<td>GNRH-I + COXi</td>
<td>1696 ± 371</td>
<td>133 ± 47</td>
<td>137 ± 47</td>
<td>537 ± 32</td>
<td>372 ± 252</td>
<td>18 472 ± 2255</td>
</tr>
<tr>
<td>GNRH-I + NOSi</td>
<td>1947 ± 317</td>
<td>637 ± 185</td>
<td>899 ± 184</td>
<td>6509 ± 2542</td>
<td>9497 ± 1072</td>
<td>1246 ± 340</td>
</tr>
<tr>
<td><strong>Day 9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5197 ± 383</td>
<td>2089 ± 297</td>
<td>161 ± 71</td>
<td>7220 ± 1855</td>
<td>8116 ± 1830</td>
<td>14 212 ± 2764</td>
</tr>
<tr>
<td>GNRH-I</td>
<td>2048 ± 240</td>
<td>3825 ± 346</td>
<td>103 ± 44</td>
<td>7442 ± 1505</td>
<td>41 452 ± 5559</td>
<td>35 421 ± 4201</td>
</tr>
<tr>
<td>GNRH-I + GNRH-la</td>
<td>5479 ± 169</td>
<td>2170 ± 447</td>
<td>179 ± 50</td>
<td>8013 ± 962</td>
<td>6472 ± 2044</td>
<td>15 608 ± 3236</td>
</tr>
<tr>
<td>GNRH-I + PLCi</td>
<td>4851 ± 394</td>
<td>1842 ± 160</td>
<td>174 ± 32</td>
<td>8135 ± 1197</td>
<td>8133 ± 1094</td>
<td>14 197 ± 2369</td>
</tr>
<tr>
<td>GNRH-I + IP3a</td>
<td>4458 ± 452</td>
<td>2051 ± 536</td>
<td>181 ± 19</td>
<td>7061 ± 1073</td>
<td>7213 ± 1241</td>
<td>19 971 ± 5048</td>
</tr>
<tr>
<td>GNRH-I + DAGa</td>
<td>4708 ± 250</td>
<td>1825 ± 341</td>
<td>191 ± 27</td>
<td>8048 ± 951</td>
<td>6956 ± 1566</td>
<td>15 230 ± 3553</td>
</tr>
<tr>
<td>GNRH-I + PKCi</td>
<td>4580 ± 664</td>
<td>2107 ± 133</td>
<td>162 ± 39</td>
<td>6053 ± 1139</td>
<td>837 ± 1039</td>
<td>15 464 ± 4083</td>
</tr>
<tr>
<td>GNRH-I + COXi</td>
<td>7604 ± 633</td>
<td>202 ± 27</td>
<td>78 ± 10</td>
<td>767 ± 192</td>
<td>318 ± 144</td>
<td>7065 ± 1662</td>
</tr>
<tr>
<td>GNRH-I + NOSi</td>
<td>7919 ± 558</td>
<td>4343 ± 521</td>
<td>205 ± 52</td>
<td>6436 ± 3051</td>
<td>43 295 ± 5218</td>
<td>1158 ± 304</td>
</tr>
<tr>
<td><strong>Day 13</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3789 ± 182</td>
<td>3304 ± 247</td>
<td>248 ± 46</td>
<td>16 440 ± 5308</td>
<td>26 599 ± 4624</td>
<td>17 549 ± 9250</td>
</tr>
<tr>
<td>GNRH-I</td>
<td>1894 ± 176</td>
<td>4614 ± 266</td>
<td>113 ± 37</td>
<td>16 636 ± 5068</td>
<td>45 060 ± 3325</td>
<td>36 432 ± 3692</td>
</tr>
<tr>
<td>GNRH-I + GNRH-la</td>
<td>3680 ± 877</td>
<td>2768 ± 485</td>
<td>255 ± 97</td>
<td>15 640 ± 2021</td>
<td>24 809 ± 3494</td>
<td>17 212 ± 468</td>
</tr>
<tr>
<td>GNRH-I + PLCi</td>
<td>3922 ± 356</td>
<td>2819 ± 492</td>
<td>272 ± 47</td>
<td>18 757 ± 4722</td>
<td>23 776 ± 4026</td>
<td>17 616 ± 1326</td>
</tr>
<tr>
<td>GNRH-I + IP3a</td>
<td>4165 ± 434</td>
<td>2816 ± 296</td>
<td>247 ± 41</td>
<td>18 126 ± 3678</td>
<td>27 674 ± 5558</td>
<td>20 435 ± 2380</td>
</tr>
<tr>
<td>GNRH-I + DAGa</td>
<td>3850 ± 391</td>
<td>3104 ± 262</td>
<td>266 ± 31</td>
<td>19 989 ± 2879</td>
<td>26 736 ± 4166</td>
<td>18 426 ± 1684</td>
</tr>
<tr>
<td>GNRH-I + PKCi</td>
<td>3892 ± 576</td>
<td>2981 ± 369</td>
<td>282 ± 60</td>
<td>20 608 ± 3325</td>
<td>31 061 ± 2442</td>
<td>18 326 ± 5029</td>
</tr>
<tr>
<td>GNRH-I + COXi</td>
<td>6108 ± 613</td>
<td>185 ± 34</td>
<td>86 ± 31</td>
<td>796 ± 482</td>
<td>292 ± 71</td>
<td>5640 ± 1982</td>
</tr>
<tr>
<td>GNRH-I + NOSi</td>
<td>6161 ± 693</td>
<td>492 ± 366</td>
<td>261 ± 70</td>
<td>15 182 ± 3319</td>
<td>40 792 ± 447</td>
<td>963 ± 164</td>
</tr>
</tbody>
</table>

Different letters indicate significantly different values (P<0.01): Greek letters among control CL days; Latin letters among experimental groups of the same CL day.

Our data strengthen the idea that GNRH-I has a direct role in the down-regulation of rabbit CL progesterone production. The in vitro experiments showed that buserelin reduced progesterone production by cultured CL collected at days 9 and 13 of pseudopregnancy, whereas this steroid synthesis was not affected by GNRH-I on day 4 CL. In addition, this GNRH-I role is also supported by the data of the co-incubation with antide, a GNRH-I antagonist, that counteracted the buserelin-induced progesterone decrease. The in vivo experiments mirrored the above-reported in vitro data; in fact, blood progesterone levels were down-regulated when buserelin was administered at mid- and late-pseudopregnancy. Even if we cannot exclude that this in vivo buserelin-induced circulating progesterone decrease is also caused by a pituitary gonadotropin down-regulation, the in vivo and in vitro coincident lack of buserelin effects during early-pseudopregnancy suggests that this progesterone decline is primarily or exclusively due to the direct action of GNRH-I agonist on pseudopregnant CL.

Recently, we demonstrated, in in vitro studies on CL of pseudopregnant rabbits, that PGF2a and PGE2 affect progesterone release differently, depending on the luteal stage (Boitti et al. 2001). PGE2 depressed NOS activity and increased progesterone production in day 4 CL but was totally ineffective in day 9 CL; on the contrary, PGF2a-up-regulated NOS activity and induced functional luteolysis in day 9 CL but had no effect on CL collected at the early-luteal phase. This physiological mechanism protects the growing CL from functional luteolysis to occur in the early-luteal stage until day 6 of pseudopregnancy, when CL shift from refractoriness.
induction of COX-2 expression and an increase in PGE2 in vitro that this luteolytic-protecting mechanism includes also the on the buserelin ineffectiveness at early-luteal stage suggest this figure available via http://dx.doi.org/10.1677/JOE-10-0109.

Many studies indicate that GNRH, complexing with cognate receptor, mainly activates PLC via G_q/11 family G proteins. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP3 and DAG (Millar 2005). Our data confirmed that this GNRH post-receptorial mechanism is present also in the rabbit CL. In fact, the effects of buserelin in decreasing progesterone release on day 9 and 13 CL were counteracted by the co-incubation with PLC and PKC inhibitors or IP3 and DAG antagonists, while the inhibitors of AC, PLA2, and PLD had no influences.

A recent study showed that in the gonadotrope cell line LBT2, GNRH produced a marked time-dependent induction of COX-2 expression and an increase in PGE2 and PGF2a production (Naor et al. 2007). These authors hypothesize a novel GNRH signaling pathway mediated by PGF2a, which acts through an autocrine/paracrine modality. The present work suggests that the GNRH-I effects on rabbit CL progesterone production are mediated by the activation of the COX-2/PGF2a/NOS pathway. In fact, buserelin increased in vitro COX-2 activity, PGF2a production, and NOS activity in both day 9 and 13 CL, coinciding with decreasing progesterone release. In addition, the co-treatment with antide, PLC and PKC inhibitors, or IP3 and DAG antagonists counteracted the buserelin actions on COX-2, PGF2a, NOS, and progesterone, while COX inhibitor counteracted those on NOS and progesterone, and, in turn, NOS inhibitor counteracted those on progesterone only. The decrease in PGE2 induced by buserelin in day 13 CL is probably due to the increased conversion of this PG into PGF2a through PGE2-9-ketoreductase enzyme activation and/or the inactivation of PGE2 synthase. A similar intracellular mechanism was reported for the PGF2a auto-amplifying loop utilized by the rabbit CL during the luteolytic process (Zerani et al. 2007). Our results also indicated that early CL refractoriness to luteolytic factors extends to GNRH-I as well, given that the all in vitro effects of buserelin on hormone productions and enzymatic activities occur only in day 9 and 13 CL.

In conclusion, this study provides the evidence that GNRH-I, in autocrine, paracrine, and/or endocrine manner, directly down-regulates progesterone production in rabbit CL that have acquired luteolytic competence, with a receptorial/post-receptorial mechanism as summarized in Fig. 9. GNRH-I couples its cognate type I receptor activating PLC and, in turn, releasing IP3 and DAG; these two intracellular messengers, through the PKC activation, start up COX-2 activity, with the consequent rise of PGF2a production; this PG induces (via paracrine, autocrine, and/or intracrine mechanism) an increase in NOS activity (Boitti et al. 2003); finally NO down-regulates progesterone levels (Boitti et al. 2000).

However, though the present data throw new light on the knowledge of the physiological mechanisms regulating luteal activity, further studies are needed to better understand the GNRH/GNRHR system fine-tuning that controls rabbit CL life span, including the presence of type II GNRHR or the involvement of PGF2a synthase and PGE2-9-ketoreductase, key enzymes of the PGF2a synthesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by a grant from the Italian Ministero dell’Università e della Ricerca (Contract n. 2005078028) and by donation of the BC Redwater Trust.

Acknowledgements

The authors gratefully acknowledge the revision of the English text by Sheila Beatty. The authors wish to thank Mrs G Mancini for her assistance in tissue collection.
References

Aten RF, Williams AT, Behrmann HR & Wolin DL 1986 Ovarian gonadotropin-releasing hormone-like protein(s): demonstration and characterization. Endocrinology 118 961–967. (doi:10.1210/endo-118-3-961)


Reeves Jl, Segun C, Lefebvre FA, Kelly PA & Labrie F 1980 Similar luteinizing hormone-releasing hormone binding sites in rat anterior pituitary and ovary. PNAS 77 5567–5571. (doi:10.1073/pnas.77.9.5567)


Received in final form 7 September 2010
Accepted 29 September 2010
Made available online as an Accepted Preprint 29 September 2010

Journal of Endocrinology (2010) 207, 289–300

www.endocrinology-journals.org