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Differential gonadotropin releasing hormone (GnRH) expression, autoregulation and effects in two models of rat luteinized ovarian cells

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Abstract

GnRH has been suggested to participate in corpus luteum function. Here we studied the expression of GnRH mRNA and peptide in two models of rat luteinized tissues: ovarian cells from PMSG–hCG treated prepubertal rats (SPO) and from intrasplenic ovarian tumors (Luteoma). A GnRH autoregulatory effect was evaluated as well as its action on cell proliferation and apoptosis.

GnRH mRNA was present in SPO, isolated corpora lutea from SPO and Luteoma from 1 week to 7 months of development. In vitro cultures of Luteoma cells expressed 2-fold higher GnRH mRNA and 10-fold higher GnRH peptide than SPO cells. Buserelin (GnRH analog) increased GnRH mRNA and peptide expression in SPO but not in Luteoma cells. While basal proliferation was very low in Luteoma cells, SPO cells showed a significant increase in cell number by both the thymidine and the MTS methods after 72 h in culture. Buserelin induced a decrease in cell number in both cell types to a similar degree. Although basal apoptosis levels were higher in SPO than in Luteoma cells, Buserelin-induced apoptosis was only detected in Luteoma cells after 48 h treatment.

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These results show that the two types of rat, luteinized tissues, Luteoma and SPO, markedly differed in some intrinsic properties and in their local GnRH systems. Luteoma cells proliferate very weakly, express and secrete high amounts of GnRH, do not show an autoregulatory effect and respond to the decapeptide with apoptosis stimulation. In contrast SPO cells proliferate significantly, secrete low levels of GnRH but possess a positive, autoregulatory mechanism and respond to GnRH stimulation with impairment of proliferation.
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Keywords: GnRH; GnRH mRNA; Proliferation; Apoptosis; Rat ovarian luteinized cells

Introduction

A complete GnRH system, including ligand, receptor proteins and their respective mRNAs, has been widely demonstrated in normal and tumoral ovaries (Aten et al., 1987; Clayton et al., 1979; Gründker et al., 2002; Olofsson et al., 1995; Peng et al., 1994; Pieper et al., 1981; Schally et al., 2001). The role of GnRH as an autocrine/paracrine regulator of ovarian function has been extensively described (Cheng and Leung, 2000; Leung and Steele, 1992). Examples of this are the increase of both GnRH peptide and its receptor during luteinization (Nathwani et al., 2000) or the GnRH-induced downregulation of the expression of both α and β estrogen receptors (Chiang et al., 2000). In addition, studies on GnRH have demonstrated that GnRH has both stimulatory and inhibitory effects on cell proliferation depending on the cell type. Antiproliferative and/or proapoptotic actions for this peptide and its analogs have been postulated in normal and tumoral ovary, prostate and breast tissues (Billig et al., 1994; Emons et al., 1996; Emons et al., 1998; Everest et al., 2001; Gründker et al., 2001; Gründker et al., 2002; Kang et al., 2000b; Kimura et al., 1999; Limonta et al., 1999; Nicholson et al., 1985). In contrast, proliferative actions for GnRH have been demonstrated in ES-2 human ovarian cancer cells (Arencibia and Schally, 2000), Jurkat cells (Azad et al., 1997), enriched gonadotropes (Childs and Unabia, 2001) and the gonadotrope cell line L beta T2 (Lewy et al., 2003). In addition regulatory effects of GnRH on its own synthesis have been described in various human ovarian cells (Kang et al., 2000a; Kang et al., 2000b; Kang et al., 2001). However, autoregulation of the GnRH system has not been demonstrated in rat ovarian cells.

Taking into consideration the important role of GnRH in rat corpus luteum function (Cheng and Leung, 2000; Leung and Steele, 1992) and the little information available regarding the expression and regulation of GnRH in rodent luteal cells, two models of luteinized rat ovarian tissue were examined in the present studies. Cells from superovulated prepubertal ovaries were selected as a classical model for luteal cells and the intrasplenic ovarian tumor, histologically defined as a Luteoma, as a model of ovarian hyperplasic tissue of predominantly luteinized cells. This Luteoma is gonadotropin-dependent, endocrinally active and grows considerably during one year of development, not evidencing malignant transformation (Chamson-Reig et al., 1997; Chamson-Reig et al., 1999a; Hockl et al., 2003; Lux-Lantos et al., 1995). GnRH analog administration induced in vivo Luteoma regression (Chamson-Reig et al., 1997; Lux-Lantos et al., 1995). In vitro stimulation of GnRH receptors present in the Luteoma induced signaling through a non-classical pathway, involving cAMP production, MAPK phosphorylation and total absence of phospholipase C activation (Chamson-Reig et al., 1999b; Chamson-Reig et al., 2003), in contrast to ovarian luteinized cells from superovulated prepubertal rats (SPO) in which the main GnRH signaling pathway was PLC activation.

In this work the expression of GnRH mRNA was evaluated in these two models of luteinized ovaries, superovulated prepubertal rat ovaries and Luteoma in different stages of development. In addition, a possible auto-regulatory effect of Buserelin, a GnRH analog, on GnRH mRNA and peptide expression was examined and GnRH actions regarding proliferation and apoptosis were analyzed in both models of rat ovarian cells.

Materials and methods

Animals

Adult female virgin Sprague–Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700 to 1900 h. They were given free access to laboratory chow and tap water. At the end of experimental procedures, animals were killed by decapitation according to Protocols for Animal Care and Use of the Instituto de Biología y Medicina Experimental, approved by the NIH.

Tumor-bearing animals: rats were bilaterally ovariectomized and one ovary was implanted into the spleen, as previously described (Chamson-Reig et al., 1997; Lux et al., 1984; Lux-Lantos et al., 1995). Luteinized ovarian tumors (Luteoma) were left to develop for the required periods of time for each experimental design.

Superovulated prepubertal female rats (SPO) were 23–25 day-old, injected with 25 IU pregnant marés serum gonadotropin (PMSG, Novormon, Syntex, Buenos Aires) and 25 IU human chorionic gonadotropin (hCG, Endocorion, Elea, Buenos Aires) 48 h thereafter. These animals were used 5 days after hCG injection, when luteinization is substantial (Chamson-Reig et al., 1997).

Cell culture

Cells from 8 week-old Luteoma, as well as from SPO, were isolated with collagenase (GIBCO BRL, Life Technologies, N.Y., USA), as described previously (Chamson-Reig et al., 1999b; Chamson-Reig et al., 2003). Briefly, 1.10^6 cells were plated in plastic 6 well culture dishes, coated with 0.5 mg/ml collagen (Sigma, St Louis, MO) and incubated in BIC-FCS: DMEM high glucose-F12 (GIBCO BRL, Life Technologies, N.Y., USA) with 2.2 g/l sodium bicarbonate, 10% fetal calf serum (GIBCO BRL, Life Technologies, N.Y., USA), Fungizone (GIBCO BRL, Life Technologies, N.Y., USA) and Gentamicine (GIBCO BRL, Life Technologies, N.Y., USA). After 24 h plating media were replaced.

For GnRH mRNA RT-PCR studies and GnRH measurements, after 8 days in culture, cells were washed once with serum free, bovine serum albumin (SIGMA, St Louis, MO) supplemented medium (DMEM-F12 with 2.2 g/l sodium bicarbonate, 0.1% BSA: BIC-BSA) and were then preincubated for 2 h with BIC-BSA. Thereafter the corresponding stimuli were added: Buserelin (a GnRH superactive agonist ([D-Ser-(-tBu)^6-des-Gly^{10}]-GnRH-*N*-ethylamide, a gift from Hoechst, Buenos Aires) in a concentration range of 0.1 to 100 ng/ml in BIC-BSA or BIC-BSA as control. After 4 h in culture at 37 °C, media were collected and frozen at −20 °C. Media and stimuli were replaced and cells were incubated for further 20 h. Thereafter media were collected as before, plates were placed on ice, cells were washed with cold phosphate–saline solution and 500 μl/well of

TRIZOL reagent were added, cells were scarped and the lisates were transferred to microfuge tubes for RNA extraction.

Total RNA preparation and RT-PCR analyses

Total RNA was prepared from 1, 2, 3, 4, 5 or 6 week-old and 3 or 7 month-old tumors, estrous ovaries, ovaries of SPO rats and from isolated corpora lutea from SPO rats by the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987) using the TRIZOL reagent (GIBCO BRL). Four different samples of each tissue were analyzed. RNA was also obtained from medial basal hypothalamus, used as a positive control of GnRH expression. In addition total RNA was obtained from SPO and 8 week-old Luteoma cell cultures. The RNA concentration was determined based on absorbance at 260 nm and its purity was evaluated by the ratio of absorbance at 260 nm/280 nm (>1.8). The RNA quality was evaluated by a denaturing MOPS gel. RNAs were kept frozen at −70 °C until analyzed.

Total RNAs from the different tissues or cells were reverse transcribed into cDNA with 200 units reverse transcriptase MMLV (USB), 0.025 μg/μl oligo dT primers, 5.3 units of RNAse inhibitor (dilution 1:5), 10 mM DTT, 0.5 mM of a mix of the 4 dNTPs in a final volume of 20 μl. The reaction was incubated at 42 °C for 50 min and was finally inactivated at 70 °C for 15 min. Two microliter were amplified by PCR.

The specific primers to detect GnRH cDNA were designed based on the published sequences of rat, human, mouse, pig, and shrew hypothalamic GnRHs: forward: 5′-GCCGCTGTTGTTCTGTTGACT-3′ (located on exon 1); reverse: 5′-TTCCTCTTCAATCAGACGTTCC-3′ (located on exon 3) (Bio Synthesis, Lewisville, TX). Based on the rat GnRH genomic sequence (accession number: M31670), these primers will amplify a fragment of 234 bp from cDNA and a fragment of 3174 bp from genomic DNA, allowing to exclude samples with genomic DNA contamination.

The primers for β-actin were obtained from Invitrogen Argentina SA, following a published sequence (Brussaard et al., 1999): forward: 5′-GGAAATCGTGCGTGACAT-3′; reverse: 5′-GGAAGGTGGACAGTGAGG-3′, amplifying a fragment of 440 bp from mRNA and 652 bp for genomic DNA. PCR reactions were carried out in a volume of 50 μl in the presence of 1U of Taq DNA polymerase (GIBCO BRL), 4 mM $MgCl_2$, 0.8 mM dNTPs and 1 μM GnRH specific primers. For β-actin, conditions were similar but 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 μM primers were used.

After 31 (GnRH) or 18 (β-actin) cycles (denaturation at 95 °C for 1 min, annealing for 45 s at 62 °C for GnRH primers and 55 °C for β-actin primers, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C after the last cycle) of amplification, 8 μl PCR reaction mix were fractioned in 1% agarose gel and stained with ethidium bromide. Controls without the addition of reverse transcriptase were carried out to test the presence of contaminating genomic DNA in the RNA samples. PCRs were also performed in the absence of primers or template as negative controls. To evaluate levels of GnRH mRNA expression in Luteoma and SPO cell cultures under basal or Buserelin (GnRH agonist) stimulated conditions, semiquantitative PCRs were performed using the expression of β-actin mRNA as control of the variation in the RNA concentration in RT reaction. To validate the assay for GnRH measurement, different amounts of total RNA were reverse transcribed, and aliquots were amplified with the specific primers for GnRH or β-actin mRNA for different number of cycles. A linear relationship between the amount of RNA and PCR products was obtained when 1 μg total RNA was

used in RT reaction and when 31 and 18 PCR amplification cycles for GnRH and β-actin, respectively, were used. The identity of the PCR product obtained with the GnRH primers (approximately 230 bp) was verified by sequencing (Universidad Nacional de General San Martin-CONICET, Argentina) showing identical sequence to GnRH cDNA of hypothalamic origin, as compared with the Gene Bank data base.

RIA for GnRH

GnRH concentration in culture media was determined as previously described (Thyssen et al., 2002). Briefly, samples were analyzed in duplicate using the anti GnRH antiserum (R1245 anti-GnRH-BSA, final dilution 1:50,000) kindly provided by Dr Nett, GnRH was iodinated with ^{125}I (NEZ 03 ^{3}H, DuPont NEN Life Sciences Products, Inc, Boston, MA) by the chloramin-T method. Intra- and inter-assay coefficient of variation were 7.1% and 11.6% respectively; the detectability limit was 1.5 pg.

To determine that Buserelin added to the culture media did not interfere in the GnRH assay, displacement curves with Buserelin added in a dose range of 1 pg to 100 μg per tube were obtained, determining that only doses over 100 ng of Buserelin displaced 125I-GnRH bound to the antibody (Fig. 1). As the concentrations of Buserelin in the well varied between 0.1 and 100 ng in 1 ml culture medium and only 100 μl from these were measured in the assay, corresponding to 0.01–10 ng, we demonstrated no interference from Buserelin, at the concentrations used, in the GnRH assay.

Cell number assays

Proliferation by the ^{3}H-Thymidine incorporation assay

Basal cell proliferation was assayed using the ^{3}H-Thymidine incorporation assay. Luteoma and SPO cells were obtained as described above and seeded (1.10^{5} cells/well) in 24-well plates in BIC-10% FCS. Cells were cultured for 24 or 72 h. During the last 24 h in culture, media were replaced by BIC-0.1% BSA and ^{3}H-Thymidine (NET027Z Thymidine, [Methyl- ^{3}H]-DuPont NEN Life Sciences Products, Inc, Boston, MA, 0.5 μCi/well) was added. After 24 h in the presence of ^{3}H-Thymidine, cells were washed with BIC. Thereafter 250 μl of distilled H_2O were added and plates were frozen to induce

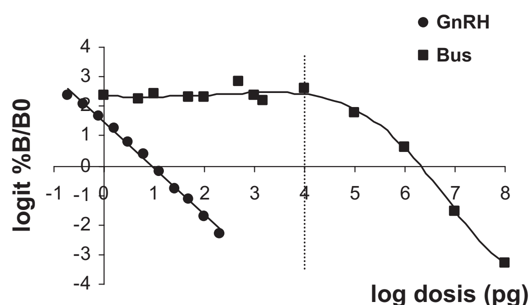

Fig. 1. GnRH RIA displacement curves. Binding of ^{125}I-GnRH to the antibody was competed by GnRH (0.15 to 200 pg/tube) or Buserelin (1 pg to 100 μg/tube). IC_{50} for GnRH: 8.6 pg. IC_{50} for Buserelin: 1.07 μg. The dotted line (·····) represents the maximum dose of Buserelin in the culture medium aliquot measured in the RIA.

cell rupture. The next day well contents were transferred to Eppendorf tubes and wells were washed and scrapped with 750 μl cold ethanol, which were then added to the Eppendorf tubes. After 20 min incubation at − 70 °C, tubes were centrifuged at 12,000 rpm at 4 °C and the supernatant was discarded. The pellets were left to dry overnight and were then solubilized with 100 μl 8 mM NaOH, Optiphase Hisafe 3 scintillation cocktail was added and samples were measured in a β counter. Experiments were repeated twice.

Evaluation of metabolically active cells by the MTS colorimetric assay

Proliferation of control luteal cells and tumor cells was also evaluated with the kit CellTiter 96^{R} Aqueous Non-Radioactive Cell Proliferation Assay (Promega, WI, USA), which is a colorimetric method for determining the number of viable cells in proliferation. It is composed of a solution of a tetrazolium (MTS), which is bioreduced by metabolically active cells into a formazan product, soluble in culture medium; its absorbance can be measured at 490 nm. The quantity of formazan produced is directly proportional to the number of living cells.

Luteoma and SPO cells were obtained as described above and plated (1.10^{5} cells/well) in P96 culture dishes in BIC-10% FCS. In a group of wells of each cell type, proliferation was assayed 2–3 h after seeding to determine cell number at time = 0, following the manufacturer's instructions. In the other wells media were replaced after 24 h incubation. Buserelin (Bus: 1.10^{-7} M) was added on the day of plating and was renewed every 24 h, control wells received culture medium.

After 72 h of culture, cell proliferation was assayed. Results of proliferation were expressed as MTS absorbance at 492 nm or as percent of the absorbance of stimulated cells relative to non-stimulated cells (control). Experiments were repeated 10 times.

Apoptosis assays

Apoptosis in SPO and ovarian tumor cells was detected with the kit Cell Death Detection ELISA (Roche, Mannheim, Germany). This quantitative measurement of cell death consists of detection of histone-associated DNA fragments in one immunoassay followed by a colorimetric reaction. SPO and Luteoma cells were obtained and plated in BIC-FCS (2.10^{5} cells/well in a 24 well culture dish), as described above, in the presence or absence of Buserelin (1.10^{-7}M) for 6, 24 or 48 h renovating the stimuli daily. An aliquot of both freshly obtained cell types (2.10^{5} cells) were centrifuged and frozen at − 70 °C to evaluate apoptosis at time 0. After the indicated times of culture, media were collected and centrifuged to obtain cell pellets containing loose cells in the media. These were immediately frozen at − 70 °C together with the dry culture dishes with the cells attached. Cell solubilization of culture dishes and pellets was performed, lysates corresponding to the same original well were reunited and ELISA was performed following the manufacturer's instructions. Results of apoptosis were expressed as the absorbance at 405 nm–absorbance at 492 nm or percent of apoptotic cells in each treatment relative to non-stimulated cells. Experiments were repeated 3 times.

Statistical analysis

All experiments were run in replicates and were repeated 3–5 times. Results are expressed as mean ± SE. The data were analyzed by one-way or two-way analysis of variance followed by Tukey

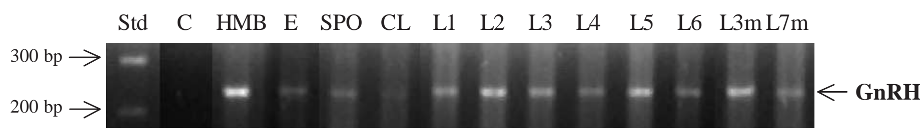

Fig. 2. Representative image of GnRH mRNA determination in SPO ovaries and Luteoma at different stages of development by RT-PCR. One microgram RNA was reverse transcribed and PCR was performed for 40 cycles. A single PCR amplification product of approximately 230 bp was obtained. Std: MW standards; C: control without cDNA in the PCR reaction; HMB: medial basal hypothalamus; E: estrous ovary; SPO: ovaries from superovulated prepubertal rats; CL: corpora lutea from SPO ovaries; L1–L6: 1 to 6 week-old Luteoma; L3m: 3 month-old Luteoma; L7m: 7 month-old Luteoma. Notice the faint band in isolated corpora lutea (CL). Four different samples of each tissue were analyzed obtaining similar results.

HSD for unequal *N* test (Statistica v 5). When only two groups were compared Studentś *t* test was used. In all cases results were considered significant when $p < 0.05$.

Results

Expression of GnRH mRNA in SPO and Luteoma at different stages of development

The expression of GnRH mRNA in SPO ovaries and in Luteoma at different stages of development was examined by RT-PCR, observing the presence of the transcript in both tissues (Fig. 2). Decapeptide

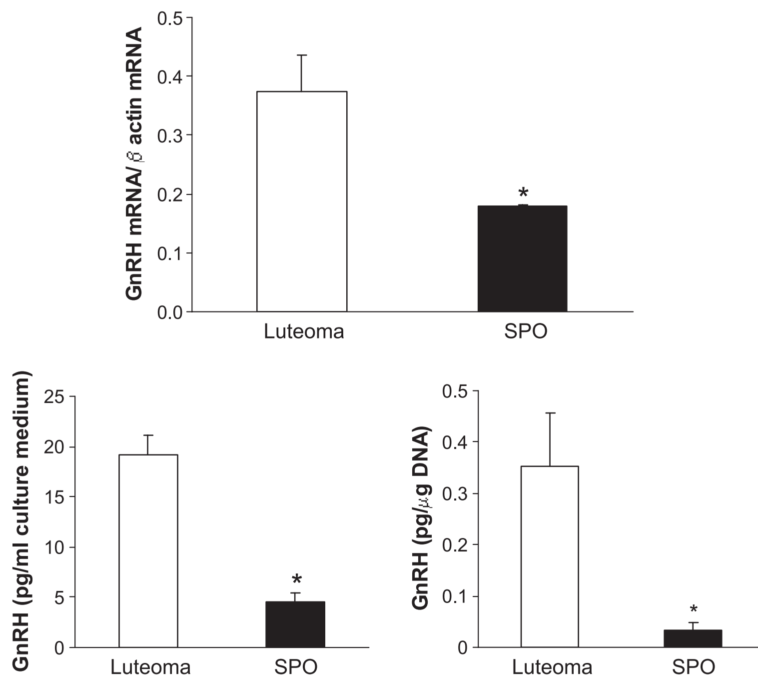

Fig. 3. Upper panel: basal levels of GnRH mRNA relative to β actin mRNA in Luteoma and SPO cells analyzed by semiquantitative RT-PCR, *: $p < 0.05$, $n = 4$. Lower panel: GnRH peptide secreted under basal conditions (pg/ml culture medium, left) or (pg/μg DNA, right), measured by RIA, *: $p < 0.01$, $n = 4$.

expression was detected in Luteoma at very early stages of tumor growth, with positive signals already after 1 week of development, to late stages of development (7 months). In addition positive signals were also obtained in ovaries of estrous rats and in isolated corpora lutea of SPO rats, confirming that GnRH mRNA is expressed in highly luteinized rat ovarian tissue. Positive controls were hytothalami from the same animals.

Expression of GnRH mRNA and peptide in SPO and Luteoma cell cultures

To determine if GnRH mRNA was also expressed in cultured cells from Luteoma and SPO ovaries, cultures were obtained and levels of GnRH mRNA were determined by semiquantitative RT-PCR while peptide titers were measured in the culture media by RIA.

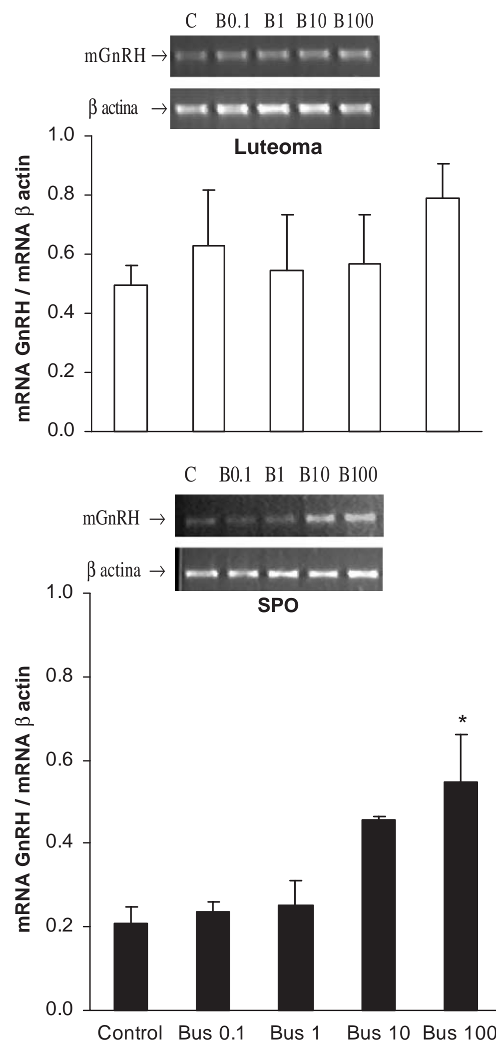

Fig. 4. Effect of Buserelin on GnRH mRNA levels relative to β actin mRNA expression in Luteoma (upper panel) and SPO (lower panel) cells determined by RT-PCR after 24 h in the presence of the stimulus (Bus: 0.1, 1, 10 and 100 ng/ml) in serum-free medium, *: $p<0.05$, $n=4$. Inserts: representative gels for each cell type.

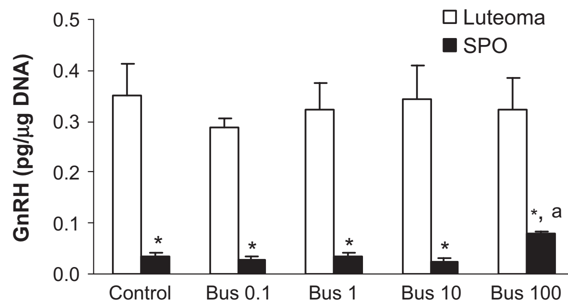

Fig. 5. Effect of Buserelin on GnRH secreted by Luteoma or SPO cells after 20 h in the presence of the stimulus (Bus: 0.1, 1, 10 and 100 ng/ml) in serum-free medium, $n = 4$ experiments. Two-way ANOVA: interaction—$p < 0.01$, *— significantly different from Luteoma cells, a— significantly different from SPO cells.

After 8 days in culture GnRH mRNA and peptide were detected in both cell types, indicating that their expression is maintained in culture. Results showed that Luteoma cells expressed twice as much GnRH mRNA in basal conditions than SPO cells (Fig. 3, upper panel). In addition, Luteoma cells secreted 4 times more GnRH peptide into the media than SPO cells when expressed per ml of incubation medium (Fig. 3, lower, left panel). Since microscopic observation of culture dishes showed higher cell

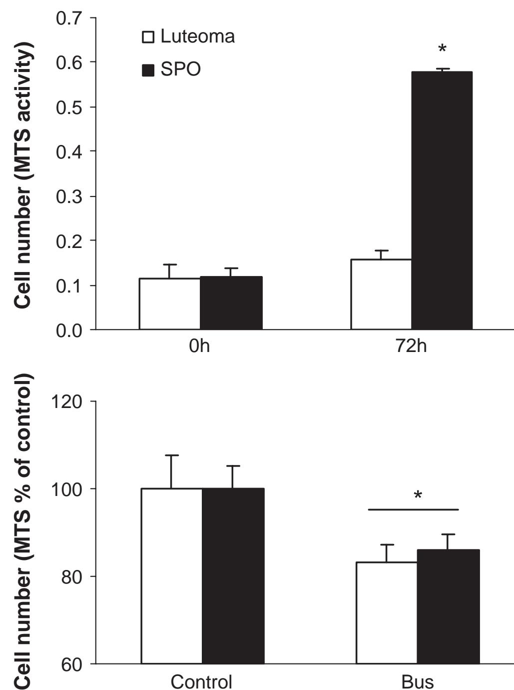

Fig. 6. Upper panel: cell number in Luteoma and SPO cultures measured by the MTS assay (abs 490 nm). *: significantly different from Luteoma cells at 72 h and from SPO cells at 0 h. Lower panel: cell number (MTS, % of control) in Luteoma and SPO cell cultures in the presence or absence of Buserelin (Bus 1.10^{-7}M) after 72 h in culture, $n = 10$. Two-way ANOVA: interaction—ns, main effect cell type—ns, main effect treatment—$p < 0.01$, *—significantly different from control. Bracket means that both cell types are analyzed as one group, as the interaction and cell type were non-significant.

density in SPO than in Luteoma wells at the time of sampling, results were also expressed as pg GnRH/μg of DNA in the well (Fig. 3, lower, right panel). When results were expressed in this way the difference in GnRH secretion between cell types was remarkable [GnRH (pg/μg DNA): SPO = 0.034 ± 0.008 vs Luteoma = 0.352 ± 0.061, $p < 0.01$].

Autoregulatory effects of Buserelin, a GnRH analog, on GnRH mRNA and peptide expression in both models of luteinized cells

In Luteoma cells, GnRH mRNA expression was not significantly modified by 24 h incubation in the presence of Buserelin in a concentration range of 0.1–100 ng/ml (Fig. 4). In contrast, the highest concentration of the peptide significantly increased GnRH mRNA expression in SPO cells ($p < 0.05$). Moreover, when GnRH secreted into the culture media was analyzed, similar results were obtained (Fig. 5). Buserelin stimulated GnRH secretion in SPO cells at the highest concentration tested after 20 h in culture. No significant effect was observed in Luteoma cells. Similar results were obtained after 4 h incubation (not shown).

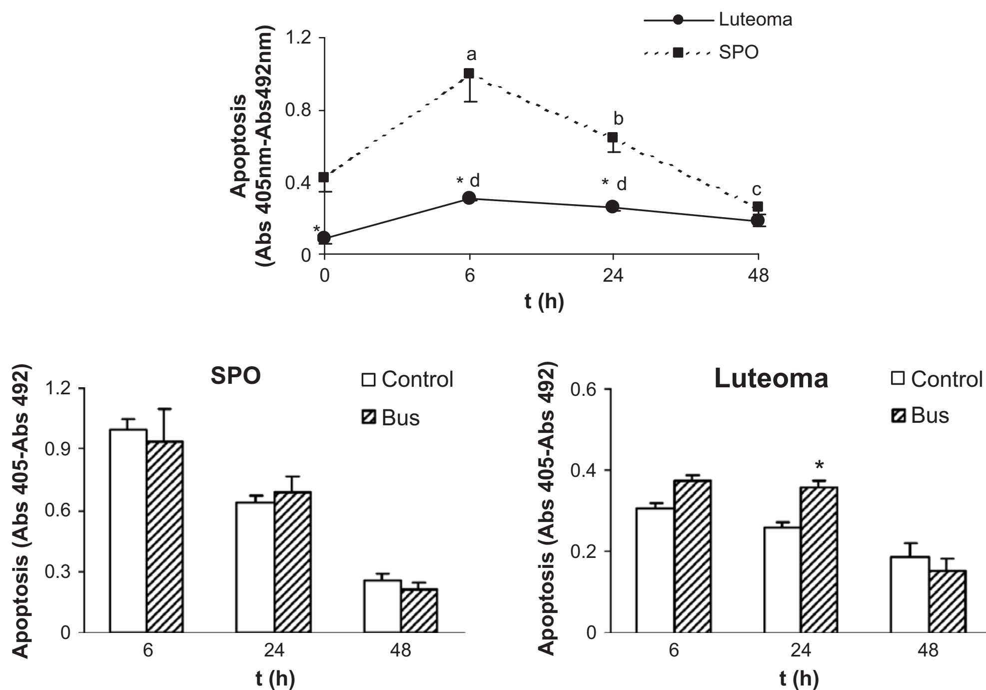

Fig. 7. Upper panel: basal apoptosis in Luteoma and SPO cells at different times after cell isolation. Two-way ANOVA: interaction— $p < 0.01$, main effect cell type—$p < 0.01$, main effect time—$p < 0.01$. *: significantly different from SPO cells at each time, a—significantly different from 0, 24 and 48 h in SPO cells, b—significantly different from 0, 6 and 48 h in SPO cells, c—significantly different from 0, 6, 24 h in SPO cells, d— significantly different from 0 h in Luteoma cells, $n = 3$. Lower panel: Buserelin-induced apoptosis (abs 405–abs 492) in SPO (left) and Luteoma (right) cells at different times after stimulus administration. Bus: Buserelin 1.10–7 M, control: spontaneous apoptosis of each cell type determined at each time studied. *: significantly different from control apoptosis in Luteoma cells, $p < 0.02$, $n = 3$.

Proliferation of SPO and Luteoma cells

As qualitative microscopic observation suggested that Luteoma cells proliferated and/or survived less than SPO cells, quantification of proliferation under basal conditions was studied. Analyzed by the ^{3}H-Thymidine incorporation method, a significantly higher proliferation rate was observed in SPO than in Luteoma cells after 24 and 72 h in culture [^{3}H-Thymidine inc. (cpm): 24 h: SPO = 3754 ± 177 vs Luteoma = 2473 ± 233, $n = 2$, $p < 0.05$; 72 h: SPO = 8663 ± 1295 vs Luteoma = 3267 ± 508, $n = 2$, $p < 0.01$]. When evaluated by the MTS activity method a five-fold increase in cell number was evidenced in SPO cells after 72 h culture while no significant variation in cell number was observed in Luteoma cells (Fig. 6, upper panel). As GnRH had been postulated to be involved in the regulation of cell proliferation, effects of Buserelin on the proliferation of both cell types was evaluated. After 72 h in culture in the presence of the analog a similar percent decrease in metabolically active cell number was determined in both kinds of cells (Fig. 6, lower panel).

Spontaneous and GnRH-induced apoptosis in SPO and Luteoma cells

As GnRH has been shown to induce apoptosis in a variety of normal and tumor cells, spontaneous and GnRH-induced apoptosis were evaluated in freshly obtained SPO and Luteoma cells and after 6, 24 and 48 h in culture. Spontaneous apoptosis was five-fold higher in freshly obtained SPO than Luteoma cells and a significant difference in the level of apoptosis between both cell types was maintained until 24 h after plating (Fig. 7, upper panel). In both cell types apoptosis increased to a maximum after 6 h in culture. In SPO cells, cell death was still elevated at 24 h, and then fell at 48 h to lower than initial levels. In contrast, in Luteoma cells apoptosis declined less markedly. In the presence of GnRH a significant increase in apoptosis was observed after 24 h incubation in Luteoma cells (Fig. 7, lower, right). No increase in apoptosis above high spontaneous levels was observed in SPO cells (Fig. 7, lower left).

Discussion

GnRH is expressed together with its receptor/s in normal reproductive organs as well as in tumors of the reproductive tract, including those of the prostate, breast, ovary and endometrium. In these systems GnRH has been postulated to function as an autocrine/paracrine signal. In addition, autoregulatory actions on its own synthesis have been demonstrated in human ovarian cells (Kang et al., 2000a; Kang et al., 2000b; Kang et al., 2001). In hormone-related tumors, as in normal tissue, activation of GnRH receptors usually decreases cell number (Grundker and Emons, 2003; Kang et al., 2003; Limonta et al., 2003), though proliferative actions have also been demonstrated (Arencibia and Schally, 2000; Azad et al., 1997; Childs and Unabia, 2001; Lewy et al., 2003). Interestingly, GnRH-induced growth suppression was also demonstrated in a gonadotrope cell line and in HEK293 cells stably expressing the GnRH receptor (Miles et al., 2004). In addition, recent data suggest that GnRH might also reduce the migratory and invasive capacity of cancer cells (Grundker and Emons, 2003; Kang et al., 2003; Limonta et al., 2003), suggesting that GnRH might be a negative regulatory factor on tumor growth.

As the decapeptide has been proposed to be involved in corpus luteum function, in this study we evaluated the expression, autoregulation and actions of GnRH in two models of rat ovarian luteinized tissues, ovaries from superovulated prepubertal rats and Luteoma, benign luteinized ovarian tumors.

By RT-PCR we demonstrated that the GnRH mRNA is expressed in Luteoma from very early on (1 week) to very late (7 months) in tumor development. In addition, we demonstrated its expression in ovaries from SPO rats and in isolated corpora lutea from SPO rats. These results demonstrate that rat luteinized ovaries synthesize and secrete GnRH, as was shown in human tissue (Gründker et al., 2002; Limonta et al., 2003), suggesting the participation of this peptide in their regulation. Moreover, both Luteoma and SPO cells expressed this transcript in basal conditions after 8 days in culture. Levels of expression were twice as much in Luteoma than in SPO cells. Both cell types secreted GnRH peptide into the culture medium; in this case Luteoma secreted 10 times as much decapeptide as SPO cells. These data on GnRH mRNA and peptide demonstrate a clear difference between both cell models, suggesting that there may be an increase in the transcription as well as in the transduction of GnRH in Luteoma cells, though we cannot discard differences in peptide turn-over or cell storage. Interestingly, our results are in agreement with Furui et al. who have recently described that ovarian cancers produce much higher amounts of GnRH than normal ovaries (Furui et al., 2002). When evaluating if GnRH had autoregulatory effects on its own production, a stimulatory action of Buserelin on GnRH production, both at the mRNA and peptide levels, was observed in SPO cells at the highest concentration of the analog tested (100 ng/ml). This effect was not evident in Luteoma cells. Previous works from Kang et al., 2000b and Peng et al., 1994 showed a biphasic autoregulatory effect of GnRH on its own expression in human ovarian cells. In this case, at the concentration range tested, only a stimulatory component was present in rat SPO cells. To our knowledge this is the first GnRH autoregulatory effect on both mRNA and peptide described in rat luteinized ovarian cells. The lack of effect in tumor cells may be due to the high decapeptide expression levels observed under basal conditions. This sustained stimulation of GnRH receptors (during 1 week in culture) may desensitize a GnRH response in tumor cells, thus impairing a regulatory action of the exogenously administered GnRH analog.

Next, the effect of GnRH on cell proliferation was examined. As previous preliminary data from cell culture observation suggested that after a few days in culture significantly less Luteoma than SPO cells were present after plating the same number of each cell type, we determined cell proliferation rates in Luteoma and SPO cells in non-stimulated conditions. While no significant proliferation in Luteoma cells was observed, a three to five-fold increase in cell number was detected in SPO cells after 72 h in culture, suggesting a markedly different proliferation and/or apoptotic rate between cell types. Nevertheless, in the presence of exogenously administered GnRH analog for the first 72 h in culture, both, Luteoma and SPO, cells showed a similar decrease in number. Our results with Buserelin on cell proliferation in SPO cells are similar to previous data with other GnRH agonists and antagonists (Mongiat et al., 2004). A possible participation of high, persistent GnRH levels in the slow proliferation rate of Luteoma cells in non-stimulated conditions may be postulated and will be examined in future studies.

As GnRH was also shown to induce apoptosis in normal and tumor ovarian cells (Billig et al., 1994; Gründker et al., 2002; Imai and Tamaya, 2000; Kang et al., 2003; Parborell et al., 2002; Wang et al., 2002; Zhao et al., 2000), we evaluated if this mechanism was involved in the decrease in cell number induced by Buserelin in rat luteinized cells. SPO cells had initial spontaneous high degrees of apoptosis after cell isolation with respect to Luteoma cells. In response to Buserelin only Luteoma cells showed an increase in apoptosis over control levels after 24 h of stimulation, suggesting that programmed cell death could participate in Buserelin antitumoral effects. The lack of proapoptotic effect of Buserelin in SPO cells is intriguing, since GnRH has been shown to promote apoptosis in corpora lutea (Andreu et al., 1998; Imai and Tamaya, 2000; Yuan and Giudice, 1997). Nevertheless, it has also been described that when spontaneous apoptosis is high, no further effect of GnRH analogs is observed (Parborell et al.,

2001). These results in the rat suggest that in Luteoma cells, which proliferate very slowly, GnRH effect on cell number must be mainly proapoptotic, while in SPO cells this effect seems to be mostly antiproliferative under these experimental conditions.

Conclusions

We conclude that GnRH is expressed in rat luteinized ovaries. In addition, the two types of luteinized cells, Luteoma and SPO, markedly differed in some intrinsic properties and in their local GnRH systems. Luteoma cells proliferate very weakly, express and secrete high amounts of GnRH, do not show an autoregulatory effect and respond to the decapeptide with apoptosis stimulation. In contrast SPO cells proliferate significantly, secrete low levels of GnRH but possess a positive, autoregulatory mechanism and respond to GnRH stimulation with impairment of proliferation. In addition, as the expression of GnRH receptor had already been demonstrated in the Luteoma (Chamson-Reig et al., 1997; Chamson-Reig et al., 1999b; Chamson-Reig et al., 2003), we now have evidence that the complete system, ligand and receptor, is present and may modulate the development of this benign rat ovarian tumor.

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