Gonadotrope oestrogen receptor-α and -β and progesterone receptor immunoreactivity after ovariectomy and exposure to oestradiol benzoate, tamoxifen or raloxifene in the rat: correlation with LH secretion

J E Sánchez-Criado*, **J Martín de las Mulas[1,*]**, **C Bellido**, **R Aguilar** and **J C Garrido-Gracia**

Departments of Cellular Biology, Physiology and Immunology, University of Córdoba, Córdoba, Spain

[1]Department of Comparative Pathology, University of Córdoba, Córdoba, Spain

(Requests for offprints should be addressed to J E Sánchez-Criado; Email: fi1sacrj@uco.es)

*(J E Sánchez-Criado and J Martín de las Mulas contributed equally to this work)

Abstract

The selective oestrogen receptor modulator (SERM) tamoxifen (TX) has agonist/antagonist actions on LH secretion in the rat. Whereas in the absence of oestrogens TX elicits progesterone receptor (PR)-dependent GnRH self-priming, it antagonizes oestrogen-stimulatory action on LH secretion. The aim of these experiments was to explore whether TX treatment-induced differential expression of oestrogen receptor (ER)α and ERβ in the gonadotrope may determine its agonist effect on LH secretion. In the first experiment, basal LH secretion, GnRH-stimulated LH secretion and PR-dependent GnRH self-priming were determined in incubated pituitaries from ovariectomized (OVX) rats treated with oestradiol benzoate (EB), TX or raloxifene (RX). Cycling rats in metoestrus or pro-oestrus were used as basic controls. As in pro-oestrus, pituitaries from OVX rats treated with EB exhibited GnRH-stimulated LH secretion, immunohistochemical PR expression and GnRH self-priming. While RX had no effect on these parameters, TX induced PR expression and GnRH self-priming. GnRH self-priming was absent in pituitaries incubated with the antiprogestin ZK299. In the second experiment, we evaluated the immunohistochemical expression of ERα and ERβ in gonadotropes of cycling rats and OVX rats treated with EB, TX or RX. We found that while ERβ expression was similar in all six groups, ERα expression was oestrous cycle dependent. Moreover, ERα expression in gonadotropes of TX-treated rats was as high as that found in pro-oestrus, while ERα expression in the gonadotropes of RX-treated rats was lower than in metoestrous or pro-oestrous pituitaries. These results suggest that, in the absence of the cognate ligand, TX, unlike RX, may regulate LH secretion through the ERα subtype in gonadotropes.

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Introduction

In the female, oestrogens are mainly produced by the ovary and retained with high affinity and specificity in target cells by an intracellular binding protein, the nuclear oestrogen receptor (ER), which modulates the transcription of target genes (Murdoch & Gorski 1991). Oestrogens regulate the synthesis and secretion of several pituitary hormones and play a key role in the regulation of reproductive cyclicity (Freeman 1988). Two classic pituitary cells for oestrogen action are lactotropes, where oestrogens stimulate synthesis and secretion of prolactin (PRL) (Ben-Jonathan 1994), and gonadotropes, where oestrogens sensitize the pituitary to gonadotrophin-releasing hormone (GnRH) and elicit GnRH self-priming (Fink 1988).

Selective ER modulators (SERMs), previously known as anti-oestrogens, are synthetic molecules which may function as agonists or antagonists depending on the target tissue, the animal species and the response measured (McDonnell 1999). Tamoxifen (TX) is a prototypical SERM (Cosman & Lindsay 1999) that displays selective biological activities (Jordan & Morrow 1999). At the rat pituitary gonadotrope level, TX reduces the GnRH-stimulated luteinizing hormone (LH) secretion elicited by oestrogens, whereas, in the absence of the cognate ligand, it induces progesterone receptor (PR) expression (Bellido *et al.* 2003) and GnRH self-priming (Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003). The mechanism through which these selective effects of TX are achieved is unknown.

Two ER subtypes have been described, α and β (Kuiper *et al.* 1998). While some tissues express predominantly

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ERα (Hiroi *et al.* 1999, Scully *et al.* 1999) or ERβ (Hiroi *et al.* 1999, Pettersson & Gustafsson 2001), other tissues, such as the rat pituitary, express both isoforms (Nishihara *et al.* 2000, Petterson & Gustafsson 2001). The ER isoforms are encoded by separate genes, differ in their ligand-binding domain primary sequences (Mitchner *et al.* 1998) and can activate transcription in response to oestrogen (Hall & McDonnell 1999, Smith & O'Malley 2004). It has been postulated that TX treatment-induced differential expression of ER isoforms in the gonadotrope may determine the SERM biocharacter of the drug (Katzenellenbogen *et al.* 1996, Watanabe *et al.* 1997, McDonnell *et al.* 2002). Therefore, the aim of the present experiments was to study whether a relationship existed between the immunohistochemical expression of ER isoforms in the gonadotrope in ovariectomized (OVX) rats treated with oestradiol benzoate (EB), TX or raloxifene (RX), and the differing LH secretory response of incubated pituitaries from rats treated with the different ligands.

Materials and Methods

Animals and general conditions

Adult cyclic female Wistar rats weighing 210 ± 15 g were used. Rats were housed under a 14 h light:10 h darkness cycle (lights on at 05·00 h) and 22 ± 2 °C room temperature, with *ad libitum* access to rat chow and tap water. Vaginal smears were taken daily and only rats showing consistent 4-day oestrous cycles were used. Rats were ovariectomized at random stages of the oestrous cycle (OVX rats) and assigned to the different groups 2 weeks later (2-week OVX rats). All experimental protocols were approved by the Ethics Committee of the University of Córdoba, and experiments were performed in accordance with the rules of laboratory animal care and international law on animal experimentation.

Drugs, treatments and experiments

OVX rats were daily injected s.c. over 3 days with 25 µg EB (Sigma Chemical Co., St Louis, MO, USA), 3 mg TX (Shughrue *et al.* 1997) (Sigma) or 1 mg RX (Tocris Cookson Ltd, Bristol, UK). Controls were injected with 0·2 ml oil. The doses of ER ligands were selected on the basis of previous studies (González *et al.* 2000, Bellido *et al.* 2003, Sánchez-Criado *et al.* 2003, 2004). Cycling rats in metoestrus and pro-oestrus were used as basic controls. At 0900 h, intact cycling rats and OVX rats on the day after treatment were decapitated and their anterior pituitaries dissected out and processed for immunohistochemistry or incubation studies. In the first experiment, we studied pituitary PR expression by immunohistochemistry and basal LH secretion, GnRH-stimulated LH secretion and PR-dependent GnRH self-priming in incubated pituitaries. In the second experiment, we studied the simultaneous expression of ER subtypes (α and β) and LH in pituitary tissue sections from all groups of rats to evaluate the differential expression of ER subtypes in gonadotropes.

Pituitary incubation protocol

Incubation of pituitaries was carried out as previously described (Sánchez-Criado *et al.* 2002, 2004). Briefly, halves of anterior pituitaries were incubated at 37 °C for 180 min, after 60 min of preincubation, with constant shaking (60 cycles/min) in an atmosphere of 95% O_2/5% CO_2. Each vial contained 1 ml Dulbecco's modified Eagle's medium, without L-glutamide and phenol red, containing glucose (4·5 g/l) and bovine serum albumin (0·1%, w/v), pH 7·4.

Sixteen randomly selected hemipituitaries from each group of rats (metoestrous and pro-oestrous cycling rats, and oil-, EB-, TX- or RX-treated OVX rats) were used. Whereas hemipituitaries from cycling rats in metoestrous and OVX rats injected with oil were incubated with medium alone, hemipituitaries from cycling rats in pro-oestrous and OVX rats injected with EB were incubated with 10 M oestradiol (E_2; Sigma) and hemipituitaries from OVX rats injected with TX and RX were incubated with 10^{-7} M TX and 10^{-7} M RX respectively. GnRH (10^{-8} M; LHRH; Peninsula Laboratory, Inc., Merseyside, UK) was added to the incubation medium of all hemipituitaries for 15 min at the beginning of the second and third hour of incubation (Bellido *et al.* 2003), while 10^{-8} M of the antiprogestin ZK299 (Shering, Berlin, Germany) (Neef *et al.* 1984) was added to the incubation medium of half of the hemipituitaries per group of rats. All medium was aspirated at the end of the first 15 min of each 3 h of incubation, replaced with fresh medium without GnRH and quantified for basal LH secretion, stimulated LH secretion and GnRH self-priming. This period corresponds to peak pituitary responsiveness to the GnRH challenge (Bellido *et al.* 2003).

RIA of LH, and GnRH self-priming

Concentrations of LH in the incubation medium were measured in duplicate by RIA using a double-antibody method with kits supplied by NIH (Bethesda, MD, USA) and a previously described microassay method (Sánchez-Criado *et al.* 1990). Rat LH-I-9 was labelled with ^{125}I using the chloramine T method. Intra-assay coefficient was 8% and assay sensitivity was 3·75 pg/tube. LH concentrations were expressed as ng/hemipituitary of the reference preparation LH-rat-RP-3. GnRH self-priming is a phenomenon in which the magnitude of the LH response to the second of two exposures of GnRH separated by an interval of 60 min is significantly greater than the response to the first exposure to GnRH. In the

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present experiment, GnRH priming was evaluated as the net increase of LH accumulation in the medium after the second challenge of GnRH with respect to LH accumulation after the first GnRH exposure.

Immunocytochemistry

Three to five pituitaries per group were fixed in 10% buffered formalin and embedded in paraffin wax. Immunocytochemical stainings were performed in dewaxed and hydrated 3 μm thick tissue sections. Sections of similarly processed tissue samples of rat pituitary (for LH antibody) and rat ovary (for ERα and ERβ antibodies) were used as positive controls. Substitution of the specific primary antibody by mouse ascitic fluid (for the ERα monoclonal antibody) or non-immune rabbit serum (for the LH and ERβ polyclonal antibodies) at the same dilution as the specific primary antibody in tissue sections of the cases under study was used as negative control. Nuclear counterstaining was performed with Mayer's haematoxylin in all cases. The number of cells immunoreactive to all four antibodies employed was counted in 15 high power fields (40×) per pituitary tissue section (five sections from each of the three to five rats of each of the six groups of rats) and is expressed as the number of positive cells/150 μm^2.

Immunohistochemical detection of PR in the pituitary

The commercial mouse monoclonal anti-human PR receptor clone PR10A9 (Immunotech, Marseille, France), diluted 1:15 000, and the avidin–biotin–peroxidase complex (ABC) technique (Vector, Burlingame, CA, USA) were used as previously described (Bellido *et al.* 2003, Sánchez-Criado *et al.* 2004).

Immunohistochemical detection of LH in the pituitary

Polyclonal rabbit anti-rat LH antibody (Biogenesis Ltd, Poole, Dorset, UK) was employed using either the peroxidase anti-peroxidase (PAP) immunocytochemical technique or the labelled streptavidin–biotin (LSAB)/peroxidase/universal kit (LSAB+; Dakocytomation, Glostrup, Denmark). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. Sections were incubated with the anti-LH antibody for either 18 h at 4 °C (PAP, 1:100 dilution) or 30 min at room temperature (LSAB+, 1:200 dilution). For the PAP technique, tissue sections were incubated with goat anti-rabbit IgG antibody diluted 1:20 and the PAP complex diluted 1:50 (Dakocytomation), both for 30 min at room temperature (Bellido *et al.* 2003). Three washes in PBS of 10 min each were applied between the last two incubations. The manufacturer's recommendations were followed for the LSAB+ technique. The immune reaction was developed using either the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dakocytomation) (LSAB+) or the chromogen amino-ethyl-carbazol (AEC; DakoCytomation) (PAP). Only immunoreactive cells with a visible nucleus were counted as positive with LH antibody.

Immunohistochemical detection of ERα and ERβ in the ovary

The monoclonal mouse anti-human ERα, clone 1D5 (Dakocytomation) diluted 1:50 was employed using either the streptavidin–biotin–alkaline phosphatase (SABAP) complex technique (StrAviGen Super Sensitive; Biogenex, San Ramon, CA, USA) or the LSAB+ technique. Tissue sections were subjected to high-temperature antigen retrieval by incubation with 0·01 M citrate buffer, pH 6·0, at 100 °C for 3 min in a stainless-steel pressure cooker and cooled at room temperature for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. The specific primary antibody was incubated for either 18 h at 4 °C (SABAP) or 1 h at room temperature (LSAB+) and, after three washes in PBS of 10 min each, the techniques were applied following the manufacturers' recommendations. The immune reaction was developed using the chromogen fast red included in the kit (SABAP) or the chromogen DAB.

Polyclonal rabbit anti-human ERβ antibody (Affinity Bioreagents, Golden, CO, USA) was employed using the LSAB+ technique. Tissue sections were subjected to high-temperature antigen retrieval by incubation with 0·01 M citrate buffer, pH 6·0, at 95 °C for 3 min in a water bath. After cooling at room temperature for 20 min, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. The specific primary antibody, diluted to 1 μg/ml, was incubated for 30 min at room temperature and, after three washes in PBS of 10 min each, the LSAB+ technique was applied following the manufacturer's recommendations. The immune reaction was developed using the chromogen DAB.

Immunocytochemical detection of ERα and ERβ in the gonadotrope

For the simultaneous immunohistochemical co-localization in a single histologic section of ERα and LH, LH expression was detected using the PAP technique, and the immune reaction was developed with DAB, while ERα expression was detected using the SABAP complex technique and the immune reaction was developed with fast red. Corresponding reagents for each individual technique (anti-LH and anti-ERα antibodies; anti-rabbit IgG and biotinylated anti-mouse IgG respectively, and the PAP and SABAP complexes respectively) were mixed (1:1) and used at twice their standardized optimal dilutions. Cells with co-localization of ERα and LH displayed a red nucleus and brown cytoplasm respectively.

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For simultaneous immunohistochemical co-localization of ERβ and LH, in a single tissue section, individual immunostainings with each antibody were performed consecutively. The ERβ immunostaining (LSAB+) was run first and developed using the chromogen DAB. The LH immunostaining (LSAB+) was run second and developed using the chromogen AEC. Cells with co-localization of ERβ and LH displayed a brown nucleus and red cytoplasm respectively. When counting the number of cells with co-localization of ERβ and LH, cells counted as positive to ERβ antibody were those with nuclear staining irrespective of cytoplasmic staining.

Statistical analysis

Statistical analysis was performed by ANOVA to test for significant differences among groups. When significant differences existed, ANOVA was followed by the Student–Newman–Keuls multiple range test to compare means. Significance was considered at the 0·05 level.

Results

LH secretion and GnRH self-priming in intact cyclic rats and SERM-treated OVX rats

Pituitaries from cycling rats in metoestrus did not respond to GnRH stimulation or exhibit GnRH self-priming. By contrast, pro-oestrous pituitaries responded to GnRH and displayed GnRH self-priming (Fig. 1A). Regardless of the treatment, pituitaries from all OVX rats had an increased basal secretion of LH relative to pituitaries from intact rats. In an oestrogen environment (pituitaries from OVX rats treated with EB and incubated with E_2) pituitaries exhibited increased responsiveness to GnRH and exhibited GnRH self-priming. TX, but not RX, induced GnRH self-priming without affecting basal or GnRH-stimulated LH secretion (Fig. 1A). Incubation of pituitaries with the antiprogestin ZK299 blocked GnRH self-priming, and neither SERM significantly altered basal rates in cycling rats in pro-oestrus and in OVX rats treated with EB and TX, without affecting basal or GnRH-stimulated LH secretion (Fig. 1B).

PR expression in pituitary gonadotropes from intact cyclic rats and SERM-treated OVX rats

Immunoreactive products to PR antibody were detected in the nuclei of anterior pituitary cells in pro-oestrous rats (Fig. 2B), OVX+EB-treated (Fig. 2D) and OVX+TX-treated rats (Fig. 2E) while no reaction was found in metoestrous rats (Fig. 2A), OVX+oil- (Fig. 2C) or OVX+RX-treated rats (Fig. 2F). Of the treatment groups that displayed PR immunoreactivity, the highest number of positive cells was found in the EB-treated OVX rats, and the lowest in intact cycling rats in pro-oestrus (Fig. 3).

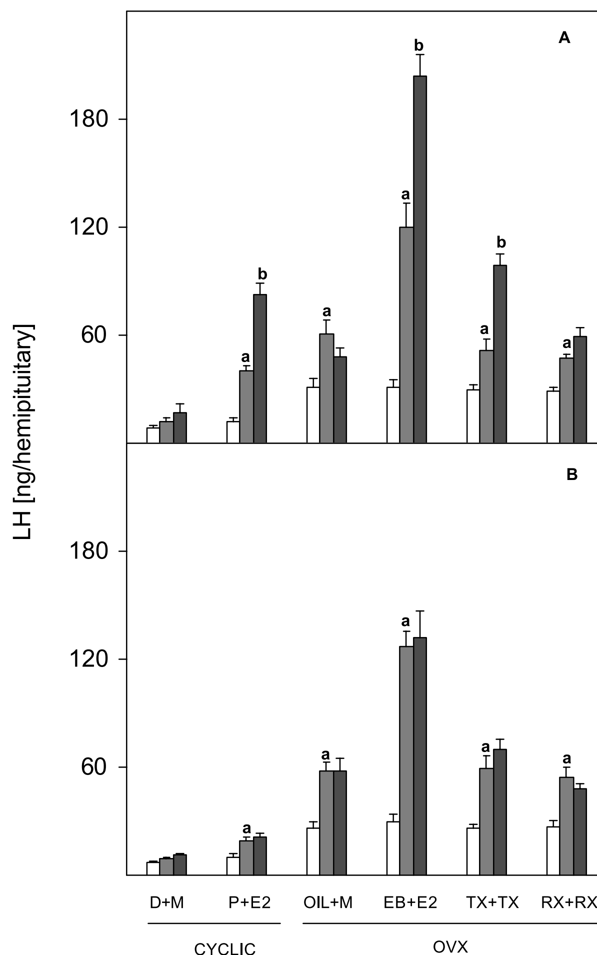

Figure 1 Secretion of LH into the incubation medium by pituitaries from intact rats in metoestrus (D) or pro-oestrus (P) and from OVX rats. Pituitaries from cycling rats in metoestrus were incubated with medium alone (M), while those from rats in pro-oestrus were incubated over 3 h with 10^{-8} M E_2. Pituitaries from OVX rats injected (s.c.) over 3 days with 0·2 ml oil, 25 µg EB, 3 mg TX or 1 mg RX were incubated with medium, 10^{-8} M E_2, 10^{-7} M TX and 10^{-7} M RX respectively. All pituitaries were challenged with two consecutive 15-min GnRH challenges (10^{-8} M) 1 h apart in the (A) absence or (B) presence of 10^{-8} M of the antiprogestin at the receptor, ZK299. Values represent means ± S.E.M. (eight hemipituitaries) of medium concentrations of LH during the first 15 min of each 3-h incubation). (Open bars) Basal LH secretion, (shaded bars) LH response to the first GnRH challenge and (solid bars) LH response to the second GnRH challenge. ^{a}P<0·01 vs basal LH concentration; ^{b}P<0·01 vs LH concentration after the first GnRH challenge (GnRH self-priming); one-way ANOVA and Student–Newman–Keuls multiple range test.

LH expression in pituitaries from intact cyclic rats and SERM-treated OVX rats

LH expression was found in gonadotropes with two different morphological appearances: (1) round to polygonal cells with rounded, centrally located nuclei (normal gonadotropes) (Fig. 4A, B and C) and (2) large, ovoid to

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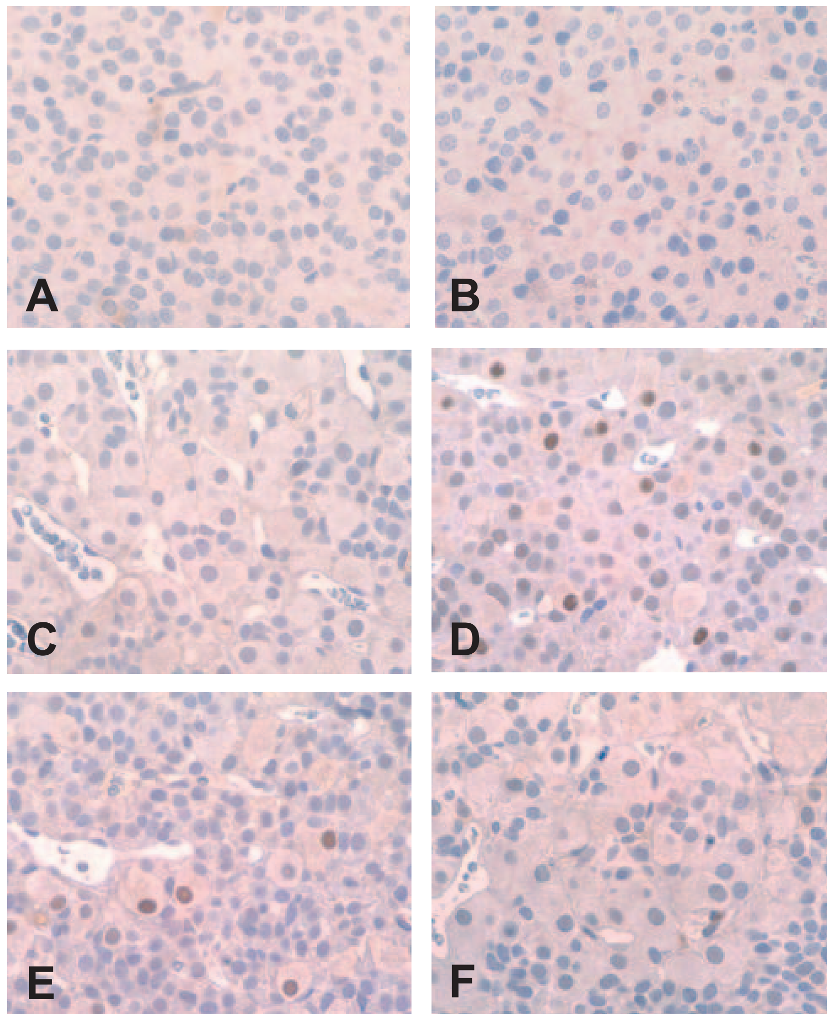

Figure 2 PR expression in pituitary gonadotropes from intact cyclic rats in (A) metoestrus and (B) pro-oestrus and in 2-week OVX rats injected (s.c.) over 3 days with (C) oil, (D) EB, (E) TX or (F) RX. See Fig. 1 legend for details of treatments. PR are seen in the cell nucleus in pituitaries from pro-oestrous cyclic, EB- and TX-treated OVX rats exclusively. The intensity of staining was lowest in pro-oestrus and highest in OVX-EB- and OVX-TX-treated rats. ABC immunohistochemical technique, nuclear counterstaining with haematoxylin (×40).

goblet cells with rounded to ovoid nuclei and foamy (Fig. 4B and C) or vacuolated (Fig. 4D) cytoplasm (OVX cells). Normal gonadotropes were present in all groups, while OVX cells were only present in OVX rats (Fig. 5). OVX cells with vacuolated cytoplasm were exclusively found in OVX+RX-treated rats (Fig. 4D). Together, normal gonadotropes and OVX cells (LH cells) accounted for about 8–9% of the total pituitary cells. The number of normal gonadotropes decreased and that of OVX cells increased significantly after OVX, and EB and TX treatments increased the number of normal gonadotropes and decreased the number of OVX cells (Fig. 5).

ERα and ERβ expression in the ovary and in gonadotropes from intact cyclic rats and SERM-treated OVX rats

In the ovary, used as the positive tissue control to assess the specificity of the anti-ER antibodies employed, staining for ERα was detected in the nuclei of theca and interstitial cells (Fig. 4E), whereas ERβ was detected in the nuclei of granulosa cells in follicles of various sizes (Fig. 4F). Ovarian androgen-producing cells and granulosa cells showed no immunoreactivity for ERβ and ERα respectively.

In the pituitary, ERα expression was found in the nuclei of normal gonadotropes and/or OVX cells in all

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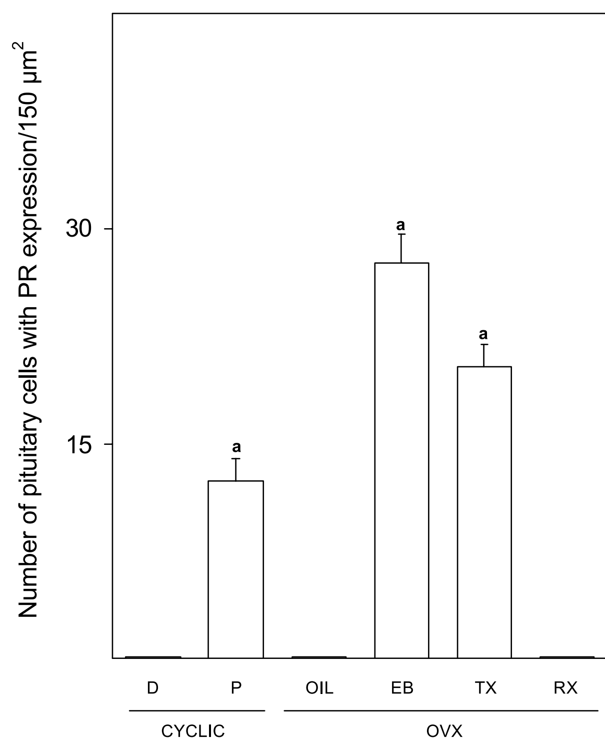

Figure 3 Number of pituitary cells with PR expression in metoestrus (D) and pro-oestrus (P) and in 2-week OVX rats injected (s.c.) over 3 days with oil, EB, TX or RX. See Figs 1 and 2 legends for details of treatments. [a]$P<0{\cdot}01$ vs metoestrus rats or OVX rats injected with oil; one-way ANOVA and Student–Newman–Keuls multiple range test.

groups of rats (Fig. 6A). The highest proportion of LH-positive cells with ERα expression was observed in pituitaries from pro-oestrous and OVX+TX-treated rats, and the lowest proportion in pituitaries from OVX+RX-treated rats (Fig. 7). ERβ was found in the nuclei and in the scarce cytoplasm of anterior pituitary cells (Fig. 6B). Only cells with positive nuclei were counted when evaluating the number of LH-positive cells showing ERβ expression. No differences were found in the percentage of LH-positive cells with ERβ expression among groups (Fig. 7).

Discussion

The present data provided evidence that the immunohistochemical expression of ERα, but not ERβ, in gonadotropes is oestrous cycle dependent, and that ERα expression was influenced differently by different ER ligands in OVX rats. The specificity of the anti-human ER antibodies employed under our standard immunohistochemistry protocol was demonstrated by the finding of ERα expression in theca and interstitial cells and ERβ expression in granulosa cells in consecutive ovarian sections from intact rats. The ovary was selected as the positive tissue control on the basis of its high levels of ERα and ERβ expression (Hiroi *et al.* 1999). The present data also revealed hitherto undescribed agonist and antagonist actions of TX and RX on OVX cells respectively.

Although few studies have measured ERβ protein in the pituitary (Kuiper *et al.* 1998, Nishihara *et al.* 2000), the gonadotrope immunohistochemical expression of ERβ found in these experiments agrees with previous findings. Thus, the disruption of ERβ gene has little effect on pituitary function in ER-knockout models (Couse & Korach 1999), and oestrogen treatment has little or no effect on ERβ in pituitary cells (Mitchner *et al.* 1999). Much more is known about the ERα subtype, which is the predominant isoform in most pituitary secretory cells (Scully *et al.* 1999). ERα is expressed at high levels in lactotropes, at somewhat lower levels in gonadotropes, and at generally very low levels in other cell types (Keefer *et al.* 1976, Mitchner *et al.* 1998, Pelletier *et al.* 2000).

In the ultrashort oestrous cycle of the rat, ovarian circulating levels of oestrogens slowly rise from late metoestrus to mid pro-oestrus (less than 2 days) and both sensitize the pituitary to GnRH and induce PR expression and GnRH self-priming through ER (Fink 1988, Freeman 1988, Bellido *et al.* 2003). All these actions of oestrogen facilitate the preovulatory release of LH in the afternoon of pro-oestrus (Fink 1988, Freeman 1988). After pro-oestrus (e.g. metoestrus in the present experiment), ERα protein levels, which are ultimately responsible for physiological pituitary responses to oestrogen, decreased and, accordingly, PR expression dropped and GnRH self-priming was abolished.

In the present study, we used 2-week OVX rats treated over 3 days with the different ligands because a 3-day administration of oestrogen, mimicking the endocrine events in pro-oestrous rats, induces an LH surge (Legan & Tsai 2003). Removal of endogenous oestrogen by ovariectomy abolished PR-dependent GnRH self-priming and induced OVX cells (Garner & Blake 1979, 1981, present results). Soon after ovariectomy, normal gonadotropes show progressive changes as time after ovariectomy increases (Garner & Blake 1981) and treatment with oestrogen reverses the effect of ovariectomy on gonadotrope morphology (Genbacev & Pantic 1975). In the present experiments, a 3-day treatment with EB reduced the number of OVX cells, induced PR, sensitized the pituitary to GnRH and induced PR-dependent GnRH self-priming. Unexpectedly, and in apparent contradiction with the effect of endogenous oestrogen in pro-oestrous pituitaries, EB treatment of OVX rats did not significantly increase the proportion of LH-secreting cells showing ERα expression. However, previous studies have shown that E_2 treatment over 3 days to OVX rats decreases ERα protein levels as part of a proteosome-directed degradation pathway (Alarid *et al.* 1999), without altering ERα mRNA (Schreihofer *et al.* 2000, Shupnik 2002).

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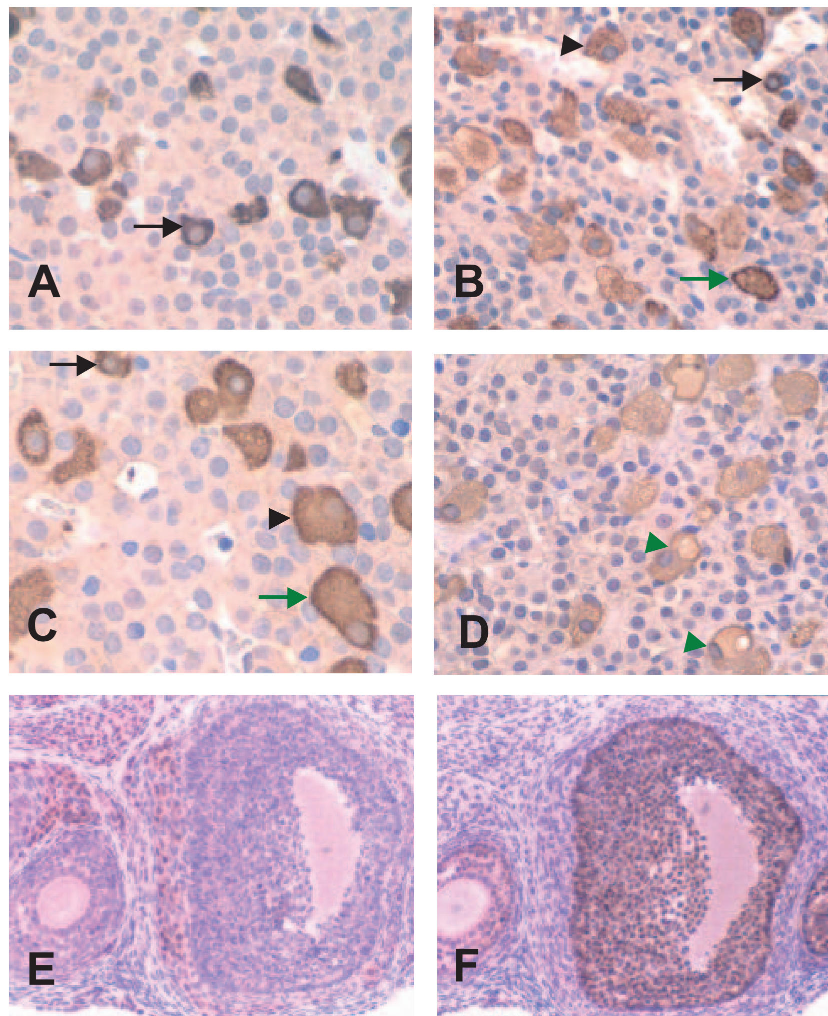

Figure 4 LH expression in the pituitary and ERα and ERβ expression in the ovary. In the pituitary, LH-positive cells from cyclic rats in (A) pro-oestrus and 2-week OVX rats injected (s.c.) over 3 days with (B) oil, (C) EB or (D) RX are shown. See Fig. 1 legend for details of treatments. LH immunoreactivity is homogeneous in the cytoplasm of round to polygonal cells (normal gonadotropes) in pro-oestrus, OVX+oil- and OVX+EB-treated rats (black arrows; A, B and C). By contrast, LH immunoreactivity has a foamy appearance in the cytoplasm of larger, ovoid to globet cells (OVX cells) in OVX rats (black arrowheads; B, C and D). Occasionally, LH immunoreactivity is located at the periphery of the cell cytoplasm (green arrows; B and C). LH-positive cells with vacuolated cytoplasm are only seen in OVX+RX-treated rats (green arrowheads; D). In the ovary, (E) staining for ERα is seen in the nuclei of theca and interstitial cells, whereas (F) staining for ERβ is seen in the nuclei of granulosa cells in follicles of various sizes. Note that ovarian androgen-producing cells and granulosa cells show no staining for ERβ and ERα respectively. LSAB+ immunohistochemical technique, nuclear counterstaining with haematoxylin (A, B, C and D×40) and (E and F×20).

Accordingly, a rapid and sustained increase of oestrogen activity prompted by EB injections over 3 days in OVX rats may have induced an earlier fall in ERα protein associated with full pituitary responsiveness at the time of the present incubation study.

As corresponds to a type I anti-oestrogen (Smith & O'Malley 2004), the triphenylethylene SERM TX displays mixed agonist/antagonist biological effects at the rat pituitary level. In the presence of oestrogen, TX reduces basal and stimulated LH and PRL secretion (González *et al.* 2000). However, in the absence of the cognate ligand, TX stimulates PRL secretion (Sánchez-Criado *et al.* 2002, Bellido *et al.* 2003) and induces both gonadotrope PR expression and ligand-independent activation of

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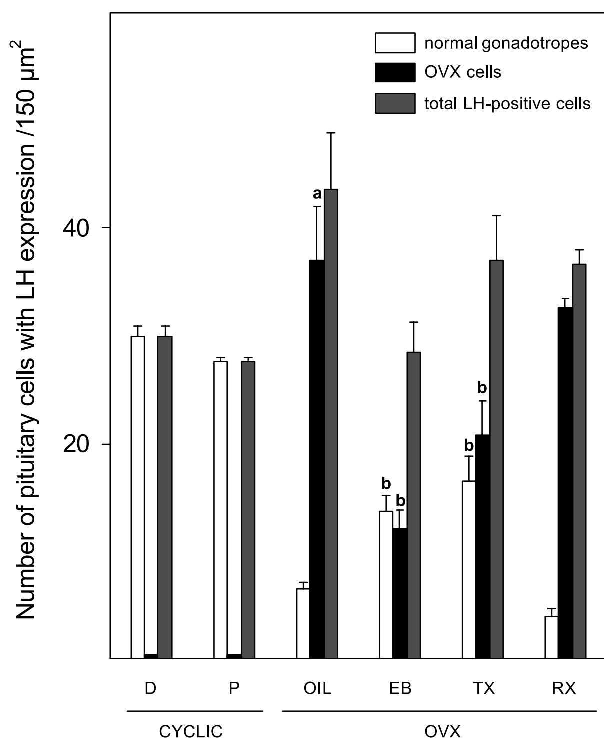

Figure 5 Number of LH-positive pituitary cells in cyclic rats in metoestrus (D) and pro-oestrus (P) and in 2-week OVX rats injected (s.c.) over 3 days with oil, EB, TX or RX (see Figs 1 and 4 legends for details of treatments and gonadotrope morphological appearance). [a]*P*<0·01 vs cyclic rats; [b]*P*<0·01 vs OVX rats; one-way ANOVA and Student–Newman–Keuls multiple range test.

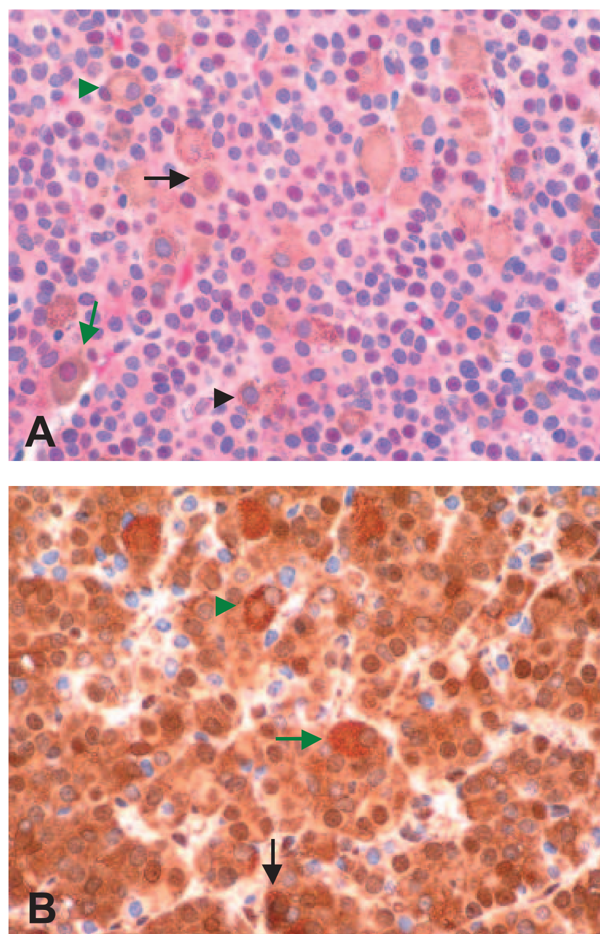

Figure 6 LH-positive cells expressing ERα or ERβ. (A) LH-positive cells (brown cytoplasm) with (red nuclei) and without (blue nuclei) ERα expression are seen in an OVX+TX-treated rat. Normal gonadotropes with (black arrow) and without (black arrowhead) ERα expression are present. In addition, OVX cells with (green arrow) and without (green arrowhead) ERα expression are also observed. Note the negative staining of the Golgi complex in an OVX cell without ERα expression (green arrowhead). (B) LH-positive cells (red cytoplasm) with (brown nuclei) and without (blue nuclei) ERβ expression are seen in an OVX+EB-treated rat. A normal gonadotrope with ERβ expression (black arrow) as well as OVX cells with (green arrow) and without (green arrowhead) ERβ expression are observed. Note the negative staining of the Golgi complex in an OVX cell without ERβ staining (green arrowhead). (A) Simultaneous PAP (LH) and SABAP complex (ERα) and (B) consecutive LSAB+ immunohistochemical techniques for ERβ and LH, nuclear counterstaining with haematoxylin (×40).

PR-dependent GnRH self-priming without affecting LH secretion (Chappell *et al.* 1999, Bellido *et al.* 2003). The present results on PR expression, which in the rat is limited to the gonadotrope (Fox *et al.* 1990), expanded our findings on SERM-induced PR (Bellido *et al.* 2003). Thus, TX increased PR expression in the the OVX rat pituitary, and reduced and increased, as did EB, the proportion of OVX cells and normal gonadotropes respectively. Importantly, the proportion of LH-positive cells expressing ERα in OVX+TX-treated rats was similar to that of cycling pro-oestrous rats and significantly higher than that exhibited by OVX+oil-treated pituitaries. In contrast, the benzothiophene SERM RX, a SERM that displays only antagonist action at the rat pituitary level (González *et al.* 2000), had deleterious effects: it failed to induce PR expression or GnRH self-priming and did not modify either the number or the type of LH-positive cells. Also, RX decreased the proportion of LH-positive cells staining for ERα. Previous reports have indicated that the type II anti-oestrogen ICI 182,780 reduces ERα protein but not mRNA levels (Schreihofer *et al.* 2000). Therefore, RX behaved as a *pure* (type II) anti-oestrogen at the rat pituitary level.

Although other intracellular selective mechanisms may also be involved in gene responses to TX after binding ER (McKenna *et al.* 1999, Katzenellenbogen *et al.* 2000, McKenna & O'Malley 2000), our data suggest that TX induces PR-dependent GnRH self-priming through ERα subtype-containing gonadotropes.

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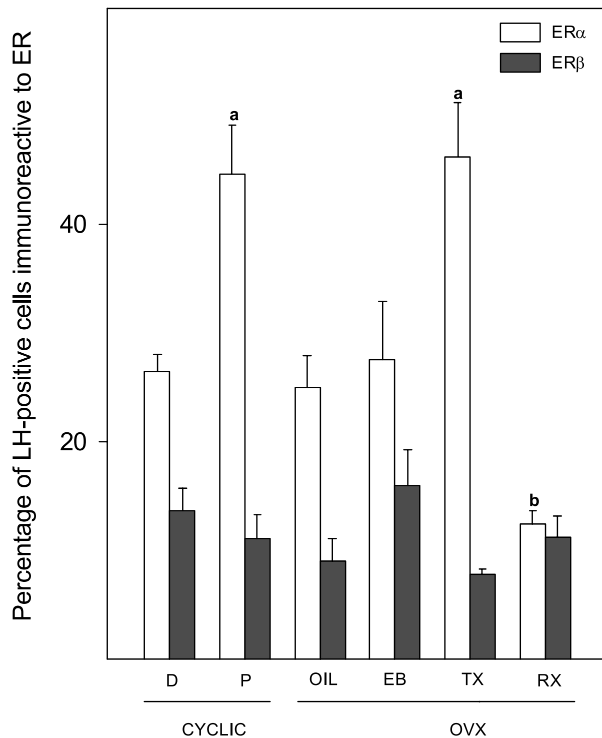

Figure 7 Relative expression of ERα and ERβ in LH-positive cells from cyclic rats in metoestrus (D) and pro-oestrus (P) and from 2-week OVX rats injected (s.c.) over 3 days with oil, EB, TX or RX (see Fig. 1 legend for details of treatments). [a,b]$P<0{\cdot}05$ vs metoestrus or OVX-oil; no differences were found among groups in the expression of ERβ; one-way ANOVA and Student–Newman–Keuls multiple range test.

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