CSIRO PUBLISHING

www.publish.csiro.au/journals/rfd *Reproduction, Fertility and Development*, 2009, **21**, 827–839

Disruption in the expression and immunolocalisation of steroid receptors and steroidogenic enzymes in letrozole-induced polycystic ovaries in rat

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Abstract. The objective of the present study was to characterise the expression and tissue distribution of steroid receptors (oestrogen receptor-α and -β (ERα, ERβ), androgen receptor (AR) and progesterone receptor (PR)) and steroidogenic enzymes (P450 aromatase (P450arom), 3β-hydroxysteroid dehydrogenase (3β-HSD) and steroidogenic acute regulatory protein (StAR)) in letrozole-induced polycystic ovaries of rats. Changes in serum hormone levels, protein expression in whole ovaries by western blot analysis and protein localisation by immunohistochemistry were determined in female rats treated with the aromatase inhibitor letrozole and compared with controls in proestrous and diestrous rats. Increases in the serum LH, FSH and testosterone concentrations were observed in letrozole-treated rats whereas serum oestradiol and progesterone levels were reduced. Protein expression as analysed by western immunoblot was consistent with the immunohistochemical data. Letrozole treatment induced an increase in the expression of AR, StAR and 3β-HSD and a decrease in ERβ. ERα, PR and P450arom showed partial changes in relation to some cycle stages. These results indicate that cystogenesis in this experimental model is characterised by changes in steroid receptors and steroidogenic enzyme expression that may be essential to proper ovarian functioning and are in agreement with similar changes observed in women with PCOS.

Additional keywords: 3β-hydroxysteroid dehydrogenase, aromatase, polycystic ovarian syndrome, StAR, steroid receptors.

Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder affecting women of reproductive age. In anovulatory women with PCOS, the prominent ovarian sign is follicular maturation arrest that results in an abnormal ovarian endocrine environment characterised by chronic anovulation, hyperandrogenaemia, increased peripheral conversion of androgens to oestrogens and characteristic morphological findings, including a sclerocystic appearance (Matalliotakis *et al*. 2006). The aetiology of the syndrome, including the mechanism of anovulation, still remains uncertain (Homburg 2008).

We and others have used a rat model, in which polycystic ovaries (PCO) are induced by treatment with a non-steroidal aromatase inhibitor (letrozole), to study ovarian morphology and metabolic profiles (Kafali *et al*. 2004; Baravalle *et al*. 2006; Mannerås *et al*. 2007). Letrozole induces PCO with striking morphological and endocrine similarities to human PCOS, including a thickened theca cell layer, anovulation and increased ovarian weight and size (Green and Goldzieher 1965; Baravalle *et al*. 2006; Mannerås *et al*. 2007).

Ovarian steroid hormones perform several important actions related to ovarian function and their effects are mediated through interaction with specific receptors (Beato and Klug 2000; Drummond *et al*. 2002; Salvetti *et al*. 2008). In this sense, ovarian steroidogenesis is regulated by the expression level of the enzymes specifically involved in each step of the process. The steroidogenic acute regulatory protein (StAR) initiates the process of steroidogenesis by transporting cholesterol from the outer to the inner mitochondrial membranes of the cell (Kahsar-Miller *et al*. 2001). Although cleavage of the cholesterol side-chain is the first enzymatic reaction in the overall process of steroid production, investigators generally consider the translocation of cholesterol by StAR across the aqueous space separating the outer and inner mitochondrial membranes to be the true

 10.1071/RD09026 1031-3613/09/070827

rate-limiting step in steroidogenesis (Clark *et al*. 1994; Lin *et al*. 1995; Stocco 1997; Silverman *et al*. 2006). It has been demonstrated that StAR expression is highly correlated with steroidogenic activity of the ovary (Kiriakidou *et al*. 1996).

Precise programming of the expression of steroidogenic enzymes in ovarian theca, interstitial, granulosa, and luteal cells is responsible for the physiological pattern of sex steroid secretion observed during the oestrous cycle (Readhead *et al*. 1983; Bao *et al*. 2000). Among the steroidogenic enzymes involved, 3β-hydroxysteroid dehydrogenase (3β-HSD) plays a key role (Mason *et al*. 1997; Ullmann *et al*. 2003). This enzyme catalyses the obligatory oxidation/isomerisation of 3β-hydroxy-5-ene-steroids into 3-keto-4-ene-steroids, thus permitting the formation of progesterone from pregnenolone, as well as the synthesis of the precursors of all androgens and oestrogens (Mason *et al*. 1997). The ontogeny and regulation of 3β-HSD in the ovary has been described at the cellular level, with evidence of distinct levels of expression in the various steroidogenic ovarian cell types (Dupont *et al*. 1992; Teerds and Dorrington 1993; Mason *et al*. 1997; Isobe *et al*. 2003). In particular, thecal cells synthesise androstenedione that is converted to oestradiol by the cytochrome P450 aromatase (P450arom) enzyme of the granulose cells (Bao *et al*. 2000).

The appearance of P450arom is a key event during the final stage of ovarian follicular development committed by two types of follicular cells, thecal and granulosa, under the stimulation of the two pituitary gonadotrophins, luteinising hormone and follicle stimulating hormone. Androgen produced by thecal cells under LH stimulation serves as an obligatory substrate for P450arom-catalysed oestrogen synthesis in granulosa cells induced by FSH (Tetsuka and Hillier 1997). Alterations in these processes could be part of the mechanism that lead to the development of follicular cysts and have been the object of speculation and research for many years, but remain poorly understood (Takayama *et al*. 1996; Kahsar-Miller *et al*. 2001; Jakimiuk *et al*. 2002; Isobe *et al*. 2003).

The present study was performed to determine how the development of PCO affects the expression of the steroid receptors P450arom, 3β-HSD and StAR in the ovaries of letrozole-treated rats. These were evaluated by western immunoblot and semiquantitative immunohistochemical analysis allowing the localisation and quantification of the various proteins analysed in each ovarian component, to determine further any association with follicular health or cysts. The potential functional significance of the differential expression of steroid receptors and steroideogenic enzymes during follicular development and cystogenesis is discussed.

Materials and methods

Animals and treatment

All procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). Eight-week-old female Wistar rats (mean bodyweight 160 g) were provided by the Center for Experimental Biology and Laboratory Animals Sciences, Faculty of Veterinary Sciences, Universidad Nacional del Litoral, Argentina. The animals were kept under a controlled light–darkness cycle (lights on between the 0800 and 2000 hours) and at a temperature of 20–24°C with free access to water and standard commercial food.

Because vaginal changes reflect the presentation of PCOS, the experimental animals were smeared daily (Salvetti *et al*. 2004; Baravalle *et al*. 2006). In this procedure, smears obtained by vaginal washing were examined under a microscope for the relative abundance of nucleated epithelial cells, cornified cells and leucocytes. Cycles with a duration of 5 days were considered to be regular. At the start of treatment, all animals presented regular cycles.

Thirty rats were divided into two groups: a control group of 20 rats that received vehicle only (0.9% NaCl solution) once daily orally and a treatment group of 10 animals administered with letrozole (Kerbizol, ASPEN, Buenos Aires, Argentina) once daily at a concentration of $1\,mg\,kg^{-1}$ p.o. dissolved to $1\,mg\,mL^{-1}$ in 0.9% NaCl solution. The treatment period was 21 days (Baravalle *et al*. 2006).

Tissue sampling

Twenty-four hours after the last dose of letrozole and after being anesthetised with a cocktail of ketamine and xylazine (40 and $4\,mg\,kg^{-1}$, respectively) administered subcutaneously, the rats were killed by decapitation; trunk blood was collected and serum was stored at −20°C until assayed. One ovary from each animal was fixed in 10% buffered formalin for 6 h at 4°C and then washed in phosphate-buffered saline (PBS). The second ovary was frozen at −80°C for subsequent use in western immunoblot analysis. Control animals were sacrificed in proestrus ($n = 10$) and diestrus ($n = 10$) to obtain small growing follicles (secondary), preovulatory tertiary as well as atretic follicles. For immunohistochemistry, fixed tissues were dehydrated in an ascending series of ethanol concentrations, cleared in xylene, and embedded in paraffin. Serial sections (5 μm in thickness) were mounted on 3-aminopropyl triethoxysilane (Sigma-Aldrich Corp., St. Louis, MO, USA)-coated slides, and dried for 24 h at 37°C (Baravalle *et al*. 2006; Salvetti *et al*. 2008).

Immunohistochemistry

Details and dilutions of antibodies used are summarised in Table 1. Each protein was assayed in at least three sections of each ovary from every individual. A streptavidin–biotin immunoperoxidase method was used as previously described (Baravalle *et al*. 2006; Salvetti *et al*. 2008). In brief, sections were deparaffinised, hydrated and then microwave pretreatment (antigen retrieval) was performed. The endogenous peroxidase activity was inhibited with 1% H_2O_2 and nonspecific binding was blocked with 10% normal goat serum. All sections were incubated with primary antibodies for 18 h at 4°C. The samples were washed and then incubated for 30 min at room temperature with appropriate preabsorbed biotinylated secondary antibodies, selected for each of the two types of primary antibodies used (mono- or polyclonal). The visualisation of antigens was achieved by the streptavidin–peroxidase method (BioGenex, San Ramon, CA, USA) and 3.3-diaminobenzidine (DAB) (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) was used as chromogen. Finally, the slides were washed in distilled

Table 1. Antibodies used for immunohistochemistry (IHC) and western blot (WB)

Antibodies	Clone	Source	Dilution for IHC	Dilution for WB
Primary antibodies				
ERα	1D5	Dako, Carpinteria, CA, USA	1 : 20	1 : 50
ERβ	Polyclonal	Zymed, San Francisco, CA, USA	1 : 100	1 : 200
AR	Clon F39.4.1	Biogenex, San Ramon, CA, USA	1 : 15	1 : 50
PR	PR88	Biogenex, San Ramon, CA, USA	1 : 20	1 : 200
StAR	Polyclonal	Abcam, Cambridge, MA, USA	1 : 250	1 : 150
3β-HSD	Polyclonal	Prof. Ian Mason, University of Edinburgh	1 : 3000	1 : 5000
P450arom	Polyclonal	Affinity Bioreagents, Golden, CO, USA	1 : 250	1 : 500
Tubulin	6G7	Developmental Studies Hybridoma Bank	–	1 : 50
Secondary antibodies				
Biotinylated anti-rabbit IgG	Polyclonal	Zymed, San Francisco, CA, USA	1 : 100	–
Biotinylated anti-mouse IgG	Polyclonal	Zymed, San Francisco, CA, USA	1 : 100	–
Anti-rabbit IgG peroxidase	Polyclonal	Amersham, Buckinghamshire, UK	–	1 : 500
Anti-mouse IgG peroxidase	Polyclonal	Amersham, Buckinghamshire, UK	–	1 : 500

water and counterstained with Mayer's hematoxylin, dehydrated and mounted.

Negative control sections were subject to the same immunohistochemical method replacing primary antibodies with mouse and rabbit non-immune serum. The specificity of the secondary antibodies was tested by incubation with primary antibodies of proven negative reaction with rat antigen: anti-CD45 (Clone: PD7/26 and 2B11; Dako, Carpinteria, CA, USA) and anti-K_i-67 (polyclonal, rabbit anti-human K_i-67; Dako). To exclude the possibility of non-suppressed endogenous peroxidase activity some sections were incubated with DAB reagent alone. Serial sections of similarly processed tissue samples of rat ovaries were used as positive controls in each assay to normalise the image analysis. Each immunohistochemical series included randomly selected slides with ovarian sections from different treatments.

Western immunoblotting

Details of antibodies used are summarised in Table 1. Rat ovarian tissues were homogenised in a immunoprecipitation assay lysis buffer consisting of 1% v/v octylphenyl-polyethylene glycol (IGEPAL CA630), 0.5% (w/v) sodium deoxycholate, 0.1% w/v SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma-Aldrich Corp.), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany). Ovarian homogenates were centrifuged at 12 000*g* at 4°C for 20 min and the protein concentration in the supernatants was estimated using the DC Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). Forty μg of protein, along with prestained molecular weight markers (Bio-Rad), were separated by SDS-PAGE (12% resolving gel). Proteins were transferred to nitrocellulose membranes (GE Healthcare Ltd, Amersham, Buckinghamshire, UK), blocked for 1 h in 2% w/v non-fat milk in Tris-buffered saline (TBS) containing 0.05% v/v Tween 20 (Sigma-Aldrich Corp.), and then incubated overnight at 4°C with specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with corresponding secondary peroxidase-conjugated antibody (Table 1). Immunopositive bands were visualised with a chemiluminescent detection kit (ECL; GE Healthcare), using ECL film (GE Healthcare) and subsequently scanned into a computer. Individual bands were quantified directly from membranes by densitometry using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). The relative density was determined using tubulin as the loading control. The signal of each protein was normalised as a percentage to those of control proestrous ovaries to produce arbitrary densitometric units of relative abundance.

Image analysis

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics). Images were digitised by a CCD colour video camera (Motic 2000; Motic China Group, Xiamen, China) mounted on top of a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan) using an objective magnification of 40×. Resolution of the images was set to 1200 × 1600 pixels. Each pixel of the image corresponded to 0.13 μm at the magnification used and each field represented a tissue area of 0.031 mm^2. The system captured each image and automatically corrected for the background. This prevented differential readings due to different lighting conditions. No further image processing was done.

The methodological details of image analysis as a valid method for quantifying expression levels are given elsewhere and have been described earlier (Wang *et al*. 1999, 2000; Ortega *et al*. 2007, 2009; Salvetti *et al*. 2007, 2008). The major strength of the imaging approach used in the present study is visualisation of the *in situ* localisation of proteins within cells of interest. In the past decade, computerised image analysis systems have been developed to obtain objective and accurate quantification of biological markers (Lejeune *et al*. 2008). This approach has been successfully applied by other researchers to quantify steroid receptors and enzymes in different tissues and validated for diagnostic, prognostic and therapeutic purposes (Shan *et al*. 1997; Zhu *et al*. 2000; Nilsson *et al*. 2003; Esslimani-Sahla *et al*. 2004,

2005; Nabi *et al*. 2004; Sharangpani *et al*. 2007; Johannesson *et al*. 2008; Lejeune *et al*. 2008).

To obtain quantitative data regarding immunohistochemical staining of StAR, 3β-HSD and P450arom in the follicular wall, at least three sections for each specimen and antibody were evaluated. The average density for each antibody reaction was calculated from at least 20 images of each area (granulosa, theca externa, and theca interna) in each slide as a percentage of total area evaluated through colour segmentation analysis, which extracts objects by locating all objects of a specific colour (brown stain).

Changes in cells expressing steroid receptors were evaluated by counting at least 500 labelled and unlabelled cells in each area (granulosa, theca externa, and theca interna) of each follicle type in each slide, and results were expressed as the percentage of the total number.

Hormone assays

FSH and LH serum levels were determined by radioimmunoassay (RIA) using a kit provided by NIDDK as previously described (Baravalle *et al*. 2006). Intra- and interassay coefficients of variance for LH and FSH were less than 8 and 12% respectively. Minimum detectable concentrations were 0.16 and 1.18 ng mL^{-1} of serum for LH and FSH respectively.

Serum oestradiol and progesterone were directly estimated by RIA using highly specific antisera kindly provided by Dr G. D. Niswender (NIDDK) (Korenman *et al*. 1974). Labelled hormones were purchased from GE Healthcare. Assay sensitivity for oestradiol was 1.7 pg and intra- and interassay coefficients of variation were 9.3% and 11.4% respectively. Assay sensitivity for progesterone was 50 pg and intra- and interassay coefficients of variation were 7.5% and 11.9% respectively (Chamson-Reig *et al*. 1999).

Serum testosterone was determined by RIA using a specific antiserum kindly provided by Dr G. D. Niswender (NIDDK), after diethyl-ether extraction. Labelled hormone was purchased from New England Nuclear (Boston, MA, USA). Regarding assay sensitivity, the lowest point in the testosterone standard curve was 12.5 pg and the lowest detectable concentration after extracting 150 μL serum was 208.3 pg mL^{-1}. Intra- and interassay coefficients of variation were 7.8% and 12.3% respectively (Chamson-Reig *et al*. 1999).

Statistics

The number of individuals chosen for each group resulted from a sample size calculation that evaluated the number of individuals necessary to produce an estimation of the immunoreactivity that would fall within 0.4 units of the real value. The formula used was: $n = Z^2 \times sd^2/d^2$, where n = sample size, Z = level of confidence (1.96 for 95%); sd = standard deviation (0.3); d = 0.4. Because we were able to reject the null hypothesis in most cases, Type 2 errors were not considered to be a problem.

A statistical software package (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL, USA) was used to perform the statistical tests. The analysis of data was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. A $P < 0.05$ value was considered to be significant. Results were expressed as mean ± s.e.m.

Results

Serum hormone levels

Serum hormone levels are shown in Fig. 1. The letrozole treatment induced a significant increase in serum LH, FSH and testosterone concentrations compared with those in proestrous and diestrous control animals ($P < 0.05$). Oestradiol showed a significant reduction in letrozole-treated rats compared with proestrous control animals ($P < 0.05$), without changes in relation to diestrous controls. Low levels ($P < 0.05$) of progesterone were detected in treated animals compared with diestrous animals. However, in this case, no differences were observed with proestrous animals.

Also, significantly lower serum levels of LH, FSH and oestradiol but higher levels of progesterone were observed in control diestrous rats in relation to proestrous animals ($P < 0.05$).

Histology

Morphologically, ovaries from the control groups exhibited follicles at various stages of development. In proestrus primordial, primary, secondary, tertiary and preovulatory follicles were observed; in diestrous only primordial, primary, secondary and small tertiary follicles as well as fresh corpora lutea were seen. Atretic follicles were observed in both control groups. In the letrozole-treated group, follicles in development could be observed, in addition to follicles showing evidence of atresia and many large cysts with thickened granulosa cell layer (Fig. 2). Corpora lutea were absent in all cases in the letrozole-treated group.

Immunohistochemistry

Steroid receptors

Summaries of the immunohistochemical expression using the specific antibodies for steroid receptors are given in Figs 2 and 3. Oestrogen receptor-α (ERα) was expressed in the cellular nuclei of the granulosa and theca interna of all follicular categories studied. In cystic follicles of letrozole-treated animals, a significantly lower expression was evidenced in granulosa cells and theca externa ($P < 0.05$). The immunostaining decreased in atresic follicles.

Nuclear oestrogen receptor-β (ERβ) expression was observed in the granulosa cells of growing follicles at all stages, from primary to tertiary follicles, decreasing in atresic follicles. Immunoexpression of ERβ was significantly ($P < 0.05$) lower in the granulosa of cystic follicles in letrozole-treated animals.

Androgen receptor (AR) was detected in the nuclei of ovarian cells, principally in the granulosa and theca interna cells of growing, atretic, and cystic follicles. The theca externa layer had no or only weak immunostaining. The percentage of positive cells was lower in the granulosa and theca layers of secondary and tertiary follicles of diestrous animals and increased in tertiary follicles of letrozole-treated animals ($P < 0.05$). The granulosa of cystic follicles showed significantly reduced immunostaining ($P < 0.05$).

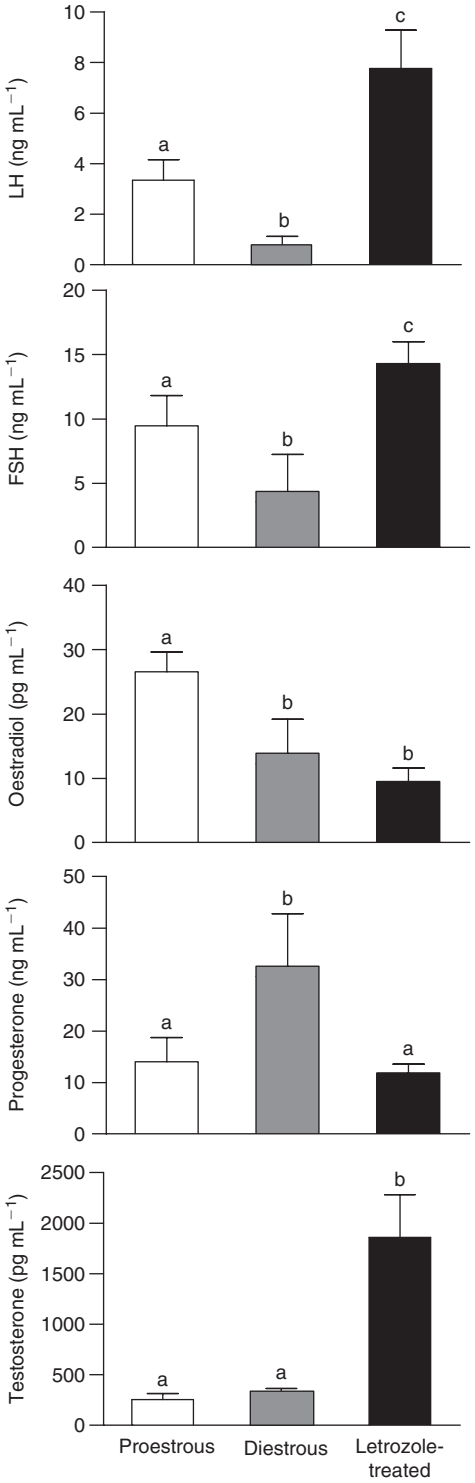

Fig. 1. Effect of letrozole treatment on serum hormone levels. The letrozole treatment induced a significant increase in serum LH, FSH and testosterone concentrations. Serum oestradiol showed a significant reduction compared with proestrous control animals. Also, low levels of serum progesterone were detected compared with diestrous animals. Values represent the mean ± s.e.m. For each hormone, bars with different superscript letters denote significant differences ($P < 0.05$).

Progesterone receptor (PR) protein was observed in the nuclei of the various ovarian cell groups, namely in the granulosa and theca interna cells of growing, atretic, and cystic follicles. The expression of PR was lower ($P < 0.05$) in the granulosa cell and theca interna of cystic follicles in letrozole-treated animals.

Steroidogenic enzymes

Summaries of the quantitative analysis of the immunohistochemical expression of the various steroidogenic enzymes are given in Figs 2 and 4. A heterogeneous staining pattern for StAR protein was evident in granulosa cells and thecal (interna of tertiary follicles) cells of healthy and cystic follicles. In letrozole-treated animals, a significantly ($P < 0.05$) higher expression was observed in granulosa and thecal cells of secondary and tertiary follicles and thecal cells of cystic follicles. Granulosa of primary follicles showed a weak immunostaining.

No specific immunostaining for 3β-HSD was found in the granulosa cells of primary follicles, or in the theca externa of tertiary and atretic follicles. A clear immunoreaction was evident in the granulosa and thecal cells of secondary and theca interna cells of tertiary follicles. In atretic follicles, an increased immunostaining was evident in thecal cells. Letrozole treatment induced a higher expression ($P < 0.05$) of 3β-HSD in granulosa and thecal cells of secondary, tertiary, cystic and atretic follicles.

The immunoreaction of P450arom was localised to the granulosa and, with a low intensity, in theca layers. In proestrous follicles increased inmmunostaning was evident in the granulosa ($P < 0.05$). Also, in letrozole-treated animals the granulosa cells of cystic follicles showed significantly reduced immunostaining ($P < 0.05$).

Antibody specificity

Specific staining was detected in all positive control tissues showing numerous immunostained cells, while a lack of staining in the negative controls demonstrated the specificity of the reactions. In western blot analysis, positive bands of the appropriate size were observed (Fig. 2).

Western immunoblot analysis

To examine protein expression in the whole ovary, immunoblot studies were performed with the same antibodies as were used for the immunohistochemistry. The antibodies revealed single positive bands of appropriate sizes in all tested homogenates for each of the proteins studied, except for PR, which showed the two isoforms corresponding to PR-B (116 kDa) and PR-C (60 kDa) (Fig. 2).

A summary of the quantitative analysis of relative protein abundance is presented in Fig. 5. ERα was significantly elevated in diestrous and letrozole-treated animals whereas ERβ showed a reduction in the treated group. With AR antibody, a reduction in diestrous animals and an increased expression in letrozole-treated animals compared with proestrous controls was evident ($P < 0.05$). Although there were no differences in the expression of PR-B, the expression of PR-C was higher in proestrous animals ($P < 0.05$). Letrozole treatment resulted in increased levels of StAR and 3β-HSD ($P < 0.05$). P450arom expression was significantly elevated in proestrous animals in relation to diestrous

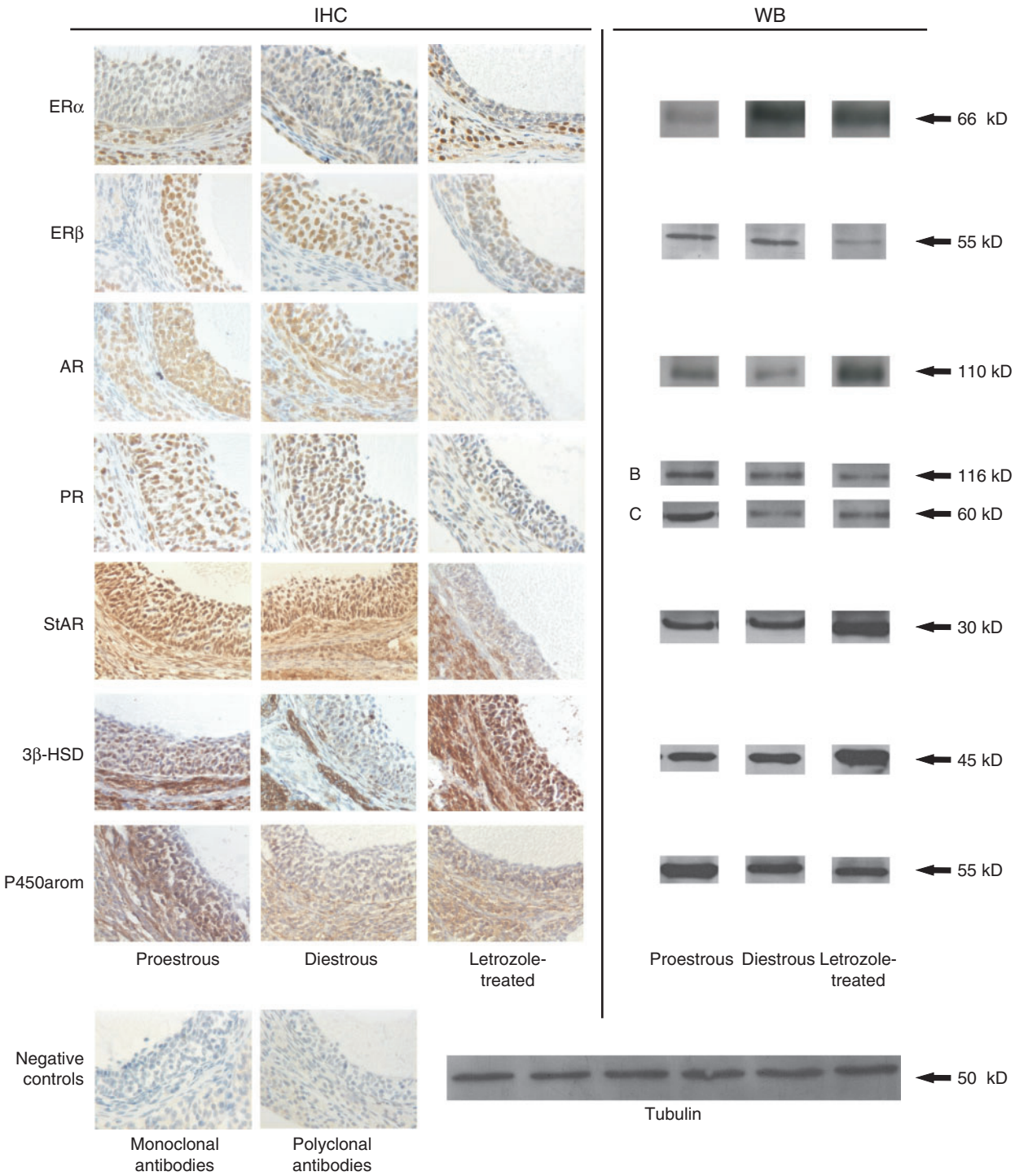

Fig. 2. Immunohistochemistry. Representative images of steroid receptor (ERα, ERβ, AR and PR) and steroidogenic enzyme (P450arom, 3β-HSD and StAR) immunostaining in tertiary follicles of control proestrous and diestrous follicles and cystic follicles of letrozole-treated animals. Negative control sections were subject to the same immunohistochemical method replacing primary antibodies with mouse and rabbit non-immune serum (400×). Western blot. Representative images showing the effect of letrozole treatment on total ovarian specific protein expression of steroid receptors (ERα, ERβ, AR and PR) and steroidogenic enzymes (P450arom, 3β-HSD and StAR), with tubulin as the loading control (showing two samples of each group). In all tested homogenates the antibodies revealed single positive bands of appropriate sizes for each of the proteins studied, except for PR, which showed the two isoforms corresponding to PR-B and PR-C.

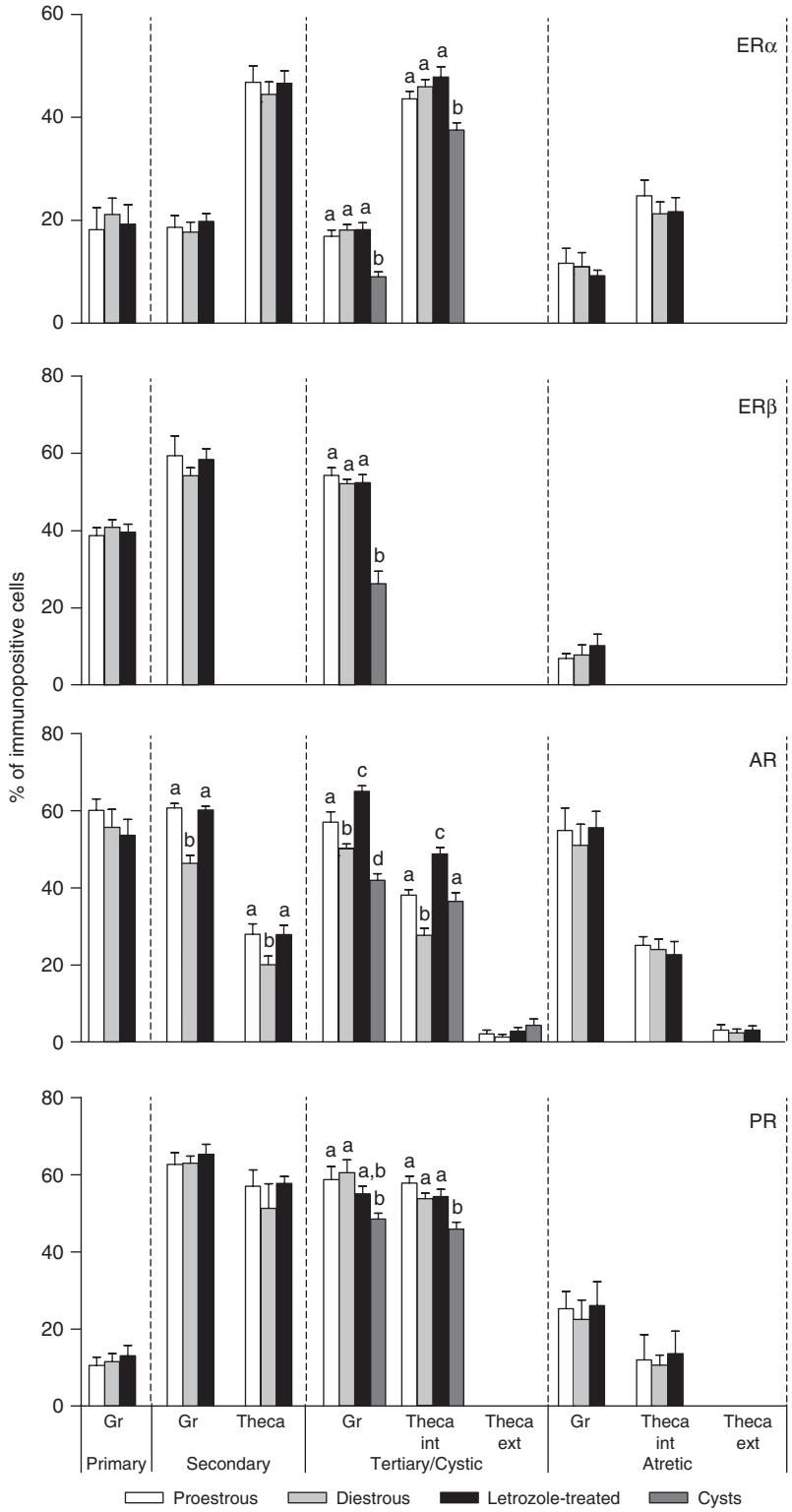

Fig. 3. Immunohistochemical staining of steroid receptors (ERα, ERβ, AR and PR) showing the percentage of immunopositive cells. In tertiary follicles, letrozole treatment induced an increase in the expression of AR, whereas in cystic follicles a decrease in ERα, ERβ, AR and PR was observed. Values represent the mean ± s.e.m. For each follicular compartment within each follicle type, bars with different superscripts letters denote significant differences ($P < 0.05$).

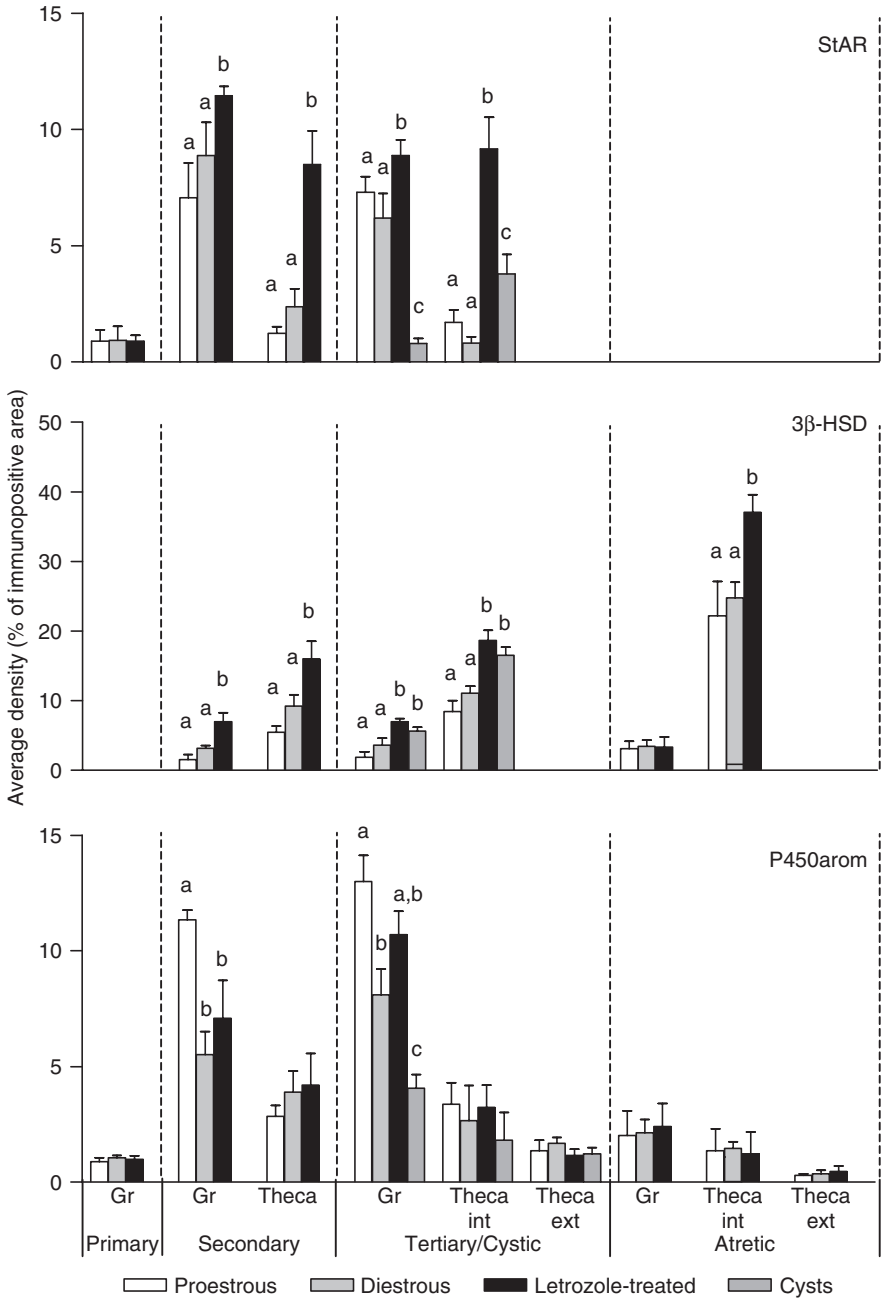

Fig. 4. Immunohistochemical staining of steroidogenic enzymes (P450arom, 3β-HSD and StAR) showing the percentage of immunopositive area. Letrozole treatment induced an increase in the expression of StAR and 3β-HSD whereas that of P450arom decreased in cysts. Values represent the mean ± s.e.m. For each follicular compartment within each follicle type, bars with different superscripts letters denote significant differences ($P < 0.05$).

and treated animals. When the primary antibody was omitted, no bands were observed (data not shown).

The expression levels of ERβ, AR, PR and the steroidogenic enzymes were consistent with the immunohistochemical data, whereas ERα did not show the same pattern, possibly due to the changes detected by immunohistochemistry, in which the changes were in certain compartments (cyst wall) and not in the whole ovary.

Discussion

Steroid receptors and steroidogenic enzymes are present throughout most stages of follicular development in a variety

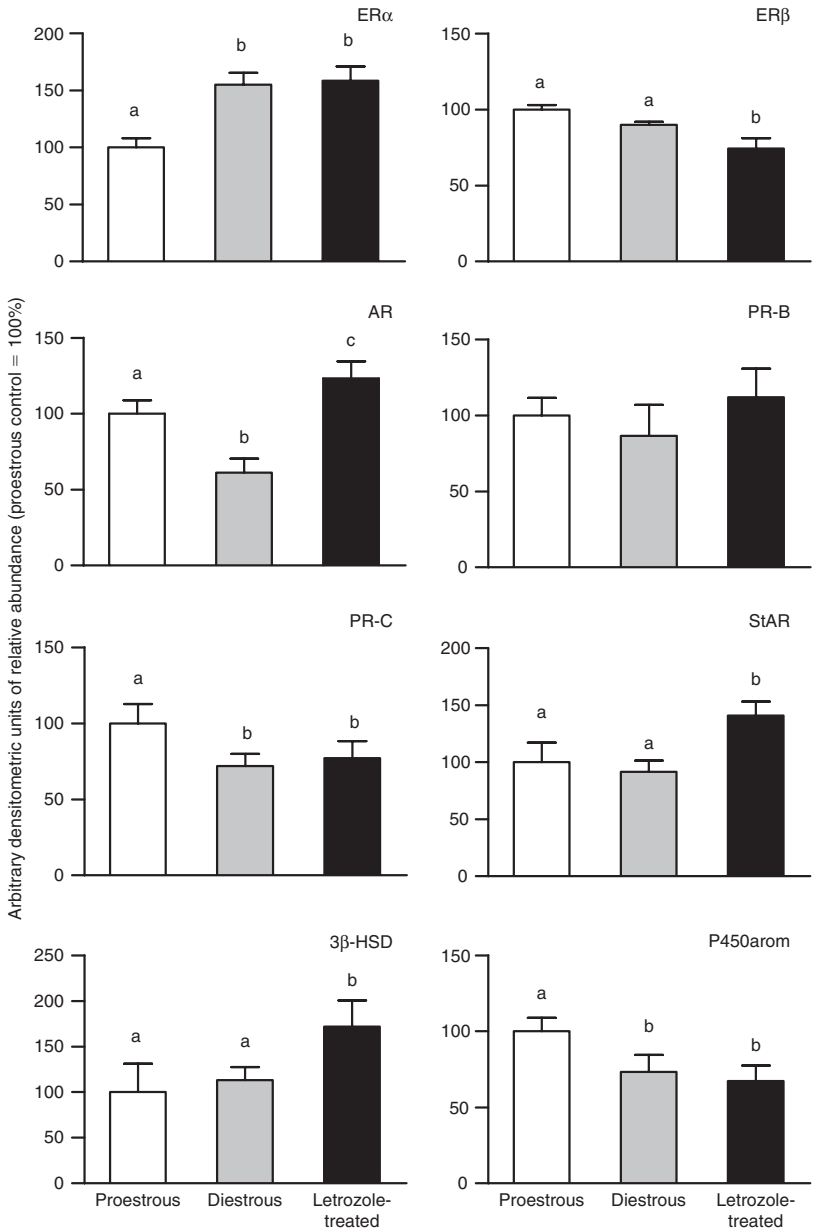

Fig. 5. Expression of steroid receptors (ERα, ERβ, AR and PR) and steroidogenic enzymes (P450arom, 3β-HSD and StAR) analysed by western immunoblot. The relative expression was determined using tubulin as a loading control and the signal of each protein was normalised as a percentage of that of control proestrous ovaries, to produce arbitrary densitometric units. Letrozole treatment induced an increase in the expression AR, StAR and 3β-HSD and a decrease in ERβ. ERα, PR and P450arom only showed partial change. Values represent the mean ± s.e.m. For each steroid receptor, bars with different superscript letters denote significant differences ($P < 0.05$).

of species, and this highlights their possible participation in the pathogenesis of PCO. In the present study, ERα and β, AR, PR, P450arom, 3β-HSD and StAR were evaluated in an experimental PCOS model, by use of western immunoblot and semiquantitative immunohistochemistry, thus allowing the localisation and quantification of the different proteins to be analysed in the each ovarian component. Findings from this study provide evidence that letrozole has differential effects on the expression of all the proteins studied.

Higher levels of gonadotrophins were observed in this PCOS model. In addition, testosterone was significantly elevated, reflecting the accumulation of androgen because the conversion of androgen substrates into oestrogens was blocked. A reduction of oestrogen in treated animals in relation to proestrous

animals was observed, but no difference was seen between treated and diestrous controls. Progesterone levels decreased, indicating anovulation, as in human PCOS (Meenakumari *et al*. 2004) and other rat models of induced PCO (Kafali *et al*. 2004; Baravalle *et al*. 2006). These hormonal profiles appear to agree with the hormonal environment described for PCOS in various species (Morin-Papunen *et al*. 2000; Marcondes *et al*. 2002; Baravalle *et al*. 2006; Mannerås *et al*. 2007).

In women with PCOS, the predominant reason for high serum LH concentrations is abnormal negative feedback on LH secretion mediated by either oestradiol or progesterone (Abbott *et al*. 2002); on the other hand, in the current model aromatase inhibition through reduction of oestrogen production in the hypothalamus and pituitary presumably enhanced LH secretion by releasing negative feedback of oestrogens. Decreased oestrogen concentrations together with increased FSH concentrations, not typical with human PCOS, may appear to be the one drawback of this model. In addition, a role for intrinsic abnormalities in ovarian steroidogenesis is supported by *in vitro* studies demonstrating that ovarian theca cells from women with PCOS produce excessive androgens resulting from increased expression of the enzymes involved in steroid synthesis (Nelson *et al*. 1999; Blank *et al*. 2006). The granulosa cells in PCOS contain functional FSH receptors and are capable of responding to the FSH signal with appropriate levels of oestradiol production (Jakimiuk *et al*. 2001). Data also suggest an important role for insulin in the pathogenesis of PCOS acting synergistically with LH to stimulate ovarian androgen production, and suppressing hepatic production of sex-hormone binding globulin, resulting in higher levels of free or bioavailable testosterone (Blank *et al*. 2006). Also, Mahajan (1988) described clearly in women various postulated steroidogenic enzyme blocks, mostly implicating higher-than-normal production of circulating androstenedione, testosterone and, in some cases, dehydroepiandrosterone. These high levels of androgens, because of their peripheral conversion to oestrogens, lead to inappropriate secretion of gonadotrophins in PCOS.

A decrease in the expression of ERβ in the granulosa cell layer of cystic follicles was observed in animals treated with letrozole compared with that in normal animals, concomitant with a decrease in total ERβ protein in treated animals. The results of ERβ immunohistochemistry concur with those we observed in rats with PCO induced by constant light exposure (Salvetti *et al*. 2008). In women with PCOS, Jakimiuk *et al*. (2002) showed a decrease in the expression of ERβ mRNA and protein in both granulosa and theca cells from follicles derived from subjects with PCOS compared with those from size-matched control follicles. In relation to ERα, the only difference identified was a decrease in its expression in granulosa and theca interna of cysts, and this is not in accordance with what has been described in other models of PCO (Salvetti *et al.* 2008), although in these animals, no changes in steroid levels were observed. The absence of ERs in the granulosa of PCO has been demonstrated in women (Takayama *et al*. 1996).

Numerous studies have shown that gonadotrophins and oestrogen downregulate granulosal expression of the ERβ isoform (Byers *et al*. 1997; Sharma *et al*. 1999) and that both oestrogen receptors show a tendency to upregulate together with increasing oestrogen levels in the follicular fluid (Berisha *et al*. 2002). In this sense, the high level of gonadotrophins and the low levels of oestradiol could explain the observed changes in ERs.

A significant increase in AR expression in treated animals was evident upon western blotting. This supports the suggestion of Mannerås *et al*. (2007), that the ovarian alteration observed in this model is mediated by the accumulation of endogenous testosterone, which also results in pronounced activation or upregulation of the AR. Consistent with this finding, administration of testosterone propionate to prepuberal rats at 5 days of age also increased ovarian nuclear AR expression (Bukovsky *et al*. 2002).

The expression of PR was lower in granulosa and theca interna of cystic follicles in treated animals. By western blot, significant decreases in expression of PR-C (but not PR-B) was confirmed in treated animals compared with proestrous animals, and this agrees with our previous results in another model of PCOS (Salvetti *et al*. 2008). Indeed, in the PR-knockout (PRKO) mouse, mature follicles do not ovulate or luteinise but persist, giving the ovaries a polycystic appearance (Lydon *et al*. 1995). PR expression and activation are involved in regulating rat granulosa cell susceptibility to apoptosis after LH receptor stimulation (Svensson *et al*. 2000). PRs are required specifically for LH-dependent follicular rupture leading to ovulation (Lydon *et al*. 1995).

In the second part of the present study, the expression profiles of steroidogenic enzymes were analysed. The expression of StAR and 3β-HSD was significantly elevated in letrozole-treated animals, while P450arom did not show differences with diestrous animals. The immunolocalisation of StAR coincides with that previously described in the rat (Thompson *et al*. 1999). In addition, based on this report that StAR expression was stimulated by PMSG, we propose that elevated levels of FSH may stimulate StAR expression in letrozole-treated animals. On the other hand, an overexpression of StAR mRNA has been described in granulosa and thecal cells of women with PCOS (Jakimiuk *et al*. 2001; Kahsar-Miller *et al*. 2001). Considering that both the human disorder and letrozole-induced PCOS exhibit increased androgen synthesis, another possible hypothesis is that an increased amount of intracellular cholesterol is necessary for androgen biosynthesis.

The pattern of immunostaining of 3β-HSD was consistent with that described previously in normal rat ovary (Juneau *et al*. 1993; Teerds and Dorrington 1993). Coinciding with data described in women with PCO (Nelson *et al*. 1999; Kaaijk *et al*. 2000) an incremental increase in 3β-HSD expression, mainly in the theca interna, was demonstrated in letrozole-treated animals. Observations *in vivo* (Matalliotakis *et al*. 2006; Homburg 2008) and *in vitro* (Gilling-Smith *et al*. 1994, 1997) suggest that augmented androgen production is a stable steroidogenic phenotype of PCO. The increased activity of 3β-HSD in theca cells of PCO maintained in long-term culture is suggestive that this is a stable property of PCOS thecal cells (Nelson *et al*. 1999). These data are consistent with the concept that increased androgen production by PCO theca cells is an intrinsic and, possibly genetically determined, property of the cells (Nelson *et al*. 1999; Abbott *et al*. 2002; Goodarzi and Azziz 2006).

Letrozole effectively blocked the production of oestrogen without exerting effects on other steroidogenic pathways

(Bhatnagar 2007). Its use in rats provided a strong inhibition of P450arom activity manifested mainly by a decrease in oestradiol and elevation in testosterone levels. The expression of P450arom protein in ovaries showed the same pattern as oestradiol levels with a reduction in the immunostaining within cystic follicles. In women with PCOS, P450arom mRNA in the granulosa of cysts is lower than in large follicles (Jakimiuk *et al*. 1998), and the enzyme protein has not been detected previously in these cells (Takayama *et al*. 1996). However, other authors suggest that expression would be normal, unless the different functional characteristics could represent the effect of altered local influencing factors (Pierro *et al*. 1997). It is known that aromatase expression is regulated by many substances acting through paracrine and autocrine routes, thus indicating that in PCOS eventual abnormalities in the secretory products of follicular cells could lead to an irregular autocrine regulation of steroid biosynthetic activity (Andreani *et al*. 1996; Pierro *et al*. 1997).

In conclusion, although much remains to be done in order to characterise further the letrozole-induced PCO model, we confirmed that it is a very useful tool to study some aspects of cystogenesis in PCOS. These results indicate that cystogenesis in this experimental model is characterised by changes in steroid receptors and steroidogenic enzyme expression that may be essential to the proper ovarian functioning and are in agreement with some changes observed in women with PCOS. Further studies are needed to assess the specific role and regulation of each of these cellular components and their participation in the pathophysiology of PCOS.

Acknowledgements

We are very grateful to Dr Pablo Beldomenico for critical reading of the manuscript. Also we thank the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases and Dr A. F. Parlow for the RIA kits, and Dr Damasia Becu-Villalobos (CONICET) for valuable RIA contributions. The technical support of Celina Baravalle in obtaining experimental animals and the provision of Letrozole by ASPEN Argentina is gratefully acknowledged. This study was supported by grants from the National University of Litoral (CAID Program 2006).

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Manuscript received 7 February 2009, accepted 17 May 2009