Reprod Dom Anim **44**, 805–814 (2009); doi: 10.1111/j.1439-0531.2008.01086.x
ISSN 0936-6768

Heat Shock Protein 70 and Sex Steroid Receptors in the Follicular Structures of Induced Ovarian Cysts

NR Salvetti[1,2], C Baravalle[1,2], GA Mira[2], EJ Gimeno[3], BE Dallard[1], F Rey[1] and HH Ortega[1,2]

[1]*Department of Anatomy and Histology;* [2]*Center for Experimental Biology and Laboratory Animals Sciences, Faculty of Veterinary Sciences, National University of Litoral;* [3]*Institute of Pathology, Faculty of Veterinary Sciences, National University of La Plata, Argentina*

Contents

The purpose of this study was to estimate the expression and relative amounts of estrogen (ER) and progesterone receptors (PR) and their isoforms as well as heat shock protein 70 (HSP70) in ovaries of rats with induced cystic ovarian disease (COD). Primary, secondary, tertiary, atretic and cystic follicles were evaluated by immunohistochemistry and total ovarian proteins were analyzed by Western blot. In the granulosa layer, growing and cystic follicles in the treated group have a higher expression of ERα than growing follicles of control individuals. In the theca interna layer, tertiary follicles presented a significantly higher expression of ERα in the treated group. An increase in total ERα protein was detected in the treated group. Granulosa cells of all growing, atretic and cystic follicles show a lower expression of ERβ in animals with COD, and the total protein expression of ERβ was lower in this group. The expression of PR was lower in the granulosa cell layer of tertiary and cystic follicles in treated animals, and theca interna layer had less intense immunostaining in this group. Although there were no differences in the expression of PR-B by Western blotting, the expression of PR-A was higher and the expression of PR-C was smaller in the treated group. An intense HSP70 immunostaining was observed in the cells of cystic follicles. By Western blotting, higher protein expression of HSP70 was detected in the ovarian samples of the control group than those of the treated ones. Ovaries of animals with COD exhibited an altered steroid receptor expression and subtype balance as compared with control animals, and an increase in HSP70 immunoexpression.

Introduction

Cystic ovarian disease (COD) is a common reproductive disorder that occurs in many species, including domestic animals and human beings (Jakimiuk et al. 2002; Silvia et al. 2002; Salvetti et al. 2004; Vanholder et al. 2006). It is characterized by the presence of persistent follicular structures in one or both ovaries for a longer time, interrupting the estral cycle and causing infertility. The pathogenesis of ovarian cyst development has not yet been established. The more accepted hypothesis is that COD is the result of an imbalance between hormones involving the hypothalamic-pituitary-gonadal axis (Silvia et al. 2002; Vanholder et al. 2006).

Many experimental models have been realized to monitor the development of this disease closely. A relatively simple method to induce COD is by exposing mature rats to an environment with constant light (Salvetti et al. 2004). Constant illumination acts on the hypothalamus to induce a fail in the preovulatory luteinizing hormone (LH) surge and prevent ovulation. The large preovulatory follicles persist and gradually become cystic (Salvetti et al. 2004).

The ovarian steroid hormones – estrogen and progesterone – perform several important functions related to reproduction through endocrine mechanisms of action. The genomic effects of these hormones are mediated through interaction with specific intracellular receptors that are members of the nuclear receptor families. Hormones binding to their receptors induce structural and functional changes in the receptor structure that associates ligand–receptor complexes with specific target genes to regulate their transcription (Beato and Klug 2000; Drummond et al. 2002).

Two major forms of estrogen receptor (ER) have been identified in mammalians: ERα and ERβ. These receptors have a differential distribution in the different organs in both male and female. The existence of subtypes may partly explain the selective action of estrogen in different target tissues, and in the same tissue during different physiological states. These two receptors bind 17β-estradiol with high affinity and specificity. Although ERβ shares many functional characteristics with ERα, the molecular mechanisms that regulate its transcriptional activity and its tissue location are different from those of ERα (Kuiper et al. 1996; Wang et al. 2000). Several studies have demonstrated the cellular distribution of ERα and ERβ in the female reproductive organs of various species (Rosenfeld et al. 1999; Pelletier et al. 2000; Wang et al. 2000; Van den Broeck et al. 2002a,b; D'Haeseleer et al. 2005). Studies using *in situ* hybridization and immunohistochemistry have demonstrated intense signals of ERβ and ERα mRNA/protein expression in follicular granulosa and uterine epithelial cells, respectively (Byers et al. 1997; Sar and Welsch 1999). Furthermore, ERα is moderately expressed in the stromal/thecal cells, and ERβ is intensely expressed in the granulosa cells of the ovary (Pelletier et al. 2000; Wang et al. 2000).

In contrast to ER, progesterone receptor (PR) has at least three isoforms, all originating from the same gene: PR-A, PR-B and PR-C (Bramley 2003). The genomic progesterone receptor (gPR) is a member of the ligand-inducible DNA-binding superfamily of nuclear transcription factors that include the receptors for the other steroid hormones (such as estrogen and androgen receptors), thyroid hormones, vitamin D, retinoic acid and a variety of 'orphan' receptors. The cDNA for the B-form of gPR codes for a 116 kDa protein containing a number of different functional domains. Two other isoforms of the human gPR arise from the use of alternate promoters within the same gene. PR-A is NH_2-terminally truncated (94 kDa); whereas PR-C (60 kDa)

lacks exon 1 and most of exon 2 (Wei et al. 1990). Several other variants of gPR, such as PR-L and PR-S, have been described (Wei et al. 1990). In general, PR-B is a stronger transactivator, and PR-A is a dominant inhibitor of PR-B and other nuclear receptors (Mulac-Jericevic and Conneely 2004). PR-C is also an NH_2-terminal truncated transcriptional product, but it is much smaller than PR-A (Wei et al. 1990). It has an intact hormone-binding domain (HBD), but it is only the second zinc finger of the DNA-binding domain, and therefore it does not directly interact with DNA. Thus, PR-C may act as a selective suppressor of progesterone action by binding the hormone in the cytosolic fraction to curtail the binding of progesterone to active receptor forms (Wei et al. 1990). The expression of both isoforms is conserved in rodent and humans, and overlaps spatiotemporally in female reproductive tissues. However, the ratios of individual isoforms vary in reproductive tissues because of developmental and hormonal status (Gava et al. 2004).

Heat shock proteins (HSPs) are highly conserved cellular stress proteins present in many species. HSPs serve two major functions. First, under physiological conditions, they act as molecular chaperones, which are involved in mediating the folding and transport of other intracellular proteins and, in some cases, their assembly into oligomeric structures (Pratt and Toft 1997). Second, they are induced in response to cellular stress, which includes changes in temperature, presence of free oxygen radicals, viral and bacterial infections, heavy metals, ethanol and ischemia (Mosser et al. 2000). Several nuclear receptors, including sex steroids, are recovered from cells in large heterocomplexes that contain HSPs. Some components of the receptor heterocomplexes are proteins with established chaperone functions (e.g. HSP90 and HSP70), and one critical function of the HSP heterocomplex is to facilitate the folding of the HBD of receptors into a high-affinity steroid-binding conformation (Neuer et al. 2000). HSP70 exists in two forms in the cytoplasm, a 73-kDa form that is constitutively expressed (HSC70; this form is associated with steroid receptors) and a 72-kDa stress-inducible form (HSP70) (Pratt and Toft 1997). Although a lot has been studied about the expression of HSP in different tissues, and with diverse treatments that cause stress, little is known on the expression of these proteins in normal ovarian structures, as well as on their relationship with steroid receptors.

The purpose of the present study was to examine the expression and relative amounts of ER and PR and their isoforms as well as HSP70 in rat ovaries with COD and to determine whether there are significant differences when compared with the ovaries of regularly cycling rats.

Materials and Methods

Animals and treatment

All the procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). Adult (16-week old) female Wistar rats were provided by the Center for Experimental Biology and Laboratory Animals Sciences, Faculty of Veterinary Sciences, National University of Litoral. Before the experiment, the animals were kept under a controlled cycle of light–darkness (lights on from 6:00 to 20:00 h), at a temperature of 18–22°C, with free access to water and commercial food (Cargill, Argentina). Lighting was provided by banks of General Electric 4 coolwhite 40 W fluorescent tubes to obtain an intensity of 350 lux at 1 m above the floor.

Thirty animals displaying at least two normal 5-day estrus cycles, just prior to treatments, were divided into two groups. Group I (control group) consisted of 10 females of the same age as the treated animals and remained in normal cycle of light–darkness conditions. Group II (continuous light group) consisted of 20 females and were placed in conditions as described earlier, except for the cycle of light that was extended to 24 h for a period of 90 days (Salvetti et al. 2004).

Smears obtained by vaginal washing were examined under a microscope to determine the relative abundance of nucleated epithelial cells, cornified cells and leukocytes (Salvetti et al. 2004). Cycles of 5-day duration were considered regular. The observation of cornified cells in the smears during a minimum of 10 serial days was defined as persistent vaginal cornification (PVC), and was considered a confirmation of follicular cystic development (Salvetti et al. 2004).

Tissue sampling

The animals of Group II were killed approximately 2 weeks after PVC was established; those in Group I were sacrificed after 12 weeks in proestrus to obtain healthy-growing follicles.

After being anesthetized with a cocktail of ketamine/xylazine (40/4 mg/kg) via subcutaneous, the rats were killed by decapitation; trunk blood was collected, and serum was stored at −20°C until being used for hormone assays. The ovaries destined for histological procedures were dissected and fixed in 10% (v/v) buffered formalin during 6 h at 8°C and were washed in phosphate buffer saline (PBS) (control, n = 5; treated = 10). For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Five micrometer-thick sections were mounted in slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and were stained with hematoxylin–eosin for a previous observation.

Ovarian tissues for Western blot were removed and immediately frozen at −80°C (control, n = 5; treated = 10). Tissues were homogenized in a radioimmunoprecipitation assay (RIPA) lysis buffer [1% (v/v) IGEPAL CA630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, 50 mM sodium fluoruro, 0.1 M PBS (pH 7)] containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets; Roche®, Mannheim, Germany) and were centrifuged at 14 000 rpm for 20 min. The proteic fraction was placed in a sterile tube and was frozen at −80°C. Protein concentrations were measured by modified Lowry protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

Immunohistochemistry

Details of antibodies used are provided in Table 1. Each antibody was assayed in at least five sections of each ovary from each individual. A streptavidin–biotin immunoperoxidase method was done as previously described (Ortega et al. 2007; Salvetti et al. 2007). After deparaffinization, microwave pre-treatment (antigen retrieval) was performed. The endogen peroxidase activity was inhibited with 1% (v/v) H_2O_2, and non-specific binding was blocked with 10% (v/v) normal goat serum. All sections were incubated with primary antibodies for 18 h at 4°C and then for 30 min at room temperature with rat-preabsorbed biotinylated secondary antibodies selected specifically for each of the two types of primary antibodies used (mono- or polyclonal). The visualization of antigens was achieved by the streptavidin–peroxidase method (BioGenex, San Ramon, CA, USA), and 3.3-diaminobenzidine (DAB; Dako, Carpinteria, CA, USA) was used as chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer's hematoxylin, dehydrated and mounted. The different follicular categories studied were classified in accordance with the Nomina Histologica (International Committee on Veterinary Histological Nomenclature 1994). Primary, secondary and tertiary growing follicles as well as atretic follicles in stage I, II or III of atresia (Hsueh et al. 1994) in both groups were analyzed. Cystic follicles from Group II were also analyzed.

To test the specificity of immunoreactions, adjacent control sections were subjected to the same immunohistochemical method replacing primary antibodies by goat non-immune serum. The specificity effect of secondary antibodies was tested by incubation with primary antibodies of proven negative reaction with rat antigen: anti-CD45 (Clone: PD7/26 and 2B11; Dako) and anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako). To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB reagent alone.

Western blotting

After protein determination, 40 mg of proteins were separated by SDS–PAGE (10% resolving gel). Proteins were transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK), followed by Ponceau S staining to test transfer. After 1 h of blocking in 2% (w/v) non-fat milk in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween buffer, blots were incubated overnight at 4°C with mono- or polyclonal antibodies detailed in Table 1. After washing, each membrane was treated with either goat anti-rabbit or anti-mouse IgG-peroxidase-conjugated secondary antibody (Table 1) for 1 h and was washed again. Finally, immunopositive bands were visualized with DAB (Dako).

Image analysis

Image analysis was performed using an Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA).

For the immunohistochemistry technique, images were digitized by a CCD colour video camera (Sony, Montvale, NJ, USA) mounted on a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of ×40. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and by calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections stained in the absence of a primary antibody. The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method (Ranefall et al. 1998; Ortega et al. 2007; Salvetti et al. 2007).

The methodological details of image analysis have been described earlier (Wang et al. 1999, 2000). Using a colour segmentation analysis tool, the total intense positively stained nuclear area (brown reaction product) was measured and expressed as a ratio (%) of the total area of cell nuclei (brown reaction product + blue hematoxylin). The image analysis score was calculated separately in each follicular wall layer (granulosa and theca interna) from at least 50 images of the following structures: secondary, tertiary, atretic and cystic follicles from ovaries of both groups. Using AutoPro macro language, an automated sequence operation was created to measure the immunohistochemical-stained area (IHCSA) for the determination of HSP70 immunohistochemistry. The IHCSA was calculated as a percentage of the total area evaluated through colour segmentation analysis, which extracts objects by locating all objects of a specific colour (brown stain). The brown stain was selected with a sensibility of 4 (maximum 5), and a mask was then applied to separate the colours permanently. The images were then transformed to a bi-level scale TIFF format. The IHCSA (black area) was calculated

Table 1. Antibodies used for immunohistochemistry and Western blot

Antibodies	Clone	Supplier	Dilution
Primary antibodies			
Estrogen receptor α	1D5	BioGenex (Santa Ramón, CA, USA)	1 : 50
Estrogen receptor β	Polyclonal	Affinity Bioreagents (Golden, CO, USA)	1 : 50
Progesterone receptor	PR88	BioGenex	1 : 50
Heat shock protein 70	BRM22	BioGenex	1 : 100
Secondary antibodies			
Biotinylated anti-rabbit IgG	Goat polyclonal	Zymed (San Francisco, CA, USA)	1 : 150
Biotinylated anti-mouse IgG	Goat polyclonal	Chemicon (Temecula, CA, USA)	1 : 100
Anti-rabbit IgG peroxidase	Goat polyclonal	Amersham Pharmacia Biotech (Argentina)	1 : 500
Anti-mouse IgG peroxidase	Goat polyclonal	Amersham Pharmacia Biotech	1 : 500

from at least 50 images of each area (granulosa, theca interna and theca externa) in each slide, for all categories of follicles being studied.

For the Western blotting method, the nitrocellulose membranes revealed with DAB were scanned at 1200 dpi (EPSON Stylus CX3500 scanner), and the images were stored in TIFF format. The levels of protein were compared in extracts from the control and treated groups and analyzed by densitometry.

Hormone assays

Follicle-stimulating hormone (FSH) and LH serum levels were determined by radioimmunoassay (RIA) using the kit provided by NIDDK (USA) as previously described (Ortega et al. 2007). The intra- and interassay coefficients of variation for LH and FSH were less than 8% and 12%, respectively. Minimum detectable concentrations were 0.16 and 1.18 ng/ml of serum for LH and FSH, respectively. Serum estradiol and progesterone were estimated by RIA using highly specific antiserum provided by Dr. G.D. Niswender (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA). Labelled hormones were purchased from Amersham. Assay sensitivity for estradiol 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.

Statistics

The number of individuals per group resulted from a sample size calculation that evaluated the number of individuals necessary to produce an estimation of immunoreactivity, which 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 null hypothesis in most cases, type-2 errors were not considered a problem.

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for performing statistical tests. The analysis of data between two groups was performed with *t*-student test. The statistical significance of differences between more than three groups of data was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. A $p < 0.05$ was considered significant. Results were expressed as mean ± SEM.

Results

Morphology

In the treated animals, the following ovarian structures were found: healthy follicles in development in addition to follicles showing evidence of atresia at different degrees; many large cysts with thickened granulosa cell layer; or large cystic follicles with scant granulosa cells; corpus luteum was absent in all cases. Hypertrophy of interstitial glands was also observed. Ovaries from control group exhibited follicles in various stages of development, including primary, secondary and tertiary follicles, as well as atretic follicles, interstitial glands and corpora lutea.

Hormone serum levels

Serum levels of hormones are shown in Table 2. No differences were found in the values for LH, FSH, estrogen and progesterone between groups.

ERα

ERα was expressed in the cellular nuclei of granulosa, theca interna and theca externa layers of all follicular categories studied. In the control ovaries, strong immunostaining of ERα was observed in the nuclei of theca interna, stromal and interstitial gland cells. In contrast, a moderate staining was observed in the granulosa cells of primary, secondary and tertiary follicles. Immunostaining was low in theca externa. Corpus luteum cells and oocytes lacked nuclear staining with ERα. There were differences in the percentage of expression between the two groups. In the granulosa layer, growing and cystic follicles in Group II had a higher expression than growing follicles in Group I except the primary follicles. In the theca interna layer, immunoreactivity was similar for the growing follicles except in tertiary follicles, which presented a significantly higher expression of this protein in the treated group ($p < 0.05$) (Figure 1; Table 3). The data also revealed the presence of weak cytoplasmic staining in the granulosa cells of all follicular types; this staining was considered as part of the background, and it was not included in the analysis. Through Western blot analysis, we could detect one band of 66 kDa with the antibody used. The expression of total ERα protein in the ovaries of Group I was higher than that in Group II (Figure 3).

ERβ

In the control group, nuclear ERβ expression was observed in the granulosa cell nuclei of growing follicles at all stages from primary to tertiary follicles. The intensity of ERβ immunostaining in the granulosa cells of atretic follicles varied according to the stages of atresia, being less strong in the most advanced stage. Thecal and stromal cells showed a weak immunostaining. ERβ was not detected in the oocytes. Immunoexpression of ERβ was significantly different in follicular structures of ovaries in the treated group. Granulosa cells of all growing as well as atretic follicles and cysts

Table 2. Hormone serum levels in GI (control) and GII (light exposed rats)*

Hormone	GI	GII
Progesterone (ng/ml)	42.27 ± 6.71	40.56 ± 6.64
Estradiol (pg/ml)	29.66 ± 10.43	23.52 ± 2.65
LH (ng/ml)	0.54 ± 0.13	0.94 ± 0.27
FSH (ng/ml)	4.10 ± 1.54	5.65 ± 1.10

*Values represent mean ± SEM.

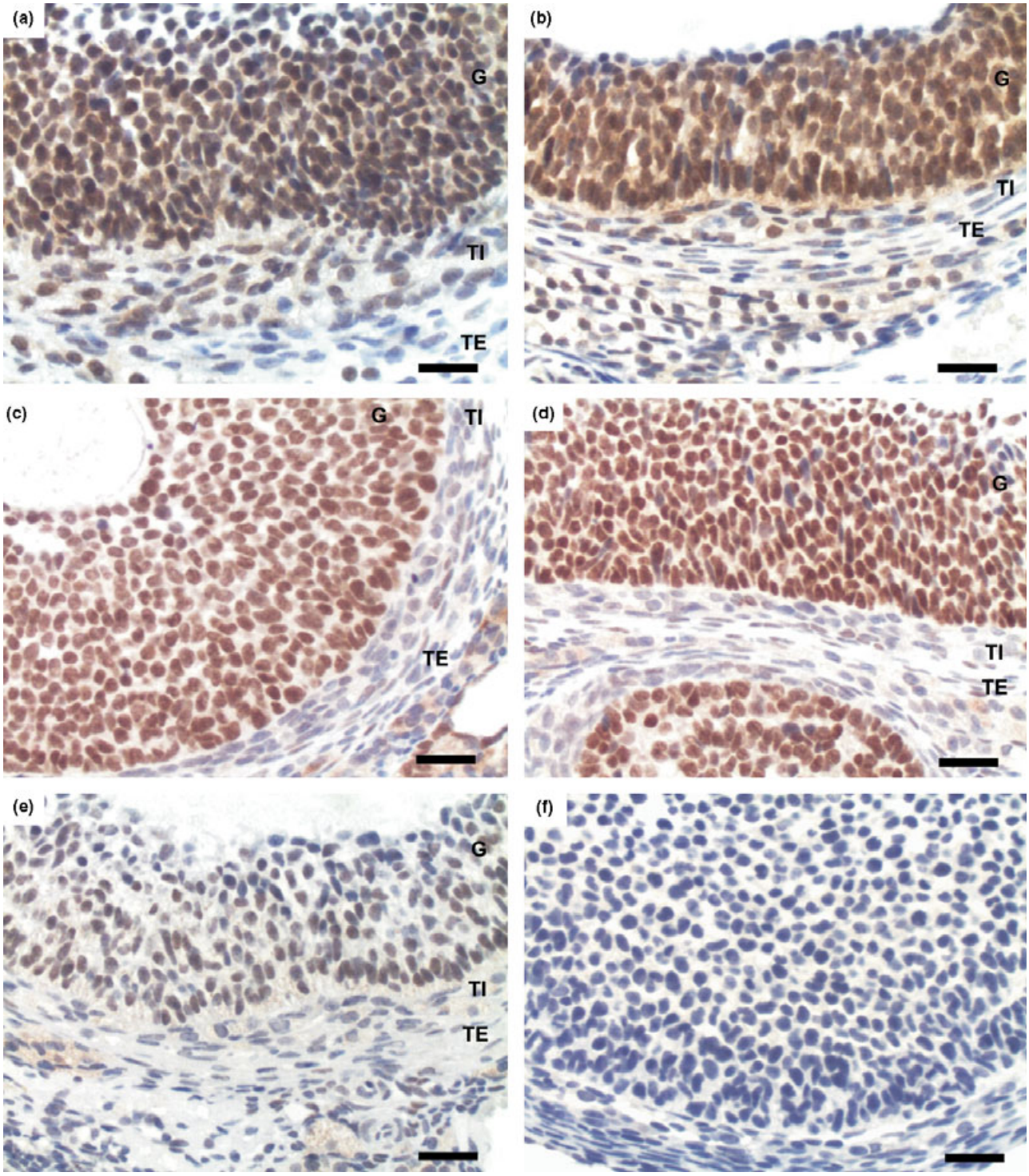

Fig. 1. Expression of ERα and ERβ in the ovary of rats with COD or control as revealed by immunohistochemistry. (a) Follicular wall of a healthy tertiary follicle from a control ovary immunostained with ERα antibody. (b) ERα immunodetection in the cystic follicular wall from an animal with COD. (c) ERβ immunostaining in the wall of a medium secondary follicle from control ovary. (d) Localization of ERβ in the follicular wall of a tertiary follicle in a control ovary. (e) Immunodetection of ERβ in the cystic follicular wall from a treated animal. (f) Negative control omitting the primary antibodies. Streptavidin–biotin method, Mayer's hematoxylin counterstain. G: granulosa; TI: theca interna; TE: theca externa. Scale bars represent 25 μm

showed a lower expression of ERβ in Group II (Figure 1; Table 4). ERβ was detected as a band in the 55-kDa zone of the nitrocellulose membrane (Figure 3). The protein expression of this receptor was lower in the treated group than in the control group.

PR

PR protein was observed in the nuclei of different ovarian cell groups, namely granulosa and theca interna cells of growing, atretic and cystic follicles. Theca

Table 3. Percentage of ERα immunostained area in the wall of different follicular categories in GI (control) and GII (light exposed rats)*

	Granulosa	Theca interna
GI		
Primary follicles	62.70 ± 9.99[a]	-
Small secondary follicles	30.60 ± 11.99[b]	-
Medium secondary follicles	30.40 ± 3.40[b]	26.20 ± 2.08[fh]
Large secondary follicles	19.13 ± 8.11[b]	18.97 ± 4.12[fh]
Tertiary follicles	21.79 ± 2.36[c]	27.06 ± 1.87[f]
Atretic follicles type I	17.13 ± 2.24[c]	19.15 ± 6.54[fh]
Atretic follicles type II	22.86 ± 2.87[c]	23.24 ± 3.58[fh]
Atretic follicles type III	21.77 ± 5.22[c]	20.67 ± 3.85[fh]
GII		
Primary follicles	61.80 ± 11.77[a]	-
Small secondary follicles	47.09 ± 16.71[de]	-
Medium secondary follicles	47.22 ± 8.72[de]	27.78 ± 7.81[fh]
Large secondary follicles	27.98 ± 2.75[ce]	16.41 ± 7.75[fh]
Tertiary follicles	50.58 ± 7.75[de]	15.42 ± 3.34[h]
Cystic follicles	39.07 ± 3.43[e]	21.02 ± 3.08[fh]
Atretic follicles type I	15.67 ± 0.62[c]	23.21 ± 6.28f[gh]
Atretic follicles type II	52.29 ± 3.77[e]	30.66 ± 13.64[gh]
Atretic follicles type III	14.96 ± 10.81[c]	32.65 ± 24.78[gh]

*Values represent mean ± SEM.
[a-h]Within a column, means with no common letters are significantly different ($p < 0.05$).

externa layer had weak or absent immunostaining. In addition, PR was detected in the lining epithelium, interstitial gland and CL cells. A weak cytoplasmic immunostaining was observed in the granulosa cells of the two groups. In the same way as ERα, this intensity of staining was considered as part of the background. The expression of PR was lower in the granulosa cell layer of tertiary and cystic follicles from Group II when compared with the tertiary follicles of control group. Theca interna layer had less intense immunostaining in the tertiary follicles of the treated group ($p < 0.05$) (Figure 2; Table 5). PR showed three isoforms corresponding to PR-B (116 kDa), PR-A (94 kDa) and PR-C (60 kDa). Although there were no differences in the expression of PR-B, there was variation in the other two isoforms; the expression of PR-A was higher in the treated group than in the control group, whereas the expression of PR-C was lower in the treated group (Figure 4).

HSP70

HSP70 was detected in all the cells that integrate the ovary in the nucleus like in the cytoplasm. In the control group, higher expression of HSP70 was detected in follicles that presented some grade of atresia, especially in the categories of atretic follicles I and II. Corpus luteum in regression showed intense immunostaining. Within the follicles, granulosa cells presented more intense staining. In the treated group, intense immunostaining was observed in the cells of cystic follicles. Atretic follicles in the treated group presented a higher immunoexpression with regard to the same categories in the control group ($p < 0.05$) (Figure 2; Table 6). By Western blotting, HSP70 protein was detected in a zone of 70-kDa. Ovarian samples from control group showed higher expression of HSP70 protein than those from the treated group (Figure 5).

Discussion

In the present study, the distribution and relative amounts of ERα, ERβ, PR and HSP70 in rats with induced COD were investigated by immunodetection methods.

A decrease in the expression of ERβ in the granulosa cell layer of growing, atretic and cystic follicles was

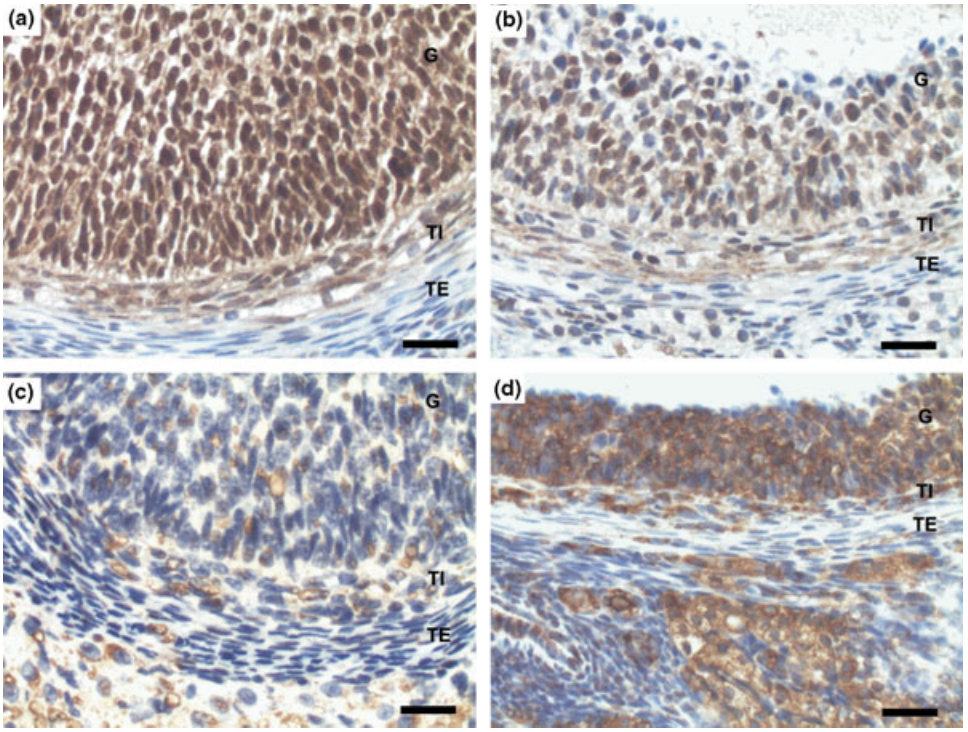

Fig. 2. Expression of PR and HSP70 in the ovary of rats with COD or control by immunohistochemistry. (a) Microphotography shows PR staining in the follicular wall of a tertiary follicle from a control ovary; (b) follicular wall of an ovarian cyst from an animal with COD. Immunostaining is observed in the nuclei like in the cytoplasm of the granulosa and theca cells. (c) HSP70 expression in the normal follicular wall showing weak cytoplasmatic immunostaining in all layers. (d) Wall of an ovarian cyst that shows very intense immunostaining in the granulosa and theca interna cells. Streptavidin–biotin method, Mayer's hematoxylin counterstain. G: granulosa; TI: theca interna; TE: theca externa. Scale bars represent 25 μm

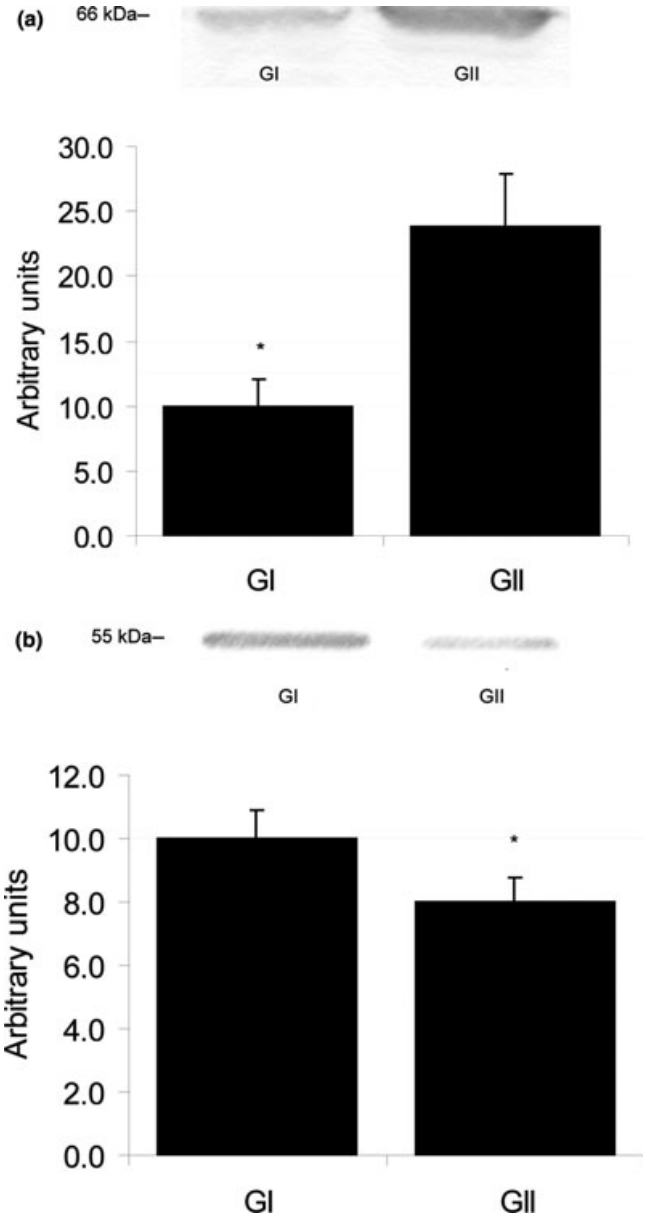

Fig. 3. Western blot analysis of the expression of ER in rat ovarian tissue extracts. (a) Quantitative analysis of ovarian ERα expression between treated and control groups. In the superior part is observed representative immunoblots from the analysis. (b) Quantitative analysis of ovarian ERβ expression between COD and normal animals. Up is observed representative immunoblots from the analysis. GI: ovaries from control rats (n = 5); GII: ovaries from rats with COD (n = 10); * significant differences at $p < 0.05$

Table 4. Percentage of ERβ immunostained area in the wall of different follicular categories in GI (control) and GII (light exposed rats)*

	Granulosa
GI	
Primary follicles	38.27 ± 4.02[a]
Small secondary follicles	53.78 ± 2.99[b]
Medium secondary follicles	60.69 ± 1.45[c]
Large secondary follicles	67.07 ± 2.14[c]
Tertiary follicles	65.20 ± 2.58[c]
Atretic follicles type I	48.10 ± 7.22[b]
Atretic follicles type II	22.45 ± 4.33[d]
Atretic follicles type III	4.77 ± 1.37[eg]
GII	
Primary follicles	21.12 ± 8.10[d]
Small secondary follicles	36.26 ± 8.37[a]
Medium secondary follicles	26.23 ± 5.90[d]
Large secondary follicles	26.83 ± 8.24[d]
Tertiary follicles	18.80 ± 5.05[f]
Cystic follicles	0.85 ± 0.28[g]
Atretic follicles type I	29.39 ± 5.48[a]
Atretic follicles type II	7.11 ± 3.73[e]
Atretic follicles type III	5.23 ± 2.40[eg]

*Values represent mean ± SEM.
[a-g]Within a column, means with no common letters are significantly different ($p < 0.05$).

Table 5. Percentage of PR immunostained area in the wall of different follicular categories in GI (control) and GII (light exposed rats)*

	Granulosa	Theca interna
GI		
Primary follicles	8.62 ± 2.17[a]	-
Small secondary follicles	1.86 ± 1.46[a]	-
Medium secondary follicles	26.40 ± 3.46[c]	1.99 ± 0.39[d]
Large secondary follicles	36.93 ± 11.51[c]	6.13 ± 3.09[de]
Tertiary follicles	35.45 ± 5.52[c]	10.29 ± 2.75[e]
Atretic follicles type I	39.32 ± 7.23[c]	1.59 ± 0.89[d]
Atretic follicles type II	20.27 ± 5.89[c]	1.40 ± 0.91[d]
Atretic follicles type III	22.35 ± 7.17[c]	4.25 ± 1.39[d]
GII		
Primary follicles	0.01 ± 0.01[a]	-
Small secondary follicles	0.19 ± 0.07[a]	-
Medium secondary follicles	26.89 ± 6.82[b]	1.08 ± 0.39[d]
Large secondary follicles	29.38 ± 17.75[bc]	1.89 ± 1.31[de]
Tertiary follicles	17.24 ± 5.04[a]	2.43 ± 0.89[d]
Cystic follicles	7.47 ± 3.02[a]	2.85 ± 0.97[d]
Atretic follicles type I	24.64 ± 9.18[c]	7.15 ± 4.05[de]
Atretic follicles type II	17.59 ± 9.58[c]	9.54 ± 5.93[de]
Atretic follicles type III	15.43 ± 7.44[a]	2.25 ± 1.02[d]

*Values represent mean ± SEM.
[a-e]Within a column, means with no common letters are significantly different ($p < 0.05$).

observed in animals with COD as compared with that in normal animals, concomitant with a decrease in the whole ERβ protein in treated animals. In relation to other follicular categories, a high expression of ERα was also found in granulosa and theca interna of cystic follicles. Total ERα found in the ovaries of COD animals by Western blotting agrees with the results obtained by immunohistochemistry. The results on ER immunohistochemistry in rats concur with that founded in bovines with COD (Salvetti et al. 2007). In women with COD, Jakimiuk et al. (2002) showed a decrease in the expression of ERβ mRNA and protein in both granulosa and theca cells in follicles derived from subjects with COD in comparison with size-matched control follicles. In relation to ERα, the only difference identified was a remarkable increase in protein expression in theca cells of cysts, coinciding partially with the results of the present study.

Numerous studies have shown that gonadotropins and estrogen down-regulate granulosa expression of ERβ isoform (Byers et al. 1997; Sharma et al. 1999), and that both estrogen receptors show a tendency to up-regulate together with increasing estrogen levels in the follicular fluid; such up-regulation is correlated to an

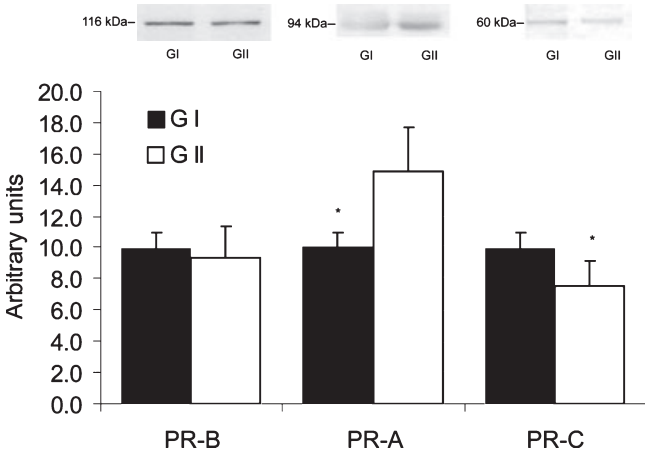

Fig. 4. Western blot analysis of the expression of PR in rat ovarian tissue extracts. Quantitative analysis of ovarian PR expression between groups is shown. In the superior part is observed representative immunoblots from the analysis corresponding to the different isoforms of the protein. GI: ovaries from control rats (n = 5); GII: ovaries from rats with COD (n = 10); *significant differences at $p < 0.05$

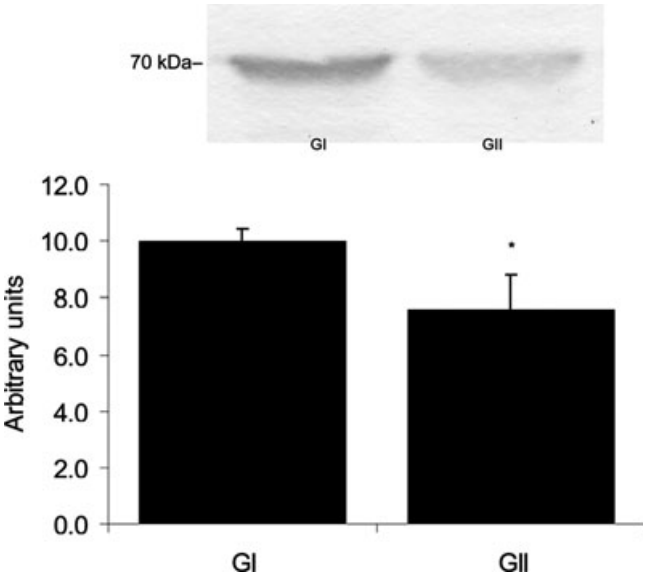

Fig. 5. Western blot analysis of rat ovaries showing bands corresponding to HSP70. The graphics shows the quantitative analysis of ovarian HSP70 expression between groups. GI: control ovaries (n = 5); GII: ovaries from rats with COD (n = 10); *significant differences at $p < 0.05$

Table 6. HSP70 IHCSA in different layers in the wall of different follicular categories in GI (control) and GII (light exposed rats)*

	Granulosa	Theca interna	Theca externa
GI			
Primary follicles	5.02 ± 4.78^{abc}	-	-
Small secondary follicles	0.62 ± 0.36^{a}	-	-
Medium secondary follicles	2.00 ± 0.54^{ab}	0.44 ± 0.11^{e}	0.60 ± 0.26
Large secondary follicles	0.96 ± 0.59^{a}	0.93 ± 0.32^{e}	0.10 ± 0.07
Tertiary follicles	4.15 ± 0.79^{ab}	1.73 ± 0.39^{e}	0.51 ± 0.17
Atretic follicles type I	9.32 ± 3.51^{abc}	4.21 ± 3.70^{efgh}	0.29 ± 0.24
Atretic follicles type II	9.60 ± 2.73^{abc}	3.78 ± 1.02^{efg}	0.27 ± 0.08
Atretic follicles type III	0.38 ± 0.08^{a}	3.22 ± 1.50^{ef}	0.18 ± 0.08
GII			
Primary follicles	8.51 ± 2.62^{abc}	-	-
Small secondary follicles	5.24 ± 3.27^{abc}	-	-
Medium secondary follicles	5.90 ± 1.08^{abc}	1.41 ± 0.32^{e}	0.38 ± 0.16
Large secondary follicles	2.09 ± 0.63^{ab}	3.06 ± 1.01^{ef}	0.18 ± 0.11
Tertiary follicles	8.89 ± 1.77^{abc}	7.03 ± 1.49^{efgh}	0.62 ± 0.16
Cystic follicles	13.84 ± 1.85^{c}	13.49 ± 0.84^{hi}	0.72 ± 0.10
Atretic follicles type I	10.82 ± 3.12^{bc}	$9.38 \pm 1.31e^{fgh}$	1.39 ± 0.53
Atretic follicles type II	22.60 ± 9.00^{d}	12.81 ± 4.23^{ghi}	1.36 ± 0.46
Atretic follicles type III	11.40 ± 5.46^{bc}	12.30 ± 5.11^{fghi}	0.90 ± 0.66

*Values represent mean ± SEM.
[a-i]Within a column, means with no common letters are significantly different ($p < 0.05$).

up-regulation in LH and FSH receptors (Berisha et al. 2002). Although there were no changes in the levels of serum gonadotropins in animals with COD, their constant levels (without recurrent variation in the characteristics of the estrus cycle) could have affected the expression of these receptors. The change in the concentration of different types of receptors in granulosa and theca cells of follicles from animals with COD could alter the ERα/ERβ ratio, causing modifications in the action or effects of estrogen on its target cells. The consequences of these changes remain to be determined; however, because ERα and ERβ have different sensitivities to 17-β-estradiol and there is the possibility for heterodimer formation to occur, even little change in the ERα/ERβ ratio may perturb normal follicle development (Pettersson et al. 1997). Then, the effects of estradiol could be an alteration in the balance of proliferation/apoptosis, an alteration in the expression of gonadotropin receptors, disturbance of enzyme action and metabolism, and others (Isobe and Yoshimura 2000a,b; Calder et al. 2001), which could affect the normal development of female cyclicity with the growth of persistent follicles.

The total expression of PR was lower in the granulosa cell layer of tertiary and cystic follicles in animals with COD, whereas theca interna layer had less intense immunostaining in tertiary follicles of this group. In rats, the LH surge acts directly to induce transient expression of PR mRNA and protein in differentiated rat granulosa cells, expressing elevated levels of LH receptor and aromatase (Natraj and Richards 1993). The absence of LH surge could explain the decrease of the levels of total PR in animals with COD.

Phenotypic studies of the PR Knockout (PRKO) mouse model in which the expression of all isoforms was inhibited provided convincing evidence of an essential role of PR in many aspects of female reproduction. Female mice lacking PRs exhibit impaired sexual behavior and neuroendocrine gonadotropin regulation, anovulation, uterine dysfunction and altered pregnancy-associated mammary gland morphogenesis (Lydon et al. 1995; Mulac-Jericevic and Conneely 2004). Indeed, in PRKO mouse, mature follicles do not ovulate or luteinize 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). Apparently, in PRKO mice, the expression of two metalloproteinases is inhibited in the

mural granulosa cells of mature follicles. One of these proteases is essential for ovulation, suggesting that this protein may represent a critical mediator of the progesterone-induced ovulatory event (Mulac-Jericevic and Conneely 2004). The reduced levels of total PR in cysts and tertiary follicles of animals with COD could indicate a reduced expression of the enzymes necessary for ovulation; this prevents ovulation and consequently contributes to cyst formation and persistence.

Animals with COD presented a higher expression of PR-A and a lower expression of PR-C in their ovaries; there were no variations detected in PR-B. PR-A is a transcriptional inhibitor of all steroid hormone receptors and a facilitator of ligand-dependent cross-talk among signaling pathways of sex steroid receptors within the cell. PR-B appears to be the transcriptional activator of progesterone-responsive genes. Thus, the PR-A/PR-B ratio in specific cell types defines the physiologic response to progesterone (Ogle 2002). There is very little information on the PR-C form. In rats, PR-C is predominantly cytosolically located after tissue homogenization; it has no transcriptional activity by itself and may render the transcriptional capabilities of PR-A and PR-B less efficient (Ogle 2002). Changes in the ratio of PR isoform expression could regulate the biological activity of progesterone and result in functional hormone withdrawal in the absence of changes in serum concentrations or total progesterone-binding activity of the reproductive tissues (Goldman et al. 2005).

The obtained results may indicate that, although the levels of total PR are lower in animals with COD, there is a high expression of PR-A isoform, which could inhibit the activity of PR-B in tertiary and cystic follicles with the consequent inhibition of the effects of progesterone on these structures.

Turning to HSP70, the present study has shown that these proteins are expressed in the follicular cells of rat ovary, but there are differences in their expression between groups and follicular types. Although HSP70 immunoreactivity was observed in all follicular cells of the categories studied, the staining intensity was stronger in granulosa and theca cells in atretic and cystic follicles. Western blot analysis showed high expression of this protein in the control ovaries, probably because of the presence of corpus luteum in regression (Khanna et al. 1995). It is necessary to highlight that there were no previous studies that showed works that indicate the normal expression of this protein in the ovarian follicles in mammals. HSP90 and HSP70 have been demonstrated to bind steroid receptors and modulate their function. HSP90 has been proposed to bind to the HBD of unoccupied receptors forming heterocomplex with HSP70, HSP40, p23, etc., and to maintain them in an inactive state (Pratt and Toft 2003). Subsequent binding of ligand to the receptor triggers the release of HSP90 and HSP70 from the receptor heterocomplex and reverses the repression. On the contrary, it has been shown that HSP90/HSP70 machinery is necessary to maintain appropriate conformation required for hormone-binding activity of the receptor (Koshiyama et al. 1995; Pratt and Toft 2003). Because the proliferation of growing follicles in proestrus is believed to be one of the responses to sex steroids, HSP70 seems to act as a repressor of the steroidal effects. Atretic and cystic follicles showed very low index of proliferation (data not published) with high levels of HSP70 immunoexpression. An increase in HSP70 expression was detected in cystic follicles according to a lower expression of ERβ and PR. Edwards et al. (1992) reported that overexpression of HSP70 by heat shock results in a marked reduction in the concentrations of PRs in breast cancer cells. Although HSP70 may be involved directly or indirectly in the down-regulation of sex steroid receptors, the detailed intracellular mechanism of down-regulation of ER and PR remains to be determined.

In summary, ovaries from Wistar rats with COD exhibited an altered steroid receptor expression associated with an increase in the HSP70 expression in ovarian cysts as compared with control rats. Further studies are necessary to fully understand and appreciate the implications of these observations.

Acknowledgements

We are very grateful to the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases and Dr. AF Parlow (Torrance, CA, USA) for the RIA kits, and to Dr. D Becu-Villalobos [National Scientific Research Council (CONICET), Argentina] for valuable RIA contribution. This study was supported by grants from the National University of Litoral (CAID Program 2002). HHO and EJG are Research Career Members, and BED, CB, FR and NRS are Fellows of CONICET.

References

Beato M, Klug J, 2000: Steroid hormone receptors: an update. Hum Reprod Update **6,** 225–236.

Berisha B, Pfaffl M, Schams D, 2002: Expression of estrogen and progesterone receptors in the bovine ovary during estrous cycle and pregnancy. Endocrine **17,** 207–214.

Bramley T, 2003: Non-genomic progesterone receptors in the mammalian ovary: some unresolved issues. Reproduction **125,** 3–15.

Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK, 1997: Estrogen receptor-β mRNA expression in rat ovary: down-regulation by gonadotropins. Mol Endocrinol **11,** 172–182.

Calder MD, Manikkam M, Salfen BE, Youngquist RS, Lubahn DB, Lamberson WR, Garverick HA, 2001: Dominant bovine ovarian follicular cysts express increased levels of messenger RNAs for luteinizing hormone receptor and 3-β-hydroxysteroid dehydrogenase $\delta(4)$, $\delta(5)$ isomerase compared to normal dominant follicles. Biol Reprod **65,** 471–476.

D'Haeseleer M, Van Poucke M, Van den Broeck W, 2005: Cell-specific localization of oestrogen receptor β (ESR2) mRNA within various bovine ovarian cell types using in situ hybridization. Anat Histol Embryol **34,** 265–272.

Drummond AE, Britt KL, Dyson M, Jones ME, Kerr JB, O'donnell L, Simpson ER, Findlay JK, 2002: Ovarian steroid receptors and their role in ovarian function. Mol Cell Endocrinol **191,** 27–33.

Edwards DP, Estes PA, Fadok VA, Bona BJ, Onate S, Nordeen SK, Welch WJ, 1992: Heat shock alters the composition of heteromeric steroid receptor complexes and enhances receptor activity in vivo. Biochemistry **31,** 2482–2491.

Gava N, Clarke CL, Byth K, Arnett-Mansfield RL, de Fazio A, 2004: Expression of progesterone receptors A and B in

the mouse ovary during the estrous cycle. Endocrinology **145,** 3487–3494.
Goldman S, Weiss A, Almalah I, Shalev E, 2005: Progesterone receptor expression in human decidua and fetal membranes before and after contractions, possible mechanism for functional progesterone withdrawal. Mol Hum Reprod **11,** 29–77.
Hsueh AJW, Billig H, Tsafriri A, 1994: Ovarian follicle atresia: a hormonally controlled apoptotic process. Endocr Rev **15,** 707–724.
International Committee on Veterinary Histological Nomenclature, 1994: Nomina Histologica, 2nd edn. Cornell, New York, p. 38.
Isobe N, Yoshimura Y, 2000a: Immunocytochemical study of cell proliferation in the cystic ovarian follicles in cows. Theriogenology **54,** 1159–1169.
Isobe N, Yoshimura Y, 2000b: Localization of apoptotic cells in the cystic ovarian follicles of cows: a DNA-end labeling histochemical study. Theriogenology **53,** 897–904.
Jakimiuk AJ, Weitsman SR, Yen HW, Bogusiewicz M, Magoffin DA, 2002: Estrogen receptor α and β expression in theca and granulosa cells from women with polycystic ovary syndrome. J Clin Endocrinol Metab **87,** 5532–5538.
Khanna A, Aten RF, Behrman HR, 1995: Heat shock protein-70 induction mediates luteal regression in the rat. Mol Endocrinol **9,** 1431–1440.
Koshiyama M, Konishi I, Nanbu K, Nanbu Y, Mandai M, Komatsu T, Yamamoto S, Mori T, Fujii S, 1995: Immunohistochemical localization of heat shock proteins HSP70 and HSP90 in the human endometrium: correlation with sex steroid receptors and Ki-67 antigen expression. J Clin Endocrinol Metab **80,** 1106–1112.
Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA, 1996: Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA **93,** 5925–5930.
Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, OMalley BW, 1995: Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev **9,** 2266–2278.
Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B, 2000: The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol **20,** 7146–7159.
Mulac-Jericevic B, Conneely OM, 2004: Reproductive tissue selective actions of progesterone receptors. Reproduction **128,** 139–146.
National Research Council, 1996: Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington.
Natraj V, Richards JS, 1993: Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology **133,** 761–769.
Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS, 2000: The role of heat shock proteins in reproduction. Hum Reprod Update **6,** 149–159.
Ogle TF, 2002: Progesterone-action in the decidual mesometrium of pregnancy. Steroids **67,** 1–14.
Ortega HH, Salvetti NR, Müller LA, Amable P, Lorente JA, Barbeito CG, Gimeno EJ, 2007: Characterization of cytoskeletal proteins in follicular structures of cows with cystic ovarian disease. J Comp Pathol **136,** 222–230.
Pelletier G, Labrie C, Labrie F, 2000: Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs. J Endocrinol **165,** 359–370.
Pettersson K, Grandien K, Kuiper GG, Gustafsson JA, 1997: Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol **11,** 1486–1496.
Pratt WB, Toft DO, 1997: Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev **18,** 306–360.
Pratt WB, Toft DO, 2003: Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med **228,** 111–133.
Ranefall P, Wester K, Bengtsson E, 1998: Automatic quantification of immunohistochemically stained cell nuclei using unsupervised image analysis. Anal Cell Pathol **16,** 29–43.
Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA, Lubahn DB, 1999: Cloning, sequencing, and localization of bovine estrogen receptor-β within the ovarian follicle. Biol Reprod **60,** 691–697.
Salvetti NR, Canal AM, Gimeno EJ, Ortega HH, 2004: Polycystic ovarian syndrome: temporal characterization of the induction and reversion process in an experimental model. Braz J Vet Res Anim Sci **41,** 389–395.
Salvetti NR, Muller LA, Acosta JC, Gimeno JE, Ortega HH, 2007: Estrogen receptors α and β and progesterone receptors in ovarian follicles of cows with cystic ovarian disease. Vet Pathol **44,** 373–378.
Sar M, Welsch F, 1999: Differential expression of estrogen receptor-β and estrogen receptor-α in the rat ovary. Endocrinology **140,** 963–971.
Sharma SC, Clemens JW, Pisarska MD, Richards JS, 1999: Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. Endocrinology **140,** 4320–4334.
Silvia WJ, Hatler TB, Nugent AM, Laranja da Fonseca LF, 2002: Ovarian follicular cysts in dairy cows: an abnormality in folliculogenesis. Domest Anim Endocrinol **23,** 167–177.
Svensson EC, Markstrom E, Andersson M, Billig H, 2000: Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin. Biol Reprod **63,** 1457–1464.
Van den Broeck W, Coryn M, Simoens P, Lauwers H, 2002a: Cell-specific distribution of oestrogen receptor-α in the bovine ovary. Reprod Domest Anim **37,** 291–293.
Van den Broeck W, D'haeseleer M, Coryn M, Simoens P, 2002b: Cell-specific distribution of progesterone receptors in the bovine ovary. Reprod Domest Anim **37,** 314–320.
Vanholder T, Opsomer G, de Kruif A, 2006: Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev **46,** 105–119.
Wang H, Masironi B, Eriksson H, Sahlin L, 1999: A comparative study of estrogen receptors α and β in the rat uterus. Biol Reprod **61,** 955–964.
Wang H, Eriksson H, Sahlin L, 2000: Estrogen receptors α and β in the female reproductive tract of the rat during the estrous cycle. Biol Reprod **63,** 1331–1340.
Wei LL, Gonzalez-Aller C, Wood WM, Miller LA, Horwitz KB, 1990: 5′-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated 'C'-receptor and unique A-receptor messages. Mol Endocrinol **4,** 1833–1840.

Submitted: 12 Nov 2007

Author's address (for correspondence): Dr. Hugo H Ortega, Department of Anatomy and Histology, Faculty of Veterinary Sciences, National University of Litoral, R.P. Kreder 2805 (3080) Esperanza, Santa Fe, Argentina. E-mail: hhortega@fcv.unl.edu.ar