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Expression of α and β Estrogen Receptors in the Chicken Ovary*

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The role of estrogens in hen reproduction is well established. However, the distribution of estrogen receptors in the chicken ovary is unknown. Therefore, the mRNA expression of α (ERα) and β (ERβ) estrogen receptors was examined within the ovaries of laying hens. Expression of ERs was determined by RT-PCR analysis. The presence of ERα and ERβ mRNAs was found in the ovarian stroma and white, yellowish, small yellow and the largest preovulatory (F3-F1) follicles. ERα and ERβ mRNAs were detected in the granulosa and theca layers of the walls of preovulatory follicles. The expression of ERα mRNA was markedly higher than ERβ mRNA in all examined ovarian compartments. Within the ovary, the relative expression of both ER mRNAs depends on the follicular diameter and the layer of the follicular wall. The results demonstrate the expression of both ERα mRNA and ERβ mRNA in all compartments of the chicken ovary, suggesting different pathways of estrogen action in the avian ovary. Much higher expression of ERα mRNA indicates that this form of estrogen receptor is predominant in the chicken ovary. The clarification of the mechanism of ERα and ERβ participation in the ovarian functions of birds necessitates further experiments examining ERs at the protein level.

Key words: Estrogen receptors, RT-PCR, ovary, chicken.

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Unlike mammals, estrogens in birds are synthesised by the theca cells of the ovarian follicles. Ovarian stroma with numerous cortical follicles and white follicles produce primarily ovarian estrogens, whereas large preovulatory yellow follicles are a source of ovarian progesterone (HUANG *et al.* 1979; RZĄSA *et al.* 2002; HRABIA *et al*. 2004a; SECHMAN *et al*. 2004).

Estrogens are key regulators of reproductive functions in birds such as gonadal differentiation and development, reproductive behaviour, synthesis of egg yolk proteins in the liver, egg white proteins in the oviduct and mobilisation of calcium for egg shell formation.

Estrogen actions are mediated by two different types of intracellular receptors, α (ERα) and β (ERβ) (SEGARS & DRIGGERS 2002). These receptors possess three major functional domains. The N terminal A/B domain contains the ligand independent transactivation function 1 (AP-1) and is a region of site specific phosphorylation by mitogen activated protein kinase (MAPK) pathways in response to growth factors and by cyclin A2-CDK2. The central DNA binding domain (DBD) acts by tightly binding the receptor to DNA. The ligand binding domain (LBD) contains the activation function-2 (AF-2) (see JACOB *et al*. 2006 for review). These receptors function primarily as ligand-activated transcription factors increasing gene transcription by direct binding of the receptors to a specific DNA target sequence (HALL *et al*. 2001; NILSSON *et al*. 2001; SEGARS & DRIGGERS 2002). Moreover, ERs act through AP-1 and SP-1, affecting transcription (SEGARS & DRIGGERS 2002). In addition, ligand-dependent or ligand-independent ER activation has been shown to be accomplished by compounds other than steroidal estrogens, such as cAMP, dopamine and growth factors through signaling pathways that involve cytoplasmic proteins or protein kinases (DRIGGERS & SEGARS 2002).

Information concerning the expression of ERs in the avian ovary is scarce. The presence of two forms of chicken ERα protein has been demon-

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strated in non-compartmented chicken ovary (GRIFFIN *et al*. 1999). Expression of ERα and ERβ mRNA in ovarian white follicles was shown in quail (ICHIKAWA *et al*. 2003). Moreover, the presence of ERα mRNA in different compartments of the laying quail ovary has been detected (HRABIA *et al*. 2004b). Recently, NI *et al*. (2007) showed ERβ mRNA in the ovary of prepubertal ducks. So far, there has been no study on the distribution of both ERs in the chicken ovary. Therefore, the present study was conducted in order to examine by RT-PCR the expression of ERα and ERβ in the ovarian stroma and the walls of all follicles in the chicken.

Material and Methods

Birds and chemicals

The experiment was conducted according to a research protocol approved by the Local Animal Ethics Committee. Hy-Line laying hens (layer strain) were purchased from the commercial farm Drobeco (Palowice, Poland). Chickens were caged individually under a photoperiod of 14L:10D (lights-on at 0800 h and off at 2200 h) with free access to water and commercial food. On the basis of recording of oviposition time, cloacal palpation and autopsy, it was found that ovulation was about 5 min after oviposition of the previous egg in the series. At the age of 40 weeks the birds were killed by decapitation (n=6) 2 h after ovulation, and the ovaries were removed, placed on ice and the following ovarian compartments were isolated: stroma with cortical follicles <1 mm in diameter (STR), white follicles (>1-4 mm; WF), yellowish follicles (>4-8 mm; YF), small yellow follicles (>8-12 mm; SYF) and 3 the largest yellow preovulatory follicles F3-F1 (F3<F2<F1). The granulosa (G) and theca (T) layers were separated from preovulatory follicles. Tissues were quickly frozen in liquid nitrogen and stored until total RNA extraction.

The chemicals were purchased from the following companies: TRI-reagent (MRC, Inc., Cincinnati, OH, USA), RevertAid M-MuLV Reverse Transcriptase, Ribonuclease inhibitor, dNTP mix, $MgCl_2$, Pol Taq DNA Polymerase, buffers, molecular weight marker – 100 bp DNA ladder (Fermentas, Vilnius, Lithuania), primers, oligo-dT_{18} (IBB, Warszawa, Poland). All other reagents were obtained from ICN Biomedicals (Aurora, IL, USA), Sigma (St. Louis, MO, USA) or POCH (Gliwice, Poland).

Total RNA isolation and RT-PCR analysis

Total RNA was extracted from the ovarian tissues using the TRI-reagent according to the manufacturer's recommendations. Total RNAs (5 μg) from each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U) and oligo-dT_{18} primers (0.5 μg). As a negative control untranscribed tissue RNA (reverse transcriptase omitted) was used. RT products (1 μl) were amplified in a Thermocycler Gradient (Eppendorf, Germany) in a 12.5 μl reaction mixture containing 1.25 μl of buffer (100 mmol Tris-HCl, pH 8.8, 500 mmol KCl, 0.8% Nonidet P40), 0.312 unit pol Taq DNA polymerase, 0.2 μmol sense and antisense primers, 0.2 mmol each dNTP, 1.5 mmol $MgCl_2$, and water. After the initial denaturation for 5 min at 95°C (ERα, 18S) or 4 min at 94°C (ERβ), amplifications profiles were applied as shown in Table 1. Amplifications were completed with an additional extension at 72°C for 7 min. Negative control (water) was included in all reactions. The primers for ERα, ERβ and 18S rRNA are described in Table 1. All PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide in 0.5x TBE buffer. The gel was examined under UV light and photographed with a digital camera. The net intensities of individual bands were measured using the Scion Image for Windows. The ratios of net intensity of examined genes to 18S rRNA were used to represent the relative level of

Table 1

Characteristics of primers and PCR conditions used in this study

Gene	GenBank	Primer sequence	PCR product	PCR conditions
ERα	X03805	F: 5'-GTGCCTTAAGTCCATCATCCT-3' R: 5'-GCGTCCAGCATCTCCAGTAAG-3'	300 bp (1522-1821)	95°C 30s, 58°C 30s, 72°C 30s, 30 cycles
ERβ	AB036415	F: 5'-TGATATGCTCCTGGCCATGAC-3' R: 5'-CTTCATGCTCAGCAGATGCTC-3'	304 bp (1374-1677)	94°C 30s, 55°C 30s, 72°C 30s, 30 cycles
18S rRNA	AF173612	F: 5'-CGCGTGCATTTATCAGACCA-3' R: 5'-ACCCGTGGTCACCATGGTA-3'	167 bp (160-326)	94°C 30s, 60°C 30s, 72°C 30s, 30 cycles

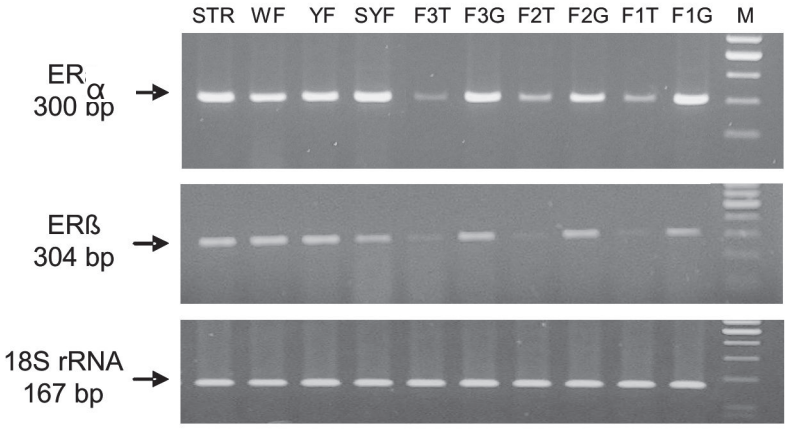

Fig. 1. RT-PCR analyses of estrogen receptor alpha (ERα) and beta (ERβ) gene expression in the chicken ovary. STR – stroma; WF – white follicles; YF – yellowish follicles; SYF – small yellow follicles; F3, F2, F1 – large yellow preovulatory follicles (G – granulosa, T – theca); M – molecular weight marker (100 bp DNA ladder). The data shown is representative of six birds.

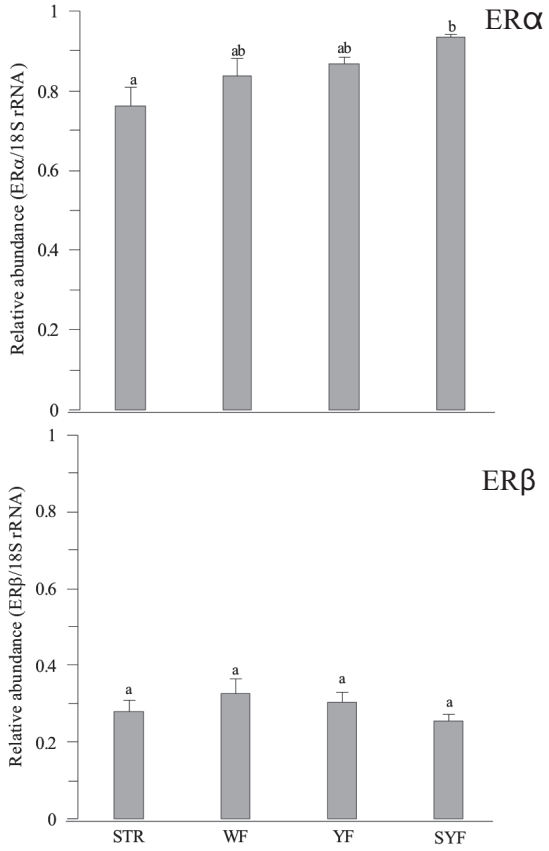

Fig. 2. Relative expression of ERα and ERβ mRNAs in the stroma (STR) and the white (WF), yellowish (YF) and small yellow follicles (SYF) of the chicken ovary. Each value represents the mean ± SEM from 6 determinations that were measured as relative density of RT-PCR products compared to 18S ribosomal RNA. Means with different letters are significantly different from each other ($P<0.05$).

target gene expression. The average abundance of six repeats was used for statistical analysis.

Data were analyzed by ANOVA followed by Tukey test. Significance of differences was considered at the level of $P<0.05$. Results are expressed as the mean ± SEM.

Results

RT-PCR analysis showed the presence of ERα and ERβ mRNAs in all examined ovarian compartments. The products were 300, 304 and 167 bp for ERα mRNA, ERβ mRNA and 18S ribosomal RNA mRNA, respectively, and corresponded to the approximate size for each as predicted (Fig. 1).

The relative level of ERα mRNA was significantly higher that ERβ mRNA in the ovarian stroma (0.76 ± 0.048 vs 0.28 ± 0.029), white (0.84 ± 0.043 vs 0.33 ± 0.036), yellowish (0.87 ± 0.017 vs 0.30 ± 0.028) and small yellow follicles (0.93 ± 0.006 vs 0.26 ± 0.017). Within these examined tissues, significantly higher expression of ERα mRNA was detected in the walls of small yellow follicles than in the stroma. In the case of ERβ mRNA, differences between stroma and the various classes of follicles were insignificant (Fig. 2).

In the granulosa and theca layers of the largest yellow follicles ERα mRNA expression was more abundant than ERβ mRNA (Fig. 3). The relative expression of ERα mRNA was significantly higher in the granulosa layer than in the theca layer in all preovulatory follicles (F3-F1), whereas ERβ mRNA was significantly higher in the granulosa layer of F3 and F2 follicles. The relative expression of ERα mRNA in the granulosa layer of F3-F1 follicles ranged from 0.86 ± 0.034 to 0.96 ± 0.046 and in the theca layer from 0.61 ± 0.05 to 0.65 ± 0.063 and did not differ between follicles. The relative level of ERβ mRNA in the granulosa layer was 0.19 ± 0.022 and 0.19 ± 0.025 in follicles F3 and F2 respectively, and significantly decreased to 0.10 ± 0.025 in the F1 follicle. In the theca layer a very low level of ERβ mRNA in F3 (0.03 ± 0.011) and F2 (0.03 ± 0.006) follicles was detected, but significantly increased in follicle F1 (0.12 ± 0.029) (Fig. 3).

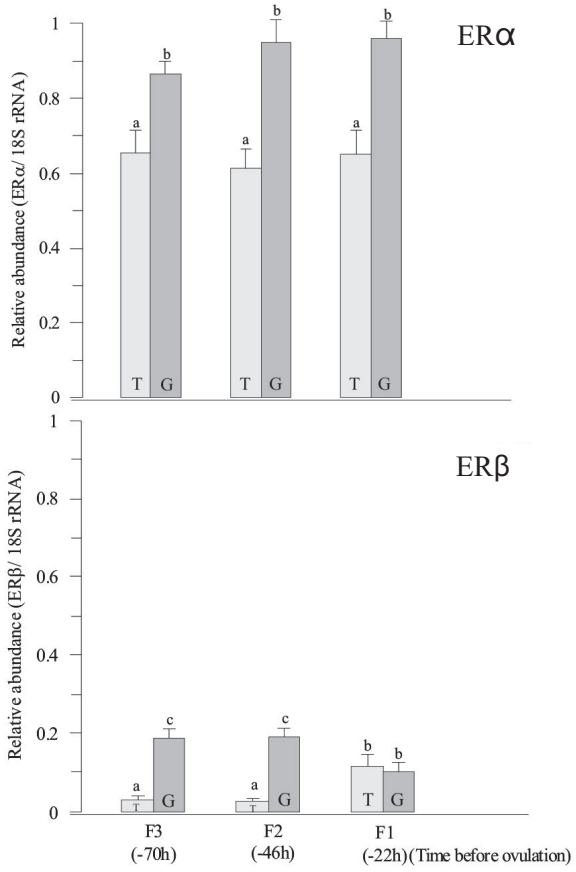

Fig. 3. Relative expression of ERα and ERβ mRNAs in the granulosa (G) and the theca (T) layers of the largest yellow preovulatory follicles F3-F1. Each value represents the mean ± SEM from 6 determinations that were measured as relative density of RT-PCR products compared to 18S ribosomal RNA. Means with different letters are significantly different from each other ($P<0.05$).

Discussion

To our knowledge the current study is the first demonstrating the distribution of ERα and ERβ mRNAs in all compartments of the chicken ovary. The presence of two transcripts of ER indicates different mechanisms of estrogen action in ovarian tissues. However, a markedly higher expression of ERα mRNA suggests that this type of estrogen receptor is predominantly involved in estrogen effects on ovarian functions in chicken. This finding corresponds to previous observations of ICHIKAWA *et al.* (2003) showing higher expression of ERα mRNA than ERβ mRNA in the reproductive tissues of adult quail. In contrary, mammalian ovary expresses mainly ERβ (BYERS *et al.* 1997; COUSE *et al.* 1997).

Estrogens may act as paracrine/autocrine regulators of ovarian functions. In chicken ovarian stroma with cortical follicles and less mature follicles, i.e. white, yellowish and small yellow follicles, the impact of estrogen may be associated with its involvement in the regulation of follicle development and differentiation. This was previously suggested for estrogens and their β receptors in the ovaries of prepubertal ducks (NI *et al.* 2007) and mice (LUBAHN *et al.* 1993; COUSE *et al.* 1997). A local type of estrogen action by ERα and ERβ during follicular development may constitute the prevention of apoptosis and stimulation of proliferation. The involvement of estrogens in these processes in reproductive tissues of birds has been documented (MONROE *et al.* 2000, 2002; JOHNSON & BRIDGEHAM 2002).

A more interesting finding was the observation of higher expression of ER mRNAs in the granulosa layer than in the theca layer of the largest F3-F1 preovulatory follicles. This suggests that estrogens through their receptors participate principally in granulosa layer functions. In contrary to previous observations in quail (HRABIA *et al.* 2004b), there were no changes in ERα mRNA expression in the granulosa layer during follicle development from F3 to F1. This discrepancy may be associated with the collection of tissues at a different stage of the ovulatory cycle of hens. Involvement of estrogens in the local regulation of granulosa function may be their participation in steroidogenesis. It was shown *in vitro* that estradiol significantly increased progesterone secretion by granulosa cells in response to LH (KAMIYOSHI *et al*. 1992; SASANAMI & MORI 1999). Another function of estrogens in granulosa cells may be transcriptional regulation of gene expression of proteins produced by the granulosa layer such as protein of the perivitelline layer in the chicken egg-envelope.

In the case of ERβ mRNA, it was found that its expression in the granulosa layer decreased, but increased in the largest follicle of the theca layer. These changes suggest the involvement of ERβ in the events connected with the final maturation of the F1 follicle. It cannot be excluded that the expression of oocyte-specific 95-kDa very low density lipoprotein/vitellogenin receptor (STIFANI *et al.* 1990) is regulated by estrogens, similarly as ligands for these receptors.

In conclusion, the present results demonstrate the expression of both ERα mRNA and ERβ mRNA in all compartments of the chicken ovary suggesting different pathway sof estrogen action in the avian ovary. Much higher expression of ERα mRNA indicates that this form of estrogen receptor is predominant in the chicken ovary. The clarification of the mechanism of ERα and ERβ participation in the ovarian functions of birds necessitates further experiments examining ERs at the protein level.

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