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Effect of 9-*cis* Retinoic Acid (RA) on Progesterone and Estradiol Secretion and RA Receptor Expression in the Chicken Ovarian Follicles

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Several lines of evidence indicate that retinoids, derivates of vitamin A, affect reproductive function in birds, however, the mechanism of their action in the ovary is still unknown. Therefore, the present study was designed (i) to show whether in the domestic hen 9-*cis* retinoic acid (9-*cis* RA), one of the retinoids, influences steroid secretion *in vitro* by white and yellow chicken ovarian follicles, and (ii) to detect expression of retinoic acid RXR receptor mRNA in these follicles. The white follicles (small: 1-4 mm, medium: 4-6 mm and large 6-8 mm in diameter) and the three largest yellow preovulatory follicles (F3-F1; 25-37 mm) were isolated from the ovary 3 h before ovulation. The granulosa layer was separated from the theca layer in the preovulatory follicles, which were subsequently divided into 4 equal pieces. The isolated whole white follicles or parts of the granulosa or theca layers were incubated for 24 h at 38°C in Eagle's medium in the following 4 groups: control, ovine LH (oLH; 10 ng/ml), 9-*cis* RA (100 ng/ml) and 9-*cis* RA + oLH. After incubation, the medium was collected for estradiol (E_2) and progesterone (P_4) determination while tissues were saved for protein assay. It was found that 9-*cis* RA affects steroid secretion from chicken ovarian follicles. It decreased E_2 secretion from white follicles and from the theca layer of the two largest (F2 and F1) preovulatory follicles. 9-*cis* RA had no effect on oLH-stimulated E_2 secretion by the white follicles and yellow F2 and F1 follicles, but it diminished E_2 secretion by F3 follicles. As regards P_4, the effect of 9-*cis* RA was opposite; it increased P_4 secretion from the granulosa layer of all preovulatory follicles. 9-*cis* RA did not change oLH-stimulated P_4 secretion by granulosa layers of F3 and F2 follicles, however, it inhibited oLH-enhanced P_4 secretion from the F1 granulosa layer. In a separate experiment, the presence of mRNA encoding RXR was found in the stroma and all follicles of the chicken ovary by means of the RT-PCR technique. The results indicate that retinoids, acting by specific nuclear receptors, are modulators of follicular steroidogenesis in the chicken ovary.

Key words: Retinoic acid, ovary, sex hormones, RXR receptor, chicken.

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Only a left ovary is present in female birds because the right one regresses during embryogenesis. The ovary of a laying chicken contains a stroma with primordial follicles (<1 mm), white follicles (1-8 mm), and yellow follicles (>8 mm; 8-36 mm). The large, rapidly growing yolk-filled follicles are arranged in a hierarchy according to size and proximity to ovulation as F1 (the largest) through F5-F7 (the smallest). Ovulation of the largest yellow follicle (F1) occurs every day except during a pause in laying. Also, an additional large white follicle is recruited to the preovulatory hierarchy each day (for review, see JOHNSON 2000).

The ovarian follicle consists of an oocyte and two surrounding layers: (1) the granulosa layer which primarily produces progestins and small amounts of androgens, and (2) the theca layer which is divided into a theca interna and theca externa. Androgens are produced mainly by interstitial cells in the theca interna, while estrogens are produced by the theca externa by cells expressing P450 aromatase (HUANG *et al*. 1979; BAHR *et al.* 1983; LEE & BAHR 1994; KATO *et al.* 1995). The two main steroids synthesized by chicken ovarian follicles are progesterone (P_4) and estradiol (E_2) which are produced by the granulosa and theca layers, respectively (NITTA *et al.* 1991, 1993; LEE & BAHR 1994; LEE *et al.* 1998). The granulosa layer of the white non-hierarchical follicles (below 8 mm in diameter) is steroidogenically incompetent and be-

comes active just prior to entering the follicular hierarchy, while the theca layer is active both in white and yellow follicles (TILLY *et al.* 1991; NITTA *et al.* 1991, 1993; SECHMAN *et al.* 2004; PROSZKOWIEC-WĘGLARZ *et al.* 2005).

Retinoids, derivatives of vitamin A, are involved in a variety of physiological processes, e.g. vision, embryogenesis, cell proliferation, maintenance of numerous tissues and reproduction (for review see CLAGETT-DAME & DELUCA 2002). The pathways for metabolism of retinol into the biologically active retinoids, all-trans retinoic acid (all-trans RA) and 9-cis retinoic acid (9-*cis* RA), involve several families of enzymes with overlapping function (NAPOLI 1999; TRYGGVASON *et al.* 2001). These two isomeric forms of RA are the most relevant to transcriptional regulation. Similarly to steroid and thyroid hormones, RA binds to intracellular nuclear receptors. All-trans RA can bind with high affinity to RAR receptors (RAR α, β, γ) but isomeric 9-*cis* RA is a main ligand for RXR (α, β, γ) and also binds to RAR receptors. Both RARs and RXRs are ligand-inducible regulators that control transcription. RXRs can form homodimers in the presence of 9-*cis* RA or can heterodimeraze with RARs and thyroid hormone receptors (TRs). Retinoic acid receptor belongs to the protein superfamily of nuclear receptors that comprises several transcriptional factors. These proteins have a very conservative structure containing specific domains. Domain AF-1 (region A/B) is an activation domain responsible for activating gene transcription, whereas region C is responsible for binding DNA at the gene promoter (RARE – retinoic acid response element). Domain AF-2 is a C-terminal ligand (RA)-binding region, which is separated by a "hinge" region that may target the receptor to the nucleus (RAGSDALE *et al.* 1991; CHAMBON 1996).

It has been shown that in immature birds administration of vitamin A stimulates development of the ovary and oviduct and advances the onset of the first oviposition (FU *et al.* 2000). On the other hand, in mature chicken a vitamin A-deficient diet leads to a decrease in egg production (BERMUDEZ *et al.* 1993). These data suggest involvement of retinoids in chicken ovarian function, however, the mechanism of this action is not known. Our previous studies *in vitro* demonstrated that the thyroid hormone, triiodothyronine (T_3), affects sex hormone secretion from chicken ovarian follicles (SECHMAN 2003; SECHMAN *et al.* 2005). Moreover, we found the presence of the thyroid hormone receptors, TRα and TRβ, in all compartments of chicken ovary (SECHMAN 2003). Since the RXR receptor heterodimerazes with the TR receptor, it is likely that 9-*cis* RA may also influence the follicular steroidogenesis of the chicken ovary. Therefore, the present study was designed to show whether 9-*cis* RA affects basal and LH-stimulated steroid hormone (P_4 and E_2) secretion from chicken ovarian follicles. Moreover, the presence of RXR receptors in the ovarian follicles was investigated.

Material and Methods

Experiment 1: effect of 9-*cis* RA on *in vitro* ovarian steroid secretion

The experiment was performed on 25 week old White Leghorn laying hens (n=20) kept in individual cages under a 14L:10D lighting regime. Hens received a commercial laying mash and water *ad libitum.* The time of egg oviposition had been recording daily at 15 min intervals between 08:00 h and 15:00 h for two weeks before the day of the experiment in order to establish the time of ovulation for each bird. Hens (n=6), laying eggs in regular sequences of at least 15 eggs per sequence, were killed 3 hrs before oviposition. White follicles (1-8 mm in diameter) and the three largest yellow follicles (F3-F1; 25-37 mm) were removed from the ovary. The white follicles were divided into three groups according to their diameter: 1-4 mm, 4-6 mm and 6-8 mm. From the yellow follicles the granulosa and theca layers were separated by the method of GILBERT *et al*. (1977). Whole white follicles and the granulosa and theca layers of the preovulatory follicles were divided into four equal groups and incubated (38°C, 24 hr) in 1 ml Eagle's medium with antibiotic (Penicilin/Streptomicin Solution; Sigma, USA) and 1% BSA (Sigma, USA) in the following groups: control, ovine LH (oLH; 10 ng/ml), 9-*cis* RA (100 ng/ml; Sigma, USA) and 9-*cis* RA + oLH. Ovine LH (oLH-26; Lot AFP5551B) was received from dr Parlow, National Hormone and Pituitary Program (NIDDK, USA). These incubations were repeated six times. Following incubation, medias were collected and frozen at -20°C till progesterone and estradiol measurement. All tissues were collected and kept at -20°C until protein measurement by the method of LOWRY *et al*. (1951). Sex steroids (E_2 and P_4) in the medium were determined by RIA methods using Spectra kits (Orion Diagnostica, Finland). The detection limits for E_2 and P_4 assays were 5.45 pg/ml and 90 pg/ml, respectively, while recoveries equaled 99.5% and 98.2%, respectively.

Experiment 2: expression of RXR receptor mRNA in the chicken ovary

In order to investigate the molecular action of 9-*cis* RA in avian ovarian function, the expression

Table 1

Parameters for oligonucleotide primers, product size, and number of cycles

Gene	Accession number	Primer sequence (5'-3'; forward, reverse)	Primer position	Size (bp)	Number of cycles
RXR	NM_205294 Gene Bank	CTACAGGGTCATCGCATCCT GATGGCACAGATGTGTTTGG	280-299 bp 477-496 bp	217	32
GAPDH	K01458 Gene Bank	GTGGAGAGATGACAGAGGTG AACAAGCTTGACGAAATGGT	635-654 bp 964-983 bp	349	33

RXR – 9-*cis* retinoid acid receptor RXR; GAPDH – glyceraldehyde-3-phosphate dehydrogenase

of mRNA encoding RXRγ was determined in the stroma and ovarian follicles by means of the RT-PCR technique. Hens (n=5) were decapitated 3 hrs before ovulation. The fragments of the stroma and the wall of the white follicles (1-4 mm, 4-6 mm and 6-8 mm), the granulosa and the theca layers of the yellow preovulatory follicles (F3-F1) and the three postovulatory follicles (P1-P3; P1>P2>P3) were dissected from the ovary. Moreover, samples of the liver, the cerebellum and the pituitary gland were isolated. The samples were snap frozen in liquid nitrogen and kept at -80°C till RNA extraction. Total RNA from each tissue was extracted according to CHOMCZYŃSKI and SACCHI (1987) with minor modifications. Briefly, tissue samples were homogenized in 1 ml TRIzol reagent (Molecular Research Center, USA) followed by addition of 0.1 ml BCP (1-bromo-3-chloropropan, Molecular Research Center, USA) and shaken instantly per 1 minute. After 15 minutes of incubation, samples were centrifuged at 12 000 g for 15 minutes at 4°C. Then, isolated RNA was washed using 0.5 ml cold isopropanol (MP Biomedicals, USA) for 2 hrs in -4°C, and after centrifugation (12 000 g, 15 minutes, 4°C) the pellets were rinsed with 1 ml 75% ethanol (Chempur, Poland). After final centrifugation (7600g, 5 minutes, 4°C) isolated total RNA was resuspended in sterile water and RNA concentration was measured at 260 nm by spectrophotometry and stored at -80°C. The quality of isolated total RNA was checked by electrophoresis in 1.5% agarose gel.

RT-PCR analysis was performed using Mastercycler (Eppendorf, USA). Five micrograms of total RNA were reverse transcribed using primer oligo dT (0.5 μg; Fermentas, Lithuania) and RevertAid M-MuLV Reverse Transcriptase (200 U, Fermentas). Afterwards each RT product served as a template in 25 μl PCR reaction containing 25 mM $MgCl_2$ (Fermentas), 2 mM dNTP Mix (Fermentas), 10xPCR Buffer (Fermentas), 5U PolTaq Polymerase (Fermentas) and 20 μM of each primer (Laboratory of DNA Sequencing, Polish Academy of Sciences). The amplification conditions were as follows: denaturation at 95°C for 30 s, annealing at 52°C (for RXR) or 51°C (for glyceraldehyde-3-phosphate dehydrogenase – GAPDH) for 30 s and primer extension at 72°C for 30 s. After final extension at 72°C for 7 min, PCR products were resolved on 1.5% agarose gel containing ethidium bromide. Chicken GAPDH gene was used as an internal control. The primer sequences and their position in the appropriate gene, number of cycles and size of predicted PCR products are shown in Table 1.

Statistical analysis

Data of the *in vitro* experiment were statistically analyzed by two-way analysis of variance ANOVA followed by Student's *t*-test.Values are expressed as the mean ±SEM from 6 determinations and considered significantly different at $P<0.05$.

Results

Effect of 9-cis-RA on *in vitro* steroid hormone secretion by chicken ovarian follicles

Estradiol secretion by white non-hierarchical follicles is presented in Fig. 1a. In the control conditions, following 24 h incubation, the highest E_2 secretion was found in 1-4 mm follicles (0.57±0.03 ng/mg protein). It was 1.6- and 2.0-fold higher than in 4-6 mm and 6-8 mm follicles, respectively ($P<0.01$). Ovine LH significantly ($P<0.01$) increased E_2 secretion in all groups of white follicles (1-4, 4-6, 6-8 mm) by 90, 80, and 124%, respectively. 9-*cis* RA caused a significant ($P<0.05$) decrease in E_2 secretion by 31% (1-4 mm), 36% (4-6 mm) and 21 % (6-8 mm). In comparison with the oLH-treated group, incubation of these follicles in medium supplemented with both 9-*cis* RA and oLH did not change E_2 secretion (Fig. 1a).

Secretion of estradiol by theca layers of the preovulatory follicles (F3, F2, F1) is shown in Fig. 1b. The theca layer of the F3 follicle in the control

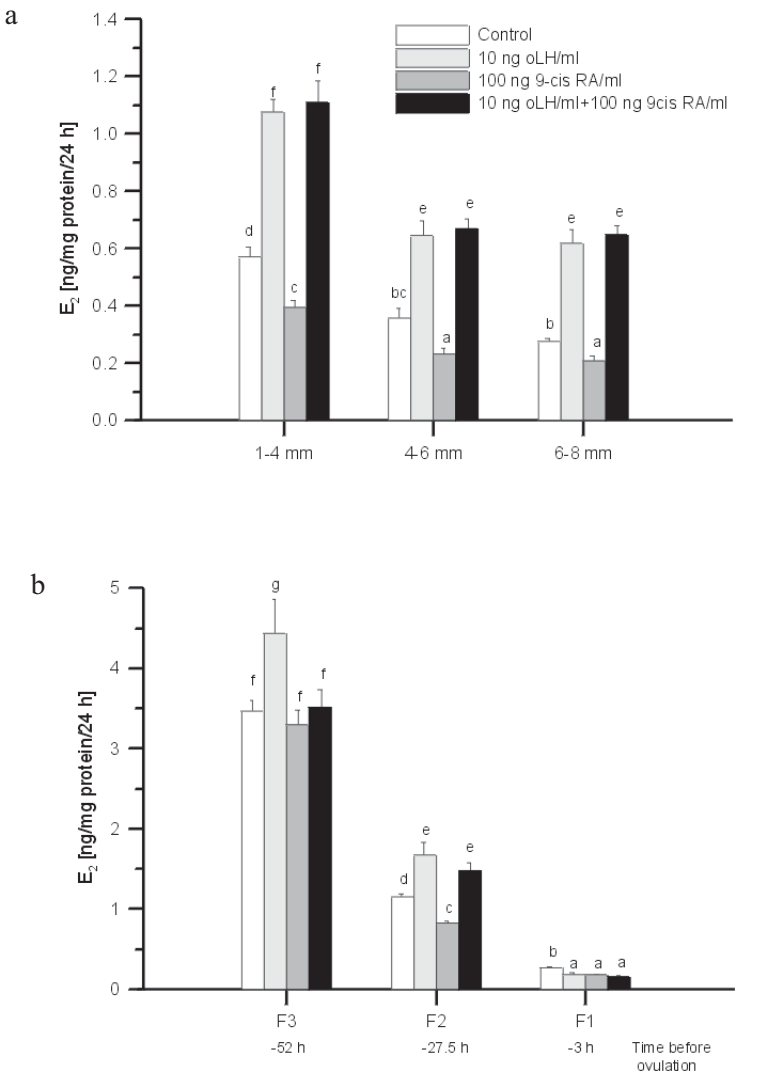

Fig. 1. Effect of 9-*cis* retinoic acid (9-*cis* RA) on estradiol (E_2) secretion *in vitro* by the white ovarian follicles (a) and the theca layer of yellow preovulatory follicles (b) of the laying chicken. Each value represents the mean ± SEM from six determinations. Values marked with different letters differ significantly ($P<0.05$).

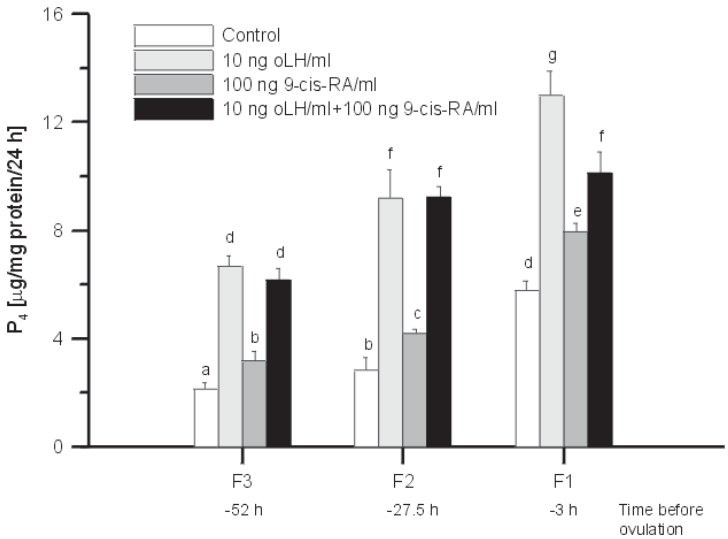

Fig. 2. Effect of 9-*cis* retinoic acid (9-*cis* RA) on progesterone (P_4) secretion *in vitro* by the granulosa layer of yellow preovulatory follicles of the laying chicken. Each value represents the mean ± SEM from six determinations. Values marked with different letters differ significantly ($P<0.05$).

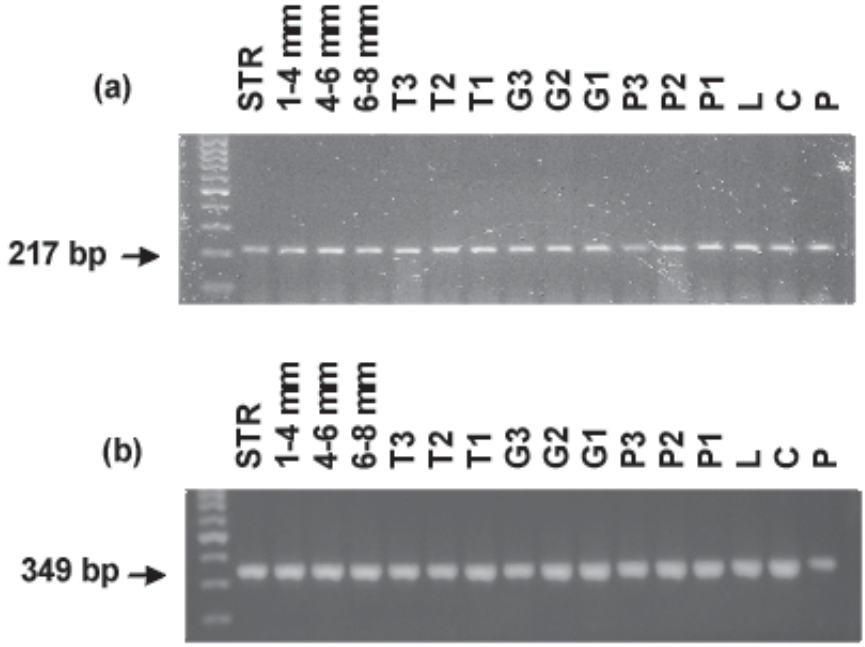

Fig. 3. Expression of the 9-*cis* retinoic acid receptor RXR gene (a), and the GAPDH gene (as an internal control); (b) in the stroma and ovarian follicles of the hen, as demonstrated by RT-PCR. Lines: STR – stroma; 1-4, 4-6, 6-8 mm - white follicles; T3, T2, T1 – theca, G3, G2, G1 – granulosa of the preovulatory follicles F3, F2, F1, respectively; P3, P2, P1 – postovulatory follicles; L – the liver; C – the cerebellum (a positive control); P – the pituitary gland.

group secreted 3.5±0.13 ng E_2/mg protein/24 h, i.e. 3- and 13-fold more than the theca layers of the F2 and F1 follicles, respectively ($P<0.01$). Ovine LH added to the medium significantly increased E_2 secretion by the theca layer of F3 (by 28%) and F2 (by 46%) follicles, but it significantly (by 33%) decreased E_2 secretion by the largest F1 follicle ($P<0.05$). 9-*cis* RA had no significant effect on E_2 secretion by the theca layer of the F3 follicle, but it significantly lowered E_2 secretion of F2 and F1 follicles by 17% and 33%, respectively ($P<0.05$). In comparison with oLH-treated follicles, the combination of 9-*cis* RA and oLH resulted from a significant ($P<0.05$) decrease in E_2 secretion (by 21%) in the F3 follicle, but they had no effect in F2 and F1 follicles (Fig. 1b).

Progesterone secretion by the granulosa layer of the preovulatory follicles is shown in Fig. 2. In control conditions, the granulosa layer of the F1 follicle secreted 5.7±0.36 μg P_4/mg protein/24 h, i.e. 2.0- and 2.7-fold more than F2 and F3 follicles, respectively ($P<0.01$). In comparison with the control group, ovine LH significantly ($P<0.01$) increased P_4 secretion from F3, F2 and F1 follicles by 213, 223 and 125%, respectively. 9-*cis* RA enhanced P_4 secretion ($P<0a.05$) from the granulosa layers of F3, F2, F1 follicles by 49, 47, 38%, respectively. In comparison with the oLH group, there were no significant differences in P_4 secretion by granulosa layers of F3 and F2 follicles incubated in medium supplemented with 9-*cis* RA and oLH. However, this combination resulted from a significant (by 22%, $P<0.05$) decrease in P_4 secretion from the F1 granulosa layer (Fig. 2).

Expression of RXR mRNA in the hen ovary

RT-PCR analysis showed that RXRγ mRNA was expressed in the stroma and all the examined ovarian follicles, i.e. in the wall of white follicles (1-4, 4-6, 6-8 mm), the granulosa and theca layers of F3-F1 follicles and in the P1-P3 postovulatory follicles. Additionally, expression of RXRγ mRNA was indicated in the liver, the cerebellum (the positive control) and in the pituitary gland (Fig. 3).

Discussion

Although the effect of vitamin A on ovarian function and laying performance has been already suggested (BERMUDEZ *et al.* 1993), the direct of the action of retinoids on the ovary in laying birds has not been examined. The present experiments were designed to evaluate whether 9-*cis* retinoic acid influences sex steroid secretion by chicken ovarian follicles. To our knowledge this is the first study that demonstrated the effect of 9-*cis* RA on steroid secretion from the ovarian follicles of chicken ovary and that showed the expression of RXR retinoid acid receptors in chicken follicles.

In the control conditions, in all examined follicles (i.e. white nonhierarchical and yellow preovulatory) a gradual decrease in estradiol secretion was observed. In the white follicles the highest secretion was found in the follicles of 1-4 mm in diameter, while the lowest in the largest ones (6-8 mm). A similar gradual decrease in estradiol secretion was observed in the yellow preovulatory follicles, where the theca layer of the F3 follicle secreted the highest amount of estradiol while the theca layer of F1 follicles – the lowest. These results are consistent with previous *in vitro* investigations by other authors (WELLS *et al.* 1985; LEE & BAHR 1994; SECHMAN 2003; HRABIA *et al.* 2004; SECHMAN *et al.* 2005). The alterations in estradiol secretion can be explained by a gradual decrease in P450 aromatase (P450arom) activity in the theca layer of the growing ovarian follicles (KATO *et al.* 1995; PROSZKOWIEC-WĘGLARZ *et al.* 2005). Addition of ovine LH to the incubating medium caused a significant increase in estradiol secretion by all classes of the white follicles (the most pronounced effect was found in 6-8 mm follicles). In preovulatory follicles ovine LH increased estradiol secretion from the theca layers of F3 and F2 follicles, but lowered it in the F1 follicle. These results are generally consistent with the ability of LH to promote steroid production from the whole follicles throughout their development (including prehierarchical follicles) (TILLY *et al.* 1991). This is associated with the expression of LH receptors (LH-R) in the theca layer of the ovarian follicles which is relatively high (JOHNSON *et al.* 1996).

The most outstanding result of the present study is an anti-estrogen effect of 9-*cis* RA in the white follicles. In all tested white non-hierarchical follicles, 9-*cis* RA decreased estradiol secretion. Moreover, a similar effect was also found in the largest preovulatory follicles (F2 and F1). These results provide evidence that 9-*cis* RA has a direct effect on avian ovarian steroidogenic function. It may affect the activity of 17α-hydroksylase ($P450_{17\alpha}$) – the enzyme responsible for androgen synthesis, or P450arom that converts androgens to estrogens in the theca layer of the follicle. The effect of 9-*cis* RA on mRNA expression of the steroidogenic enzymes has not been studied yet, but it can not be excluded that 9-*cis* RA inhibits transcription of $P450_{17\alpha}$ and/or P450arom in the avian ovarian follicles or may exert a non-genomic effect on estrogen production. 9-*cis* RA had no effect on LH-stimulated estradiol secretion by the white follicles as well as by the two largest preovulatory follicles (F2 and F1). On the other hand, it is interesting that 9-*cis* RA decreased the LH-stimulated estradiol secretion by the F3 follicle. These data indicate that the effect of 9-*cis* RA on LH-stimulated estradiol secretion in the yellow preovulatory follicles depends on the maturational state of the latter. It can not be excluded that RA may affect LH-R gene expression in ovarian follicles. MINEGISHI *et al.* (2000) found that *in vitro* RA inhibits LH-R gene transcription in rat granulosa cells and stimulates production of destabilizing factors for LH-R mRNA. Recently, it has been reported that retinoids differentially regulate steroid biosynthesis in human ovarian theca cells (WICKENHEISSER *et al.* 2005), and their effect is associated with the regulation of the key steroidogenic enzymes. Further studies are needed in order to elucidate the exact nature of the molecular mechanism of retinoid action in the theca layer of the chicken ovarian follicles.

It has been established that preovulatory follicles are the main source of progesterone in the hen ovary (HUANG & NALBANDOV 1979; BAHR *et al.* 1983; JOHNSON 2000). In the present study, the granulosa layer of the F1 follicle secreted the highest amount of progesterone. It was 2.0- and 2.7 fold more than follicles F2 and F3, respectively. A similar profile of progesterone secretion *in vitro* was also observed in previous studies (HUANG & NALBANOV 1979; ONAGBESAN *et al.* 1999; SECHMAN 2003; HRABIA *et al.* 2004). Addition of oLH to the incubation medium increased progesterone secretion by the granulosa layer of the preovulatory follicles; the smallest effect was noticed in the F1 follicle. This can be explained by a rapid decline of LH-R expression in the granulosa layer of the F1 follicle (JOHNSON *et al.* 1996; ZHANG *et al.* 1997).

9-*cis* RA increased progesterone secretion from the granulosa layers of all examined ovarian follicles, however, it did not influence LH-stimulated P_4 secretion by F3 and F2 follicles, and in the F1 follicle it even decreased LH-enhanced hormone secretion. BAGAVANDOSS and MIDGLEY (1987) also observed a stimulating effect of RA on *in vitro* progesterone accumulation in luteal cells of the rat ovary. In the granulosa layer of the chicken preovulatory follicles, 3β-hydroxysteroid dehydrogenase (3β-HSD) is the key enzyme responsible for progesterone synthesis by the Δ^4 pathway (ARMSTRONG *et al.* 1977; NITTA *et al.* 1993; LEE *at al.* 1998; JOHNSON 2000). It is possible that retinoic acid through its nuclear receptor induces 3β-HSD gene transcription, resulting from higher progesterone production. A decline in progesterone secretion by the F1 granulosa layer following combined 9-*cis* RA and LH treatment can be explained by the negative effect of RA on LH-R expression in granulosa cells. A smaller amount of LH-R in the granulosa cells leads to a lower sensitivity to LH and decreases progesterone production. Such an assumption can be supported by the investigations of HATTORI *et al.* (2000) who demonstrated that RA is a potent inhibitor of FSH receptors expression in porcine granulosa cells.

In vertebrates, the molecular actions of retinoids are transduced through nuclear RAR receptors, which bind mainly all-trans RA, and RXR receptors, which bind 9-*cis* RA (THALLER 1992; MANGELSDORF *et al.* 1993; MARILL *et al.* 2003). So far, RARα (MICHAILLE *et al.* 1995), RARβ (NOHNO *et al.* 1991) and RXRγ (SELEIRO *et al*. 1994] receptor cDNA have been cloned and detected in chicken embryonic retina, nervous system and liver. In the present study, in order to explain the molecular mechanism of 9-*cis* RA action in the chicken ovary, the expression of mRNA encoding RXRγ was determined in the stroma and ovarian follicles by means of the RT-PCR technique. The analysis showed that RXRγ receptor for 9-*cis* RA is present in all ovarian compartments, i.e. in the stroma with cortical follicles <1 mm, white follicles (1-8 mm), and the theca and granulosa layers of the preovulatory follicles (F3-F1). The expression of RXR receptor mRNA was also found in the postovulatory follicles, and all control samples (i.e. in the pituitary gland, the cerebellum and the liver). These results suggest that 9-*cis* RA acting via nuclear receptors modulates ovarian steroidogenesis in the follicles of the chicken ovary.

In conclusion, the present results clearly demonstrate that 9-*cis* retinoid acid is a potent regulator of estradiol and progesterone synthesis and/or secretion from chicken ovarian follicles. It inhibits estradiol and stimulates progesterone secretion from the theca and granulosa layers, respectively. The lack of the effect of 9-*cis* retinoic acid on LH-stimulated steroidogenesis in almost all investigated ovarian follicles indicates direct genomic action of retinoids in the cells of theca and granulosa layers. This assumption is supported by the expression of RXR mRNA in all the compartments of the chicken ovary. Further studies are necessary to explain the molecular mechanism of retinoid action in the ovary of the laying chicken.

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