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Immunolocalization of estrogens and progesterone receptors within the ovary of the lizard *Uromastyx acanthinura* from vitellogenesis to rest season

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Abstract: The sites of action and the physiological role of estrogens and progesterone in the ovary are poorly understood in Reptiles. We have undertaken a systematic study of the immunoexpression of classical estrogen receptor (ER or ERα) and progesterone receptor (PR) in the female lizard during the reproductive cycle. During vitellogenesis, ER was not expressed in vitellogenic follicles whereas PR was weakly detected in the nucleus of some follicular cells and well expressed in the internal theca cells. The follicular and theca cells were immunopositive for ER in the previtellogenic follicles, the signal in both was cytosolic. PR was strongly expressed in the follicular cells, the signal was localised in the nucleus. In the post-reproductive period, ER was detected in the previtellogenic follicles in the same manner as in the breeding period. The staining for PR was expressed in both the nucleus and cytoplasm of follicular cells and theca cells. In the sexual rest, the previtellogenic follicles were all negative for ER and PR immunoexpression. These findings suggest that the main action of estrogens in the ovary is not mediated by ER. The expression of cytosolic PR only in the post-reproduction period, at the same time at the progesterone synthesis, supports the hypothesis which stipulates an exclusive nuclear localization in the absence of progesterone.

Key words: Folliculogenesis - Estrogens receptor - Progesterone receptor - Ovary - Lizard

Introduction

Steroid hormones are important regulators of reproductive processes in female mammals. Estrogens and progesterone receptors mediate respectively the action of estrogens and progesterone by regulating transcription target genes. Estrogens possess an intrafollicular action by stimulating in synergic manner with FSH the aromatase activity [1,2]. They increase granulosa proliferation [3] and they are essential to GnRH receptor expression in the growing ovarian follicles [4]. Ovary expresses both classical estrogens receptor (now referred to as ERα) and ERβ but ERα has been only detected in the theca cells of follicles of different species including rats, mice and primate [5-11] or totally absent [12,13]. Progesterone plays a major role in controlling ovulatory and pregnancy [14]. The receptors involved in ovarian function are (PRA) and PRB. The importance of progesterone receptors in the female reproductive function is revealed by the infertility of PR knockout mice [15]. Despite their normal ovarian structure, these mice are unable to ovulate even not after exogene stimulation.

The knowledge of the distribution of ER and PR in Reptiles ovaries is limited at this stage. Therefore, the present study used the lizard ovarian follicles to further elucidate the role of these receptors in folliculogenesis by using immunohistochemical method. The immunolocalization of ER and PR was investigated throughout the annual reproductive cycle. And in order to obtain a better understanding of local action of gonadal steroid hormones in the lizard ovary, it would be helpful to analyse immunohistochemically the relationship between the expression of steroids and that of their receptors, to determine which types of cells produce

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which types of steroid hormones, and which types of cells may be regulated by these steroids in the lizard ovarian tissue during the normal reproductive cycle.

Materials and methods

Tissue samples. This study used a Saurian Reptile (*Uromastyx acanthinura*), captured in the arid region of north-western Sahara from 1999 to 2004. 44 female adults were used, 20 females were captured during the reproductive period (late May), 6 females in post-reproduction period (July) and 18 females in the sexual rest season (Winter). The animals were anaesthetised with intraperitoneal injection of 6% saline pentobarbital (10^{-3}ml/g of weight). The removed ovaries were fixed with Bouin's fixative (for receptors research) and fresh frozen into liquid nitrogen and cut with a cryostat (for hormones research). The tissues fixed in Bouin's fixative were then dehydrated in graded alcohols and embedded in paraffin. Sections 5µm thick were cut and floated onto Super Frost slides.

Immunohistochemistry. Immunolocalization of ER and PR has required heat-induced antigen retrieval including treatment of sections with heating at 95°C for 1h in the retrieval solution (1 volume of 10× *target retrieval* added at 9 volume of phosphate-buffered saline). It was determined using commercial peptide antibodies supplied by DAKO. ER and PR antibodies were a mouse monoclonal antibodies, they were used at a dilution of 1:100. The antibody anti-ER, Clone 1D5, specifically reacts with ERα but the antibody anti-PR, Clone 1A6, reacts with PRA and PRB forms. The sections were incubated for 30 min with the specific antibody and then revealed by using an Avidine Biotine Peroxidase detection system (LSAB2 kit) (indirect method). To visualize the sites of peroxidase activity, the sections were incubated in 3,3'-diaminobenzidine (DAB). The investigation of estrogens progesterone, testosterone and aromatase using specific antibodies (mouse monoclonal anti-estradiol, rabbit polyclonal anti-progesterone, anti-testosterone and anti-aromatase antibodies) was also applied by the indirect immunohistochemical method. Peroxidase was developed by 3-amino-9-ethyl-carbazole (AEC).

Results

For all vitellogenic females captured late may, ovaries possess both vitellogenic and previtellogenic follicles. The vitellogenic follicles of 10-20 mm in size were predominant in the ovary. Each one contained a granulosa generally composed of a single layer of flattened cells partially immunostained for estradiol (Fig. 1), while granulosa showed intense staining of progesterone (Fig. 2). These follicles appeared unstained for ER (Fig. 3). In contrast with these data, the granulosa cells exhibited a faint reactivity for PR into the nucleus of some cells and appreciable immunoreactivity into the cytoplasm of internal theca cells (Fig. 4).

Among the previtellogenic follicles, primary and secondary follicles are found. They reached 1.5 mm in maximal diameter. Their granulosa was stratified with three cellular categories, small, intermediate and piriform cells. All granulosa cells were estradiol (Fig. 5), testosterone and aromatase immunoreactive but the signal appears particularly more intense in the piriform cells. The theca interna was unstained. Similarly to their steroidogenic activity in favour of estradiol biosynthesis, previtellogenic follicles express RE. RE was strongly detected in the cytoplasm of the piriform cells (Fig. 6). The staining was only just detectable in the small, intermediate and internal theca cells with a cytoplasmic signal. The RP was strongly expressed in the granulosa cells, the staining was exclusively localised in the nucleus (Fig. 7).

In all the females captured during the post-reproductive period only previtellogenic follicles were present. Among these follicles there are numerous primordial and primary follicles with a maximal diameter for 2 mm. The primordial follicle was unstained for any steroid hormones investigated. For receptors, only the RP was expressed in the follicular epithelium. The majority of the primary and secondary follicles shows an estradiol and testosterone immunoreactivity, but with a faint staining in comparative with the vitellogenesis period. In addition, the progesterone appears in the piriform cells (Fig. 8). RE is detected in these follicles in the same manner as in the vitellogenesis period with a cytosolic expression essentially localized in the piriforme cells. In the young primary follicle, the RP is detected in both the cytoplasm and nucleus of the some small cells of the granulosa (Fig. 9 and 10). The intermediate and piriform cells were partially stained, the nuclear signal was rarely observed in the piriform cells. The theca expresses the cytosolic and nuclear fractions. The big primary and secondary follicles show a strong expression for the PR (Fig. 11), it's more detected in the nucleus of the basal small cells then apical small cells of the granulosa. PR was also clearly visible in the piriform and theca cells. At the level of the intermediate cells, the cytosolic signal of the PR appears only in the follicle at the secondary stage.

In the sexual rest, ovary contains previtellogenic follicles essentially at secondary and tertiary stages. The maximal diameter of the tertiary follicles reach 3 mm. The steroidogenic activity was altered in all the previtellogenic follicles; any positive staining was observed for all steroid hormones investigated (Fig. 12) and for their specific receptors, RE and RP.

Discussion

Our present immunohistochemical observations indicate that the expression of ER and PR and the secretion of their specific hormones in the ovary of Uromastyx acanthinura are well correlated with the reproductive cycle. But during ovarian activity, the expression of ER and PR is not always correlated with the presence of the hormones. In fact, during vitellogenesis, ovary possesses both vitellogenic and previtellogenic follicles and the granulosa of the vitellogenic follicle produces more progesterone than estradiol. The progesterone can take action in the same manner as in the

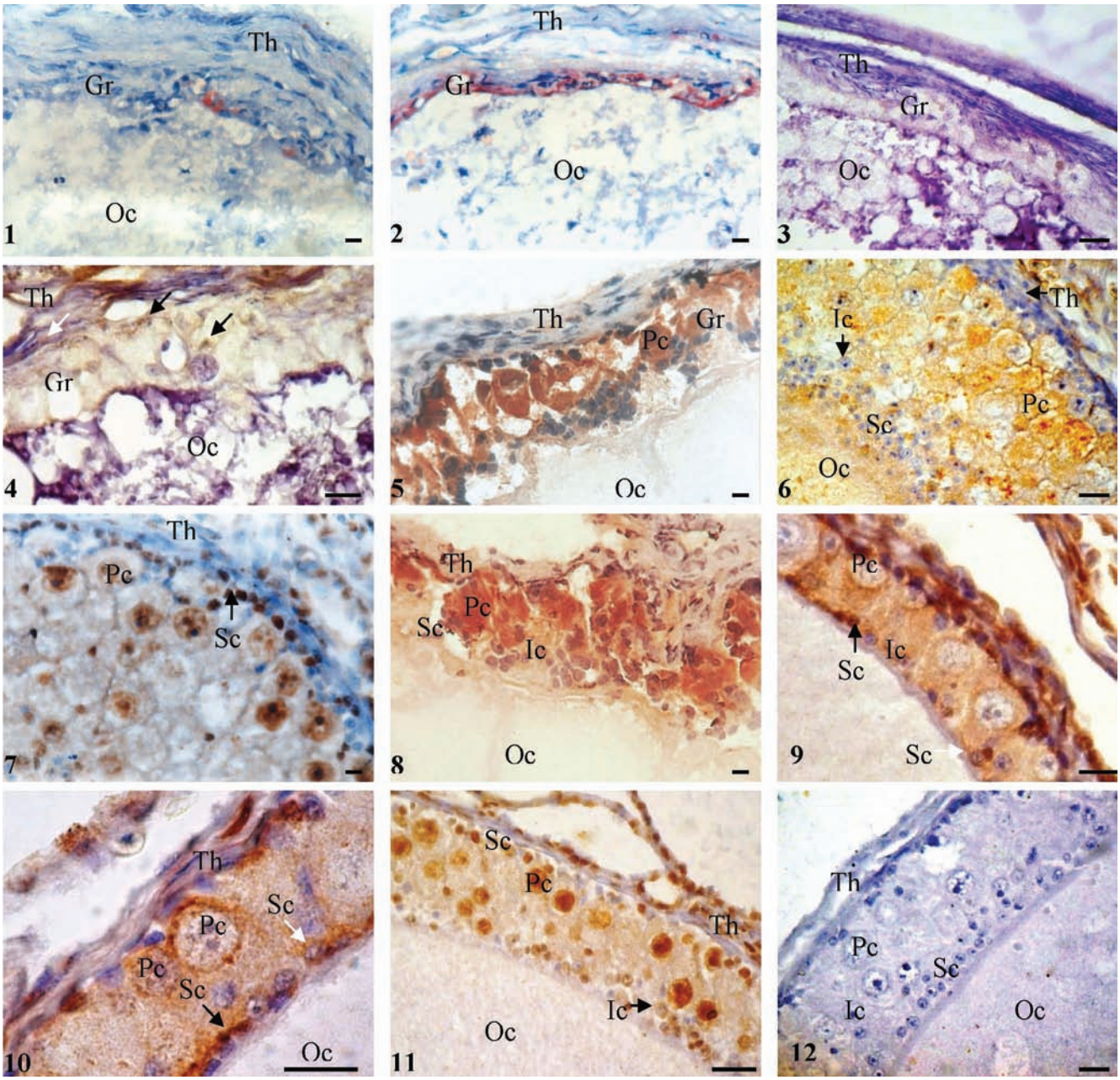

Fig. l. Immunolocalization of vitellogenic follicle estradici; partial stainin shows in granulosa (Gr). **Fig. 2**. Immunolocalization of vitellogenic follicle progesterone; ali granulosa cells are stained. **Fig. 3**. Immunolocalization of vitellogenic follicle ER; showing negative staining. **Fig. 4**. Immunolocalization of vitellogenic follicle PR; with faint staining in th? nucleus of some granulosa cells (black arrows). Internai theca cells (Th) were stained, the signal was appreciable into the cytoplasm (white arrow). **Fig. 5**. Immunolocalization of estradici in a primary follicle taken on the vitellogenesis period; with positive staining in granulosa cells and essentially in the piriform cells (Pc). **Fig. 6**. Immunolocalization of ER in a secondary follicle taken on the vitellogenesis period; RE was strongly detected in the cytoplasm of the piriform cells. **Fig. 7.** Immunolocalization of PR in a secondary follicle taken on the vitellogenesis period; a strong staining was detected in the nucleus of the granulosa cells. **Fig. 8**. Immunolocalization of primary follicle progesterone taken on the post-reproductive period; with positive staining in the piriform cells. **Fig. 9**. Immunolocalization of young primary follicle PR taken on the post-reproductive period; staining was cytosolic (white arrow) and nuclear (black arrow) in the small cells (Se) (detail in **fig. 10**). The piriform cells show a cytosolic staining. The theca was positive. **Fig. 11**. Immunolocalization of secondary follicle PR, taken on the post-reproductive period; strong staining was detected in the nucleus of the small, piriform and theca cells. The intermediate cells (le) show a cytosolic staining. **Fig. 12**. Immunolocalization of tertiary follicle estradici taken on the sexual rest period; showing negative staining. Oo: oocyte. Bar of calibration = 10 μm (Fig. 1, 2, 3, 4, 5, 6, 7, 8, 11, 12), = 5 μm (Fig. 9, 10).

level of mammalian's preovulatory follicles by inhibiting the mitotic activity [16] and apoptosis [17,18]. Thus by regulating proliferation and luteinization, the progesterone regulates the number of luteal cells and their steroidogenic potential [16], but the progesterone synthesis in *Uromastyx acanthinura* does not coincide

with the presence of PR; the PR staining is clearly weaker in comparison with previtellogenic follicles. This receptor is necessary for the ovulation; the in vitro and in vivo studies show that this expression in the granulosa is restricted to preovulatory follicles after the ovulatory discharge [5,17-20]. Progesterone receptor antagonist treatment reduces the level of both PRA and PRB [20], activates caspase 3 activity [17,20] and decreases the level of ovulations [20]. These results indicate the participation of the two isoforms in the control of ovulation by allowing the granulosa cells survival.

As for the previtellogenic follicles, they represent a great source of estradiol and their piriform cells are the seat of the synthesis. Estradiol is essential for the hepatic vitellogenin production and for oviduct development. Estradiol exerts an autocrine and paracrine action in the previtellogenic follicles. A part of this action is mediated by the ER which is revealed in the piriform cells and at lesser degree in the small follicular and internal theca cells. At the level of the piriform cells, the action of estradiol via ER can be related to their steroidogenic activity. In the primate ovary, ER was only detected in the granulosa of antral follicle which expresses aromatase [21-25]. The synergic role of estradiol with FSH in the aromatase activation is a well established fact [1]. The ER expression in the piriform cells may be also related to their exocrine secretory activity; they produce and transfer glycoproteins and lipids to oocyte via intercellular bridge [26,27].

The positive estrogens effect on the granulosa proliferation was reported [3]. In Uromastyx acanthinura, the estrogenic message transduction by ER in the small follicular cells, known for their mitotic capacity, is not obvious; the staining is only just detectable. It would seem that the estradiol action in this process is rather mediated by ERβ given that a great mitotic activity is revealed in this period of the reproductive cycle [27].

The immunohistochemical identification of the ER in the primate ovaries indicates that the ER expression into the granulosa cells was nuclear [21-23,25,28] whereas in our specie ER was cytosolic. In rat, hamster and pig, the two fractions of ER, nuclear and cytosolic, are present but the cytosolic fraction is more important [29]. According to Guiochon-Mantel and Milgrom [30], ER is essentially localized in the nucleus in the absence of estrogens. In addition, at the hypothalamic and hypophysal level, the estradiol injection induces the increase of the cytosolic fraction of the ER [31]. In our lizard the only cytosolic signalization remains without explanation, but referring to above data, the cytosolic ER would correspond to the occupied ER. Its variation during the reproductive cycle would translate a functional action. Other numerous studies show on the contrary that the theca cells are the major sites of ER expression in different species including rats, mice and primate [5-11] or total absence of the ER in the ovary [12,13].

The intense PR expression in the theca and follicular cells of the previtellogenic follicles had an estradiol, but not progesterone which proves a paracrine regulation of the progesterone. Vitellogenic follicles are the synthesis site. The progesterone would prevent apoptosis as in mammalian ovary [32,33].

During post-reproductive period, the chronological coincidence of the progesterone in the granulosa cells of previtellogenic follicles with the cytosolic PR signalization supports again Guiochon-Mantel and Milgrom result's [30]. The progesterone can be responsible for the partial alteration of estradiol synthesis. Her inhibitory effect on estradiol production [34,35] and on ovarian development [36] in the Reptiles was well documented. The lizard *Podarcis sicula sicula*, expresses a second peak of the progesterone in the post-reproduction period [37]. According to these authors, progesterone would be responsible for the refractory state. The variable distribution of the two PR fractions in the granulosa cells depending on the state of folliculogenesis suggests an autocrine and paracrine action of the progesterone at different degrees. The progesterone regulates the cellular survival [32,33] and stimulates its own synthesis [34].

In summary, these findings, from use of immunohistochemistry, suggest that the localization of lizard ovarian ER is not restricted to theca cells. The strong ER expression in the piriform cells during sexual activity suggests a functional role of this isoform; however, the presence of the only cytosolic fraction in Uromastyx acanthinura remains unexplained. PR intensively expressed in both follicular cells and theca cells of previtellogenic follicles demonstrates a paracrine action during vitellogenesis, an autocrine and paracrine role in post-reproductive period. The capacity of these follicles to produce progesterone and to express the cytosolic PR fraction suggests that this fraction appears when steroid hormone is present.

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