Acta vet. scand. 1994, *35,* 185-191.

Immunocytochemical Localization of Aromatase in The Ovary of Superovulated Cattle, Pigs and Sheep

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[1]Research Institute of Animal Production, Department of Reproduction and Embryology, and [2]Departments of Pathology, and [3]Physiology, Veterinary University, Kosice, Slovak Republic, [4]Department of Anatomy and Physiology, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark and Endocrine Biochemistry Department, Medical Foundation of Buffalo, New York, USA.

Laurincik, J., L. Kolodzieyski, V. Elias, P. Hyttel, Y. Osawa, A. Sirotkin: Immunocytochemical localization of aromatase in the ovary of superovulated cattle, pigs and sheep. Acta vet. scand. 1994, 35, 185-191. – The localization of aromatase, an enzyme converting androgens to estrogen, in the ovaries of superovulated cattle, pigs and sheep was studied immunocytochemically in the preovulatory and postovulatory period using anti-human placental aromatase cytochrome P-450 antiserum. Immunostaining for aromatase was detected in the granulosa cells of preovulatory follicles of all species studied. Theca interna cells were stained in preovulatory follicles in the pig but not in cattle and sheep. Interstitial gland cells, cumulus cells and oocytes were unstained in all species. In cattle and pig the corpora lutea were unstained whereas they displayed staining in the sheep. Preantral and small antral follicles were unstained during both the preovulatory and postovulatory period in all species.

It is concluded that granulosa cells of preovulatory follicles are the main residence for aromatase activity in superovulated cattle, pig and sheep, whereas the activity of theca interna and corpora lutea is species specific.

follicle; corpus luteum; endocrine.

Introduction

The aromatization of the androgens, androstenedione and testosterone, to estrone and estradiol-17β is the final step in the biosynthesis of estrogens in the ovary. There has been disagreement about the site of this process within the follicle. Some investigators have postulated that androgen production occurs in the theca cells, while the subsequent aromatization to estrogens is granulosa cell-specific i.e. the "two-cell theory" *(Falck* 1959). Immunohistochemical studies support this theory as aromatase localization has been demonstrated in rat granulosa and luteal cells *(Ishimura et al.* 1989, *Yoshinaga-Hirabayashi et al.* 1990) as well as in granulosa cells of preovulatory follicles in golden hamster, guinea pig and cow *(Zhong et al.* 1989).

However, several reports indicate that theca cells may also exhibit aromatase activity *(Younglai & Short* 1970, *Channing & Coudert* 1976, *Makris & Ryan* 1977, *Evans et al.* 1981, *Armstrong et al.* 1981, *El-Maasarany et al.* 1991). Other immunocytochemical studies in several species support this notion by demonstrating localization of aromatase predominantly in the theca interna *(Matsuda et al.* 1984).

Furthermore, it has been suggested that stimulation by exogenous gonadotropins may influence aromatase activity in rat *(Zimniski et al.* 1987, *Brandt et al.* 1988, *Yoshinaga-Hirabayashi et al.* 1990) and steroid hormone metabolism in porcine *(Ainsworth et al.* 1980) and human *(Aguado & Ojeda* 1985) ovaries. However, data concerning aromatase activity in superovulated farm animals are still not available.

It was the aim of the present study to demonstrate the precise localization of aromatase activity in preovulatory and postovulatory ovaries by means of the anti-human placental aromatase cytochrome P-450 antiserum in superovulated cattle, pigs and sheep.

Material and metdods

Animals

Cattle: Cyclic heifers (n = 11, 15-17 months old) were synchronized for estrus with 0.75 mg cloprostenol (Oestrophan, Spofa, Praha, CSFR). Animals, which exhibited a discernable corpus luteum on day 10 (n = 8), were treated with a total dose of 20 mg FSH-P (Folicotropin, Spofa, Praha, CSFR) given every 12 hours over a period of 4 days in decreasing doses: 1×6, 1×4, 4×2, 2×1 mg, respectively. Luteolysis was induced by administration of 0.75 mg cloprostenol 48 h after the initial FSH-P injection *(Laurincik et al.* 1993). Four animals were slaughtered at standing heat *(Laurincik et al.* 1991). The remaining 4 animals were slaughtered 7 days after ovulation, which was monitored by ultrasound scanning *(Laurincik et al.* 1993).

Pig: Gilts (n = 10) at the age of about 6 months (weight 60-80 kg) were synchronized by daily Zink-metallibur (Evertas P, VUBVL, Pohori-Chotoun, CSFR) (0.125 g in 2 kg of the complete feed mixture for 15 days). On day 16 of the treatment, 1200 i.u. of PMSG (Antex, Leo, Denmark) were injected intramuscularly. At day 19 five animals were slaughtered. The remaining 5 animals were slaughtered 7 days after ovulation *(Laurincik et al.* 1994).

Sheep: Cyclic ewes (n = 16; Slovak merino; 40-43 kg in weight) during the breeding season were treated with intravaginal sponges (20 mg chlorsuperlutin, Agelin, Spofa, CSFR) for 13 days during the breeding season. At sponge withdrawal all ewes received an intramuscular injection of 400 i.u. PMSG (Antex, Leo, Denmark). Vasectomised rams were run with ewes for heat detection throughout the experiment. Ten ewes were slaughtered at 72 h after the heat. The remaining 6 ewes were slaughtered 6-7 days later.

Aromatase localization

The ovaries were cut into pieces containing preovulatory follicles, corpora lutea, and small antral and preantral follicles. The tissue blocks were frozen in liquid nitrogen, and cryosections (6 μm) were cut at –25°C and fixed in a paraformaldehyde atmosphere for 10 min. After fixation, the sections were rinsed in phospate buffered saline (PBS) and incubated with rabbit anti-aromatase cytochrome P-450 antiserum (1:500) for 72 h at 4°C *(Zhong et al.* 1989, *Ishimura et al.* 1989, *Yoshinaga-Hirabayashi et al.* 1990). After washing in PBS, the sections were treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig G (Sevac, Praha, CSFR) for 2 h at room temperature and examined with Nikon light microscope equipped with a high pressure mercury lamp.

Control sections were treated with normal rabbit serum instead of the antiserum.

All the procedures for the purification of antigen (aromatase), the production of the antibody, and the specificity of the antibody have been previously described *(Osawa et al.* 1987a,b).

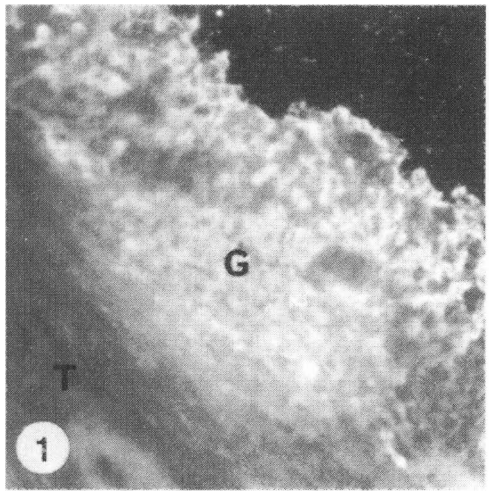

Figure 1. Bovine aromatease activity. All granulosa layers (G) of preovulatory follicles displayed immunostaining while theca interna (T) was unstained. (x460).

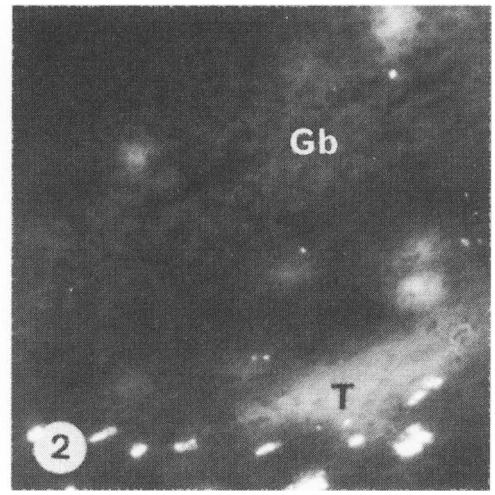

Figure 2. Porcine aromatase activity. In preovulatory follicles especially granulosa cells towards the basal lamina (Gb) as well as certain areas of the theca interna (T) showed immunostaining. (x460).

Results

Cattle

Immunostaining for aromatase was observed in the granulosa cells of preovulatory follicles. In these follicles all layers of granulosa cells showed positive staining (Fig. 1). Theca interna cells, interstitial gland cells, cumulus cells and oocytes displayed lack of staining as did corpora lutea. The granulosa cells of smaller follicles (< 3 mm) were also unstained in both preovulatory and postovulatory ovaries.

Pig

In preovulatory follicles the granulosa cells located near the basal lamina displayed more intensive staining for aromatase than those located towards the antrum, which showed very weak or negative staining. In some preovulatory follicles, staining was also observed in some areas of the theca interna (Fig. 2). Interstitial gland cells, cumulus cells and oocytes were unstained as were most cells of the corpora lutea. The granulosa cells of smaller follicles (< 3 mm) were also unstained in both preovulatory and postovolatory ovaries.

Sheep

Preovulatory follicles showed a size-dependent staining pattern for aromatase. In large follicles (> 6 mm) all granulosa cell layers were stained. In smaller follicles (< 5 mm) the granulosa cells located towards the antrum displayed strong staining, while those located near the basal lamina were unstained (Fig. 3). Theca interna cells, interstitial gland cells, cumulus cells and oocytes were unstained while most cells of the corpora lutea were stained (Fig. 4). The granulosa cells of smaller follicles (< 3 mm) were unstained in both preovulatory and postovulatory ovaries.

No positive staining was recognized in the control sections (Fig. 5).

Discussion

Estradiol and estrone are the major estrogens synthetized by the ovarian follicle. The aro-

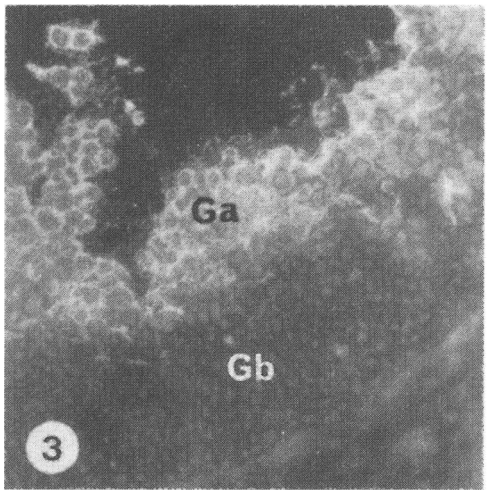

Figure 3. Ovine aromatase activity. In smaller antral follicles (< 5 mm) the granulosa cells located towards the antrum (Ga) displayed immunostaining while those near the basal lamina (Gb) were unstained. (x460).

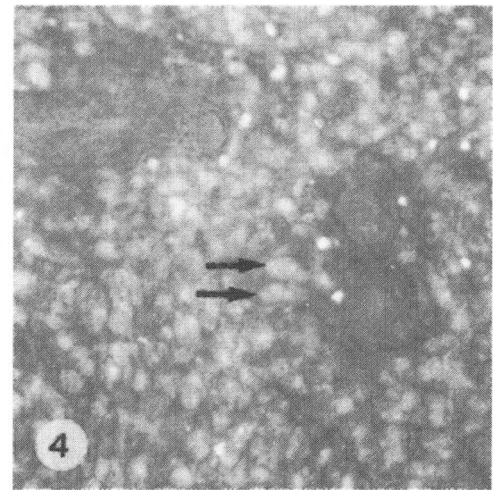

Figure 4. Ovine aromatase activity in corpora lutea. Most cells displayed immunostaining (arrows). (x460).

matizing enzymes converting androgen to estrogen are located in the microsomes of certain cell types *(Areco & Ryan* 1967). It is generally accepted that theca and granulosa cells act synergistically in estrogen production. The so-called "two-cell theory" was first proposed by *Falck* (1959) and later substantiated in rat *(Fortune & Armstrong* 1978, *Hsueh et al.* 1984) and farm animals by observations in sheep *(Moor* 1977), pig *(Gèrard et al.* 1979, *Ainsworth et al.* 1980, *Evans et al.* 1981) and cow *(Fortune & Hansel* 1979, *Dieleman et al.* 1983, *McNatty et al.* 1984, *Zhong et al.* 1989). This theory asserts that the theca interna cells, under the influence of LH, produce androgens that are transported to the granulosa cells where they are converted into estrogen by aromatizing enzymes which are stimulated by FSH.

There are, however, also several reports stating that theca cells may contribute to ovarian estrogen production in some species *(Younglai & Short* 1970, *Channing & Coudert* 1976, *Makris & Ryan* 1977; *Evans et al.* 1981, *Armstrong et al.* 1981). In this perspective the present immunological studies support the concept of localization of the aromatase activity in the granulosa compartment at least in cattle and sheep and in the theca interna cells in the pig. In accordance with this staining pattern, it has previously been documented that theca interna cells from porcine follicles serve as an additional source of estrogen synthesis *(Haney & Schomberg* 1981, *Stoklosova et al.* 1982).

Interestingly in sheep, not all antral follicles exhibited the same degree of immunostaining. *Zoller & Weisz* (1978) have demonstrated that this heterogenity may reflect functional differences among granulosa cells. As suggested by *El-Maasarany et al.* (1991) these differences may reflect which follicles are recruited for ovulation and which will become atretic.

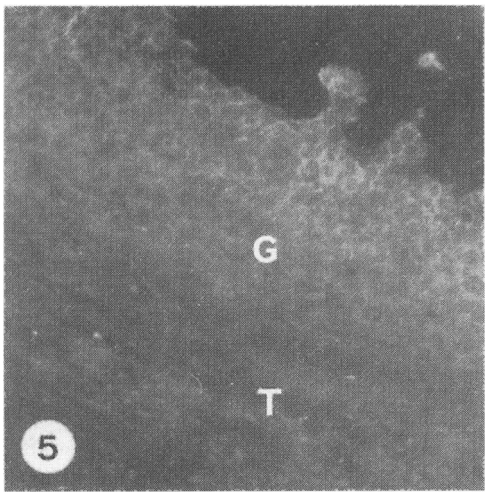

Figure 5. Control. Paralell control section from the ovine follicle presented in Fig. 3. No immunostaining was detected (G: granulosa cells; T: theca interna). (x460).

In the rat only some cells of the corpus luteum displayed immunocytochemical staining for aromatase while the majority of cells were unstained indicating that aromatase activity in the granulosa cells is not maintained for longer periods after ovulation *(Ishimura et al.* 1989). These data are, however, inconsistent with those published by other authors in rodents where corpora lutea display positive reaction *(Matsuda et al.* 1984, *El-Maasarany et al.* 1991). In the present experiment staining of the cells of the corpora lutea was demonstrated in sheep whereas corpora lutea in cattle and pigs were practically unstained. This notion supports the existence of certain species' differences concerning the duration of aromatase activity following ovulation.

Gonadotropin administration is reported to increase the degree of immunostaining for aromatase in antral follicles of immature rats *(Zimniski et al.* 1987, *Brandt et al.* 1988, *Yoshinaga-Hirabayashi et al.* 1990). In accordance, the present experiment demonstrated aromatase activity in all granulosa cell layers in contrast to earlier reports in non-stimulated cattle and sheep, where the aromatase localization was restricted to the granulosa cells lining the basal lamina *(Zhong et al.* 1989). This difference may thus be an effect of the superovulatory treatment.

It is concluded that granulosa cells of preovulatory follicles are the main residence for aromatase activity in bovine, porcine and ovine ovaries. Additional aromatase activity in the theca interna cells was demonstrated in the pig. Localization of aromatase in corpora lutea was demonstrated only in the sheep.

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Sammendrag

Immunocytokemisk lokalisation af aromatase i ovariet hos superovuleret kvæg, svin og får

Den vævsmæssige lokalisation i ovariet af enzymet aromatase, som konverterer androgener til østrogen, er beskrevet i den præ- og postovulatoriske periode hos superovuleret kvæg, får og svin. Enzymet er lokaliseret ved immunocytokemi under brug af anti-human placental aromatase cytochrom P-450 antiserum. Der observeredes positiv immunreaktion i stratum granulosum i præovulatoriske follikler hos alle arter, mens theca interna kun udviste immunreaktion hos svin. Cellerne i cumulus oophorus og selve oocyten udviste ikke immunreaktion hos nogen af arterne. Cellerne i corpus luteum udviste positiv immunreaktion hos får, hvorimod de ikke reagerede hos kvæg og svin. Prænatrale og små antrale follikler udviste ingen immunreaktion hos nogen af arterne hverken præ- eller postovulatorisk.

Det kan konkluderes, at stratum granulosum i præovulatoriske follikler er den gennemgående lokalisation for enzymet aromatase hos superovuleret kvæg, får og svin, mens enzymets tilstedeværelse i theca interna og corpus luteum er artsspecifik.

(Received August 4, 1993; accepted March 9, 1994).

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