Cell-specific variations and hormonal regulation of immunoreactive cytochrome P-450_{scc} in the rat ovary*

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Summary. Specific rabbit antibodies to the bovine cholesterol side-chain cleavage cytochrome P-450 (P-450_{scc}) were used to cross-react with the enzyme in the rat ovary. The luteal cells of cyclic, pregnant, and pseudopregnant rats were immunostained. P-450_{scc} was also expressed in the interstitial cells of prepubertal and cyclic adult rats, and in the thecal cells lining the preovulatory follicles. In cyclic females, RU 486 and oestradiol increased the intensity of P-450_{scc} immunostaining. The granulosa cells of ovarian follicles whatever their stage of development, including preovulatory follicles, were not labelled, except after ovulation. The intensity of immunostaining of thecal and interstitial cells decreased during early pregnancy or pseudopregnancy, and disappeared after Day 9, whereas these cells were intensely labelled 24 h after parturition. The immunostaining of thecal and interstitial cells was again detected in 18-day pregnant rats, treated with the antiprogestosterone RU 486. It is therefore concluded that both oestradiol and progesterone are involved in P-450_{scc} regulation.

Keywords: cytochrome P-450_{scc}; immunohistochemistry; rat; ovary; pregnancy

Introduction

The thecal, interstitial and luteal cells of the rat ovary, at least in certain physiological conditions, can convert cholesterol to steroids (Brodie, 1983; Waterman & Simpson, 1985), and contain the key enzyme for the side-chain cleavage of cholesterol, namely the cytochrome P-450_{scc} (P-450_{scc}; EC 1.14.15.67) (Zlotkin *et al.*, 1986). This enzyme is found in the mitochondria of steroidogenic endocrine cells as part of a 3-enzyme system, which also includes ferredoxin (an iron sulphur protein) and ferredoxin reductase (a flavoprotein) (Simpson, 1979; Lieberman *et al.*, 1984).

The theca interna cells and interstitial cells have been implicated in the production of androgens from cholesterol, thus providing the substrates for the production of oestradiol by granulosa cells (Erikson *et al.*, 1985). The theca interna and granulosa cells acquire their steroidogenic properties while ovarian follicles mature to the preovulatory stage (Richards *et al.*, 1986). After ovulation, the luteal cells of the corpus luteum, which evolve mainly from granulosa cells, but also from thecal cells, can perform de-novo biosynthesis of progesterone and oestradiol (Brodie, 1983; Richards *et al.*, 1986).

Antibodies to the adrenal P-450_{scc} of cattle have been prepared and their antigenic specificity has been carefully demonstrated. They have been shown to interact with the rat enzyme in cultured rat granulosa cells (Trzeciak *et al.*, 1986), and they have been used for the immunolocalization of P-450_{scc} in rat adrenal cortex and brain (Le Goascogne *et al.*, 1987).

Little is known about the occurrence and localization of P-450_{scc} as detected immunohistochemically in the rat ovary, under various physiological and experimental conditions, except its

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expression during follicular development in PMSG-treated immature rats (Zlotkin *et al.*, 1986; Trzeciak *et al.*, 1986). We have therefore investigated the presence of P-450_{sec} in the ovaries of rats during the oestrous cycle, pregnancy, pseudopregnancy, parturition and abortion.

Materials and Methods

Animals. Female rats of the Sprague–Dawley strain were obtained from IFFA-CREDO (L’Arbresle, France). They were exposed to 12 h light/day (lights on at 08:00 h), and fed *ad libitum*. They were prepubertal (1 month of age), young adult, cyclic, pseudopregnant, pregnant, or post-partum females (Table 1). The day of insemination was designated as Day 1 of pregnancy. Parturition of untreated controls occurred consistently on the morning of Day 23. Treatment of cyclic, pregnant or pseudopregnant females consisted of oestradiol benzoate (50 µg (cyclic females), 100 µg daily for 2 days (other females)), the steroidal antioestrogen RU 39411 (100 µg daily for 2 days) and the progesterone antagonist RU 486 (10 mg/kg, 48 h before death), given alone or in combination. Another group of cyclic females received progesterone (3 mg daily for 9 days). These compounds were kindly provided by Roussel-Uclaf (Romainville, France), and injected subcutaneously in sesame oil. The females were killed on Day 18 of pregnancy or Day 9 of pseudopregnancy.

Table 1. Numbers of female rats in the various treatment groups

Reproductive stage	Control	E ₂	P ₄	RU 486	RU 486	RU 486	RU 486	RU 486 + RU 411
					+ E ₂	+ P ₄	+ RU 411	+ E ₂
Prepubertal, 28–30 days	4	—	—	4	—	—	—	—
Cyclic, 60 days	6	6	4	6	—	4	—	—
Pseudopregnant 9 days	4	—	—	5	—	—	—	—
Pregnant 3 days	1	—	—	—	—	—	—	—
6 days	1	—	—	—	—	—	—	—
7 days	2	—	—	—	—	—	—	—
9 days	4	—	—	4	—	—	—	—
17 days	2	—	—	3	—	—	—	—
18 days	2	—	—	2	3	—	4	4
Post partum 2 days	2	—	—	—	—	—	—	—

E₂ = oestradiol-17β; P₄ = progesterone.

Ovaries were fixed by immersion at room temperature. Several fixatives were compared. The best immunohistochemical detection of P-450_{sec} was obtained after fixation with Carnoy’s fixative (absolute ethanol/chloroform/glacial acetic acid, 6:3:1, by vol.) or in methanol–glacial acetic acid (3:1, v/v). The results reported were obtained with Carnoy’s fixative for 2–24 h. After dehydration in a graded ethanol series and 1-butanol, specimens were embedded in paraffin wax and 7 µm thick sections were prepared.

Immunochemical reagents. Rabbit antibodies to bovine cytochrome P-450_{sec} were prepared by immunization with the protein purified from adrenocortical mitochondria of cattle (Seybert *et al.*, 1979; DuBois *et al.*, 1981). The specificity of the antiserum to P-450_{sec} was as previously described (Le Goascogne *et al.*, 1987). Biotinylated goat antirabbit immunoglobulin G and avidin–biotin–peroxidase complex (Vectastain reagents) were obtained from Vector Laboratories, Burlingame, CA, U.S.A.

Immunoperoxidase staining of ovarian sections. Deparaffinized and rehydrated sections were rinsed with PBS and incubated in 3% goat non-immune serum. The anti-P-450_{sec}–IgG was used at the dilution of 20 µg/ml PBS, at room temperature for 2 h. The biotinylated goat secondary antibody was added at a dilution of 1:200 for 30 min, followed by the avidin–biotin–peroxidase complex, at a dilution of 1:100, for 30 min. The peroxidase activity was revealed by 3,3’-diaminobenzidine tetrahydrochloride (0.5 mg/ml) in the presence of H₂O₂ (0.01% in Tris buffer pH 7.6). Sections were not counterstained. They were rinsed, dehydrated and mounted. Controls were run on adjacent sections placed

on the same histological slide, and included IgG from non-immune serum (40 µg/ml), dilution of specific antibodies down to extinction of staining, and presaturation of specific antibodies with purified bovine P-450_{sec} antigen or rat liver acetone powder (Sigma, L'Isle d'Abeau, France).

Steroid hormones and gonadotrophins. Blood was collected in heparinized syringes, centrifuged for the separation of plasma, and the plasma was kept frozen. Oestradiol and progesterone were later assayed by radioimmunoassay after partition chromatography on celite microcolumns. Rabbit antibodies were provided by Roussel Uclaf (93320 Romainville, France): antioestradiol antiserum 3341 and antiprogestosterone antiserum 3297 were used. Assay specificities have been previously reported (Mortel *et al.*, 1981). The interassay variability for oestradiol was 8.2% at a mean value of 230 pg oestradiol/ml. The lower limit of assay sensitivity was taken as 30 pg/ml. The interassay variability for progesterone was 6.7% at 35.2 ng/ml. The lower limit of assay sensitivity was taken as 200 pg/ml. LH and FSH were measured using the radioimmunoassay kits provided by the NIADDK (Bethesda, MD, U.S.A.). The rabbit antiserum to LH was NIADDK-anti-rat LH-S-10. NIADDK-rat LH-I-6 was radioiodinated by the chloramine T procedure. The standard preparation was NIADDK-rat LH-RP2. The rabbit antiserum to FSH was NIADDK-anti-rat FSH-11. NIADDK-rat FSH-I-6 was radioiodinated by the chloramine T procedure. The standard preparation was NIADDK-rat FSH-RP2. The inter- and intra-assay coefficients of variation and the sensitivities of the assays were 18%, 4.8% and 0.1 ng/ml and 12%, 5.2% and 0.8 ng/ml for the LH and FSH assays, respectively.

Results

The immature rat ovary

Our results essentially confirm the report of Zlotkin *et al.* (1986). In untreated immature females, no labelling of P-450_{sec} was observed in follicular granulosa cells. By contrast, immunostaining of P-450_{sec} occurred in interstitial cells, some of the theca interna cells, and the hypertrophied theca of atretic follicles (data not shown).

The cyclic rat ovary

The immunohistochemical staining of P-450_{sec} was positive in the interstitial cells throughout the ovary (Fig. 1a). However, it was not uniformly distributed. Most interstitial cells in the central region of the ovary were less immunostained than those in the vicinity of large antral follicles at the periphery. The staining of the interstitial cells tended to be more intense at pro-oestrus and oestrus than at metoestrus and dioestrus.

The thecal cells of preovulatory antral follicles formed a continuous immunostained lining between the unlabelled basal lamina and theca externa (Fig. 1b), whereas the staining of thecal cells of small preantral follicles was either nil, or very sparse and faint (Fig. 1a). This was a consistent observation.

The granulosa cells of intact follicles, whatever their developmental stage, from primary to Graafian follicles, or atretic follicles, were never stained with the P-450_{sec} IgG (Figs 1a, b). When rupture of mature follicles occurred at oestrus, the granulosa cells were faintly but definitely immunostained by anti-P-450_{sec} IgG (Figs 2a, b). An intense staining was characteristic of luteal cells in corpora lutea of the ovaries of cyclic females (Fig. 2c). The strictly cytoplasmic localization of P-450_{sec} was clearly observable in luteal cells. The regressed corpora lutea of remote cycles were characterized by the proliferation of connective tissue, with persisting strong expression of P-450_{sec} in the remaining luteal cells (Fig. 2d).

Specificity controls included presaturation of specific antibodies with purified P-450_{sec} (Fig. 1c) or with rat liver powder (data not shown), and their replacement by non-immune IgG (Fig. 1d).

Pregnancy and pseudopregnancy

The intensity of immunohistochemical staining with the P-450_{sec} IgG became fainter with the progression of pregnancy or pseudopregnancy and disappeared completely after the 9th day in the thecal and interstitial cells (Fig. 3a). By contrast, the luteal cells of pregnancy were intensely

Fig. 1. Distribution of immunostained P-450_{sec} in the ovary of a rat at oestrus. **(a)** One small antral follicle is surrounded by interstitial gland cells (I), which intensely express P-450_{sec}, whereas there is no P-450_{sec} labelling of the granulosa (G) and theca (T) cells (with the exception of sparse cells in the theca interna). **(b)** The luteal cells of one corpus luteum (CL) are intensely stained in their cytoplasm. The cells of the theca interna (T) surrounding a Graafian follicle express the P-450_{sec}, whereas there is no labelling of granulosa cells (G). **(c)** Specificity control: P-450_{sec} IgGs were preincubated with excess purified antigen. **(d)** Specificity control: non-immune IgGs were used instead of anti-P-450_{sec} IgG. Scale bar = 100 μ m.

stained. When cyclic females were treated with progesterone (3 mg/day) for 9 days, the staining of the thecal and interstitial cells was markedly decreased to the level observed in pregnant or pseudopregnant females at the 9th day (data not shown).

Fig. 2. Distribution of immunostained P-450_{sec} in the ovary of a rat at oestrus. (a) One ruptured follicle, showing distinct immunostaining of theca (T) and granulosa (G) cells. (b) Higher magnification of the granulosa cells of the same follicle, showing faint but distinct staining of granulosa cell cytoplasm. (c) Luteal cells in a corpus luteum of the preceding cycle. (d) Luteal cells in a regressed corpus luteum of a remote cycle, invaded by connective tissue. Scale bar = 100 μ m (a), 20 μ m (b, c, d).

Hormonal treatments

Treatment with RU 486 for 48 h resulted in the reappearance of the immunohistochemical staining of P-450_{sec} in the thecal and interstitial cells of females pregnant for 18 days (Fig. 3b) or pseudopregnant for 9 days. This effect was not observed after 24 h. In progesterone-treated females, the decreased immunoreactivity of thecal interstitial cells was also reversed by RU 486 (data not shown). The stimulatory effect of RU 486 in 18-day-pregnant rats was counteracted by

Fig. 3. Immunostained P-450_{sec} in the ovary of an 18-day-pregnant rat after treatment with oestradiol and of RU 486. **(a)** Control. Unlike the luteal cells, there was no labelling of granulosa (G), thecal (T), and interstitial (I) cells. **(b)** At 48 h after RU 486 was injected, the interstitial (I) and thecal (T) cells were intensely immunostained. Granulosa cells (G) and the stromal connective tissue were not labelled. **(c)** At 48 h after treatment with RU 486 plus RU 39411, the immunostaining of thecal and interstitial cells observed with RU 486 alone did not occur. **(d)** Oestradiol counteracted the inhibitory effect of RU 39411 on the induction by RU 486. Thecal and interstitial cells were again stained. Scale bar = 50 μ m.

the antioestrogen RU 39411, and this effect of the antioestrogen was reversed by oestradiol (Figs 3c, d). The plasma concentrations of oestradiol, progesterone, LH and FSH were measured 24 h after the injection of RU 486. This time interval was selected because, although most rats had vaginal bleeding, only about 10% of the fetuses had been born, whereas between 30 and 48 h the greatest numbers of fetuses were delivered (Garfield *et al.*, 1987), and consequently the ovarian

Table 2. Plasma concentrations of oestradiol, progesterone, LH and FSH, in female rats

Treatment	No. of rats	Oestradiol (pg/ml)	Progesterone (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Late gestation*					
Control	4	71 ± 16	115 ± 11	1.6 ± 0.2	24.5 ± 1.6
RU 486	4	78 ± 9	126 ± 7	1.5 ± 0.6	34.2 ± 7.4
Cyclic adults†					
Control	6	40 ± 4	8.3 ± 5.8	2.2 ± 0.4	35.4 ± 3.9
RU 486	6	52 ± 5	18.5 ± 6.2	2.9 ± 0.3	30.0 ± 3.3
Oestradiol	6	102 ± 16	47.3 ± 8.5	2.9 ± 0.7	31.2 ± 3.9

Results are expressed as mean ± s.e.m.

*Pregnant females were untreated (controls) or treated with RU 486 (10 mg/kg) at Day 16 of pregnancy and killed 24 h later.

†Cyclic adult females (day of cycle unknown) were treated with RU 486 (10 mg/kg) or with oestradiol benzoate (50 µg) 48 h before being killed.

changes might result from delivery rather than from an effect of RU 486. In these conditions, no difference was observed between control and treated pregnant females (Table 2).

In intact non-pregnant females, the antiprogestrone RU 486 slightly enhanced the intensity of staining of the thecal and interstitial cells compared to untreated controls (Figs 4a, b). The staining of P-450_{sec} was even stronger and its area was larger after the oestradiol treatment (Fig. 4c). Neither RU 486 nor oestradiol produced any change of the plasma concentrations of LH and FSH 48 h after a single injection of either compound (Table 2). However, RU 486, and even more markedly oestradiol benzoate, tended to increase the plasma values of progesterone.

The post-partum rat ovary

At 24 h after delivery, the intensity of immunohistochemical staining of luteal cells was slightly lower than in corpora lutea at the 18th day of pregnancy. The interstitial gland cells and the cells of the theca interna, which remained unlabelled during mid- and late gestation, were intensely stained with the P-450_{sec} IgG (Fig. 5).

The oocytes

Unexpectedly, the specific P-450_{sec} antibodies detected an antigen in rat oocytes at all stages of follicular development. As no staining occurred when anti-P-450_{sec} IgG was preincubated with rat liver powder (data not shown), we concluded that the staining observed was not specific.

Discussion

The present work confirms that antibodies produced against bovine P-450_{sec} can be used for the immunohistochemical localization of the rat enzyme (Trzeciak *et al.*, 1986; Le Goascogne *et al.*, 1987). Sufficient numbers of animals were investigated in the main treatment groups to ensure the reproducibility of the observations, not only when the immunostaining of P-450_{sec} was shown either to appear or to disappear compared to the control groups, but also when changes of staining density only occurred.

Our data are in agreement with a previous report showing that, in immature rat follicles (Zlotkin *et al.*, 1986), P-450_{sec} was confined to the belt-like regions of the interstitial cells and some of the theca interna cells, whereas granulosa cells were clearly devoid of P-450_{sec}. In PMSG-treated

Fig. 5. Immunostained P-450_{sc} in the ovary of a post-partum rat. At 24 h after parturition, the thecal (T) and interstitial (I) cells were heavily immunostained. a = tunica albuginea, G = granulosa cells. Scale bar = 50 μ m.

immature rats, follicles had attained the preovulatory stage after 48 h, and the granulosa cells in all preovulatory follicles expressed a high P-450_{sc} content (Zlotkin *et al.*, 1986). However, we found a different labelling pattern in spontaneously ovulatory cyclic females. The granulosa cells were devoid of P-450_{sc}, even in mature Graafian follicles. Expression of P-450_{sc} was found only in granulosa cells of ovulated follicles. Also, in PMSG-treated immature rats, P-450_{sc} labelling was observed throughout the ovary in thecal and interstitial cells associated with follicles at any phase of follicular maturation (Zlotkin *et al.*, 1986), whereas in cyclic females staining for P-450_{sc} was observed in some cells of the theca interna of larger antral follicles, and formed a continuous belt only in Graafian follicles. Similar results have been reported for the cow ovary (Rodgers *et al.*, 1986a).

Therefore, it is likely that the PMSG-induced follicular maturation in immature rats is not exactly representative of the events occurring during the oestrous cycle, because untimely expression of P-450_{sc} seems to take place in the thecal and granulosa cells of developing follicles. However, it is also possible that the sensitivity of the technique of Zlotkin *et al.* (1986), who used frozen sections, an homologous antiserum and a second fluorescent antibody, was better than ours and could detect P-450_{sc} in cells with low expression of the enzyme. Indeed, in the cow ovary, a slight staining of the membrana granulosa of some large antral follicles has been reported (Rodgers *et al.*, 1986a), and autoradiographs of P-450_{sc} immunoblots indicate the presence of the protein in

Fig. 4. Immunostained P-450_{sc} in the ovary of a cyclic rat treated with oestradiol or RU 486. (a) Control. (b) RU 486 (48 h). (c) Oestradiol (48 h). The intensity of immunostaining of thecal (T) and interstitial (I) cells is increased by RU 486 and even more by oestradiol, including the hyperplastic theca interna of atretic follicles (at). CL = corpus luteum. Scale bar = 100 μ m.

small antral follicles (Hedin *et al.*, 1987), although in smaller amounts than in preovulatory follicles and corpora lutea. In this report, however, granulosa cells were not separated from the theca interna. Filter hybridization assays have indicated that P-450_{sec} mRNA is low in granulosa cells and thecal cells of small antral follicles and increased in both tissues of preovulatory follicles (after treatment with a low dose of hCG) (Goldring *et al.*, 1987).

The expression of P-450_{sec} was greater in mature corpora lutea than in young ones, a result previously reported for cow ovaries (Rodgers *et al.*, 1986b). However, in regressed corpora lutea from previous rat oestrous cycles, expression of P-450_{sec} in the few luteal cells remaining in the invading connective tissue tended to increase. The regression of the corpus luteum is accompanied by a decrease of steroid dehydrogenase and by an increase of phosphatase and aminopeptidase activities (Deane *et al.*, 1962; Lobel & Levy, 1968). Apparently, the morphologically identifiable luteal cells continue to express the P-450_{sec} enzyme. These results suggest that, once P-450_{sec} mRNA and protein are induced in luteal tissue, they may be constitutively expressed (Goldring *et al.*, 1987).

The pregnant rat ovary has been widely used as a model of functional and morphological differentiation of theca and granulosa cells (Carson *et al.*, 1981). Pregnancy in this species is normally of 23 days duration and encompasses several distinct phases of endocrine activity, Days 13–18 being characterized by low serum LH concentrations, maximal luteal activity, and an absence of ovulatory follicles. After Day 18, LH concentrations gradually increase through to Day 23 and the first ovulation occurs immediately after parturition. Light and electron microscopy, and measurements of steroid hormones, have indicated that the theca interna cells isolated from small antral follicles on Day 16 of pregnancy are devoid of steroidogenic activity (Carson *et al.*, 1981). Our immunohistochemical results are in agreement with these observations: the thecal and interstitial cells on Day 18 of pregnancy did not express the P-450_{sec} antigen. Goldring *et al.* (1987) have shown that, whereas the corpus luteum expressed high levels of P-450_{sec} mRNA throughout pregnancy, the mRNA level in residual tissue was very low. The down regulatory role of progesterone in pregnant animals is suggested by the disappearance of P-450_{sec} immunostaining in females treated with progesterone for 9 days. Progesterone has been reported to inhibit the sensitivity of the follicles to gonadotrophins (Morishige *et al.*, 1973). In females treated with RU 486, the increase of P-450_{sec} immunostaining in cyclic ovaries and its appearance in ovaries of pregnancy might be related to the antagonism of progesterone action.

Oestradiol had an effect similar to RU 486 in cyclic females. Oestradiol may act directly on the ovary to induce P-450_{sec} synthesis (Toaff *et al.*, 1983), to sensitize stromal interstitial cells to LH, or it may exert a positive feedback on LH secretion. Indeed, LH has an obligatory role in the initiation of preovulatory follicular growth in the pregnant rat (Bogovich *et al.*, 1981). In the present work, no significant changes of gonadotrophins were observed in cyclic females 48 h after treatment with RU 486 or oestradiol, whereas plasma progesterone concentrations were increased. In 18-day-pregnant females, RU 486 treatment had no effect on plasma hormones 24 h later. The immunostaining of P-450_{sec} was detected only after 48 h. However, the published work on P-450_{sec} regulation in the rat ovary by combined oestrogen and FSH has indicated that P-450_{sec} was not detectable, in granulosa cells, until 24 h after the start of FSH injections (Goldring *et al.*, 1987). Therefore, any effect of RU 486 on P-450_{sec} mediated by gonadotrophins should be indicated by an increase of LH at 24 h before the appearance of immunodetectable P-450_{sec}. Moreover, RU 486 did not influence plasma gonadotrophins, in our cyclic females, 48 h after injection, and has no effect on plasma gonadotrophins throughout the luteal phase (Asch & Rojas, 1985). We therefore tentatively conclude that the steroids are acting directly on the ovary. Oestrogen and progesterone have opposite effects on the amounts of thecal–interstitial P-450_{sec}. Oestradiol increases the intensity of immunostaining of thecal–interstitial cells, whereas progesterone markedly decreases the staining of these cells. After Day 9 of pregnancy or pseudopregnancy, the effect of progesterone seems to predominate. The progesterone antagonist RU 486 unmasks the inductive effect of oestradiol. The antioestrogen in turn counteracts the latter effect.

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