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Steroid levels and the spatiotemporal expression of steroidogenic enzymes and androgen receptor in developing ovaries of immature rats

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ABSTRACT

Immunoexpression of 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 (P450c17), androgen receptor (AR), and steroid contents were studied in the ovaries of immature female Wistar rats killed between postnatal days 1 and 30. During days 1–7, ovarian somatic structures lacked AR, 3β-HSD and P450c17, except for the surface epithelium, which featured the presence of these three proteins, suggestive of its androgen responsiveness and steroidogenic function. On day 10, AR appeared in many somatic structures, including the granulosa layers, which coincided with the P450c17 immunoexpression in some theca/interstitial cells, and an increase in ovarian androgen concentration. On the following days a further rise in ovarian androgen and progesterone contents paralleled an increase in 3β-HSD and P450c17 immunoexpression in the theca layer cells and primary interstitial cells. However, the development of the follicles constituting the first follicular wave was aberrant, since they lacked AR expression until the preantral stage and were characterized by a delayed onset and much lower expression of the thecal P450c17. They could not ovulate, since ovarian content of estradiol was too low to evoke the LH surge. The clusters of the secondary interstitial cells found on day 30 exhibited predominant expression of 3β-HSD over P450c17, suggesting more intensive progesterone than androgen synthesis in these structures.

Introduction

In the rat, the formation of primordial ovarian follicles is almost entirely postnatal and some of them initiate growth within the first few days after birth. They constitute the first wave of growing follicles that is thought to ensure the first ovulation in the reproductive life (e.g. Rajah et al., 1992; Hirshfield and DeSanti, 1995; Guigon et al., 2003). This relatively uniform development creates a useful model for studying a variety of processes involved in follicular growth and differentiation, as well as atresia and formation of the secondary interstitial cells. However, it is known that the rate of development of the first wave of follicles in juvenile rats is more rapid (about 35 days) than that in adult cycling animals (about 60 days) (Hirshfield, 1991; McGee et al., 2000). Additionally, as indicated by Hirshfield and DeSanti (1995), the medullary follicles, which are the first to initiate growth in the neonatal rat ovaries, differ in their morphology and proliferation patterns from the cortical ones developing during the mature reproductive lifespan. We assumed that more differences exist between equivalent ovarian follicles growing in the neonatal rats and in older or mature ones, as these follicles develop in a quite different ovarian environment resulting in particular from differences in the levels of pituitary gonadotropins and ovarian steroid concentrations.

The steroidogenic environment of neonatal ovaries has been poorly defined so far, since only serum steroid levels, comprising also adrenal output, but not ovarian steroid concentrations, were measured in immature rats (Parker and Mahesh, 1976; Mathews et al., 1987; Guigon et al., 2003; Zapatero-Caballero et al., 2004). There are numerous data on steroid release and responsiveness to gonadotropins of neonatal and immature rat ovaries, studied in short-term incubations or organ cultures (e.g. Lamprecht et al., 1979; Carson and Smith, 1986; Mathews et al., 1987; Gelety and Magoffin, 1997). However, applied *in vitro* methods allowed establishing ovarian steroidogenic capabilities, but not *in vivo* ovarian steroid synthesis, output or concentrations. Measurements of steroid contents determined in the present study in homogenates from neonatal and immature rat ovaries provided such data.

There are reports on the immunolocalization of steroidogenic enzymes in fetal and neonatal ovaries of various species. Greco and Payne (1994) examining mouse fetal ovaries detected 3β-HSD

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mRNA, but did not find the expression of P450scc and P450c17 in the majority of investigated gonads. In contrast, developing female gonads collected from sheep fetuses showed expression of 3β-HSD, P450scc and P450c17 already around the time of morphological sexual differentiation (Quirke et al., 2001). Very early ovarian expression of P450c17 was also found in human fetuses (Cole et al., 2006). Schwartz and Roy (2000) provided data on the expression of P450scc and P450c17 in the neonatal hamster ovary. With regard to the rat, Juneau et al. (1993) and Le Goascogne et al. (1991) provided data on ovarian ontogeny of 3β-HSD and P450c17, respectively. Gelety and Magoffin (1997) determined the immunolocalization of both P450scc and P450c17, and histochemical staining for 3β-HSD in ovaries of female neonatal rats. However, the latter authors described the investigated enzymes as localized exclusively to the theca interna cells of the developing follicles.

It has already been established that normal folliculogenesis requires androgen production for both androgen receptor-mediated action and its conversion to estrogens. In the cyclic rats, the granulosa cells of differentiating follicles exhibit characteristic sequential changes in AR distribution (Tetsuka et al., 1995; Szołtys and Słomczyńska, 2000). Preantral ovarian follicles express a great number of ARs in their granulosa layer, but during further follicular differentiation the number of ARs declines and androgens are converted with an increasing efficiency to estrogens owing to the rising concentration of cytochrome P450arom (Tetsuka and Hillier, 1997). However, so far the onset of androgen production in the immature animals was considered as an indicator of differentiation of the theca interna cells starting to supply substrates for estradiol production, while the expression of AR, necessary for androgen action, was not taken into consideration.

The objective of our study was to analyze the spatiotemporal expression of ovarian AR, P450c17 and 3β-HSD during rat ontogenesis and to establish some correlations between the onset and expression patterns of the investigated protein. We were also interested in establishing some differences in the expression pattern of the investigated proteins existing in follicles growing in the immature ovaries compared to those developing in adulthood. Of interest to the present study was also the transformation of atretic follicles into the secondary interstitial cells with regard to steroidogenic enzyme immunoexpression. Our studies included the analysis of ovarian steroid concentrations on the same days selected for immunohistochemical studies.

Materials and methods

Animals and tissue preparation

Experiments were performed in accordance with Polish legal requirements under license given by the Local Ethics Committee at the Jagiellonian University. The groups of experimental female Wistar rats included neonatal, immature and prepubertal rats kept in a 12 h light:12 h darkness schedule. The day of birth was designated as day 1. The pups were housed with their lactating mothers until day 21 when weaning took place. The animals were killed sequentially on days 1, 5, 7, 10, 14, 21 and 30. Excised ovaries ($n = 4$ or more) were subjected to steroid content analysis or they were used ($n = 3$ or more) for immunohistochemistry. The rats attained sexual maturity at the age of about 36 days.

Steroid content analysis

Steroid concentrations were determined in homogenates of ovaries using specific radioimmunoassay as described previously by Szołtys et al. (1994). Progesterone was measured using [1,2,6,7-^{3}H]progesterone (specific activity 96 Ci/mmol; GE Healthcare, Amersham International, Little Chalfont, Bucks., UK) as a tracer and an antibody induced in sheep against 11α-hydroxyprogesterone succinyl: BSA (a gift from Professor Brian Cook, University of Glasgow, Glasgow, Scotland). The lower limit of sensitivity of the assays was in the order of 20 pg. Coefficients of variation within and between assays were below 5.0% and 9.8%, respectively.

Androgens were measured using [1,2,6,7-^{3}H]testosterone (specific activity 81 Ci/mmol; GE Healthcare, Amersham Int.) as a tracer and rabbit antibody against testosterone-3-0-CMO:BSA (a gift from Dr. Bela Ričařova, Institute of Radiology, Czech Academy of Sciences, Prague, Czech Republic). The lower limit of sensitivity was in the order of 5 pg. Cross-reaction of this antibody was 18.3% with dihydrotestosterone, 0.1% with androstendione and less than 0.1% with other major ovarian steroids. Since this antibody also significantly bound dihydrotestosterone, the measured steroids were referred to as androgens rather than testosterone. Coefficients of variation within and between assays were below 5.0% and 9.7%, respectively.

Estradiol-17β was determined using [2,4,6,7-^{3}H] estradiol (specific activity 104 Ci/mmol: GE Healthcare, Amersham Int.) as a tracer and rabbit antibody against estradiol-17-O-carboxymethyloxime: BSA (a gift from Professor Roman Rembiesa, Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland). The lower limit of sensitivity of the assays was 5 pg. Cross-reaction was 1% with keto-estradiol-17β, 0.8% with estrone, 0.8% with estriol, 0.01% with testosterone and less than 0.1% with major ovarian steroids. Coefficients of variation within and between assays were below 4% and 7.5%, respectively. For estradiol and androgen assays, appropriate aliquots of homogenates were extracted with 2.5 ml ethyl ether, and for progesterone with 2.5 ml *n*-hexane. All samples were assayed in duplicate.

Immunohistochemistry

The ovaries were fixed in 10% buffered formalin overnight, routinely embedded in Paraplast (Monoject Scientific Division of Sherwood Medical, St. Louis, MO, USA). Sections (7 μm) were mounted on slides coated with 3-amino-isopropyl-triethoxysilane (Sigma–Aldrich, Poole, UK). Immunohistochemistry was performed as previously described by Szołtys et al. (2010). Briefly, sections were deparaffinized, rehydrated and subjected to microwave oven (750 W) antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 12 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in Tris buffered saline (TBS) for 20 min. To block non-specific binding, sections were incubated in 5% normal goat serum in TBS for 30 min. Sections then were incubated overnight with either a polyclonal rabbit anti-human AR antibody (NCL-ARp; Novocastra Laboratories, Newcastle upon Tyne, UK), at a 1:10 dilution, or with a polyclonal anti-recombinant mouse 3β-HSD (a gift from Professor Anita H. Payne, Stanford University Medical Center, CA, USA), at a dilution 1:1000, or with a polyclonal rabbit anti-bovine P450c17 (a gift from Professor Alan J. Conley, University of California, Davis, CA, USA) at a 1:100 dilution. The following day, the sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratory, Burlingame, CA, USA) at a 1:300 dilution in TBS with 0.1% Tween 20 (TBST) for 1.5 h, and streptavidin-horseradish peroxidase complex (ABC/HRP, Dako/AS, Glostrup, Denmark), at a1:100 dilution in TBS for 40 min. Bound antibody was visualized with TBS containing 0.01% H_2O_2 0.05% diaminobenzidine (DAB; Sigma–Aldrich, St. Louis, MO, USA) and 0.07% imidazole for 1 min.

Negative controls were performed by substituting the primary antibody with nonimmune rabbit immunoglobulin G (IgG). Sections were dehydrated through a series of graded ethanol solutions,