Immunolocalization of cytochrome P450 17alpha-hydroxylase/c17-20 lyase in the ovary of pregnant pigs and fetal gonads

Katarzyna Knapczyk-Stwora, Magdalena Sternak, Małgorzata Durlej, Maria Słomczyńska
Department of Endocrinology, Institute of Zoology, Jagiellonian University, Krakow, Poland

Received: 24 September 2010; accepted: 30 March 2011

SUMMARY
The study was designed to localize P450 17alpha-hydroxylase/c17-20 lyase (P450c17) in the ovaries of pregnant pigs and fetal gonads. Immunoreactivity of P450c17 was investigated in the porcine ovaries (follicles and corpora lutea; CL) collected on days 10, 18, 32, 50, 70 and 90 post coitum (p.c.), and fetal gonads (testes and ovaries) on days 50, 70 and 90 p.c. The presence of P450c17 in ovarian follicles was demonstrated on all examined days of pregnancy but was restricted to theca interna cells. In CL, P450c17 was detected on all examined days of pregnancy but only in small luteal cells. In the female porcine fetuses, P450c17 immunostaining was found in oocyte nests and granulosa cells of primary ovarian follicles, while in the male fetuses in fetal Leydig cells. In conclusion, the immunolocalization of P450c17, detected in the ovaries of pregnant pigs and fetal porcine gonads, indicates the potential sites of androgen synthesis. We suggest that androgens may play a role in the maintenance of pregnancy and in the development of prenatal gonads in pigs. Reproductive Biology 2011 2: 71-82.
Key words: P450c17, ovary, fetal ovary, fetal testis, pig, immunohistochemistry

1Corresponding author: Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland; e-mail: katarzyna.knapczyk@uj.edu.pl
INTRODUCTION
Steroid hormones play an important role in the growth and differentiation of reproductive tissues as well as in the maintenance of pregnancy and fertility [6]. 3β-hydroxysteroid dehydrogenase (3β-HSD) and P450 17α-hydroxylase/c17-20 lyase (P450c17) are important enzymes in the steroidogenic pathway leading to progesterone and androgen synthesis [3, 26]. P450c17 is a member of the cytochrome P450 group of oxidases [21]. This enzyme is a single protein possessing both hydroxylase and lyase activities, and catalyzes the conversion of pregnenolone and progesterone to their corresponding androgen products, dehydroepiandrosterone and androstendione, respectively [26].

It is generally accepted that progesterone is a crucial hormone for mammalian gestation [28]. In pigs, the corpus luteum (CL) is required to support pregnancy throughout the whole gestational period [10, 22]. Our previous study [16] clearly showed the ovarian presence of 3β-HSD, the enzyme involved in follicular and luteal synthesis of progesterone during pregnancy. We also showed the androgen receptor (AR) immunoexpression in granulosa, theca interna, stromal and luteal cells of porcine ovaries collected on day 10, 18, 32, 71, and 90 of gestation [7]. Consequently, we hypothesize that androgens, in addition to progesterone, may contribute to the regulation of pregnancy in pigs. Thus, the first aim of the study was to determine, via examination of P450c17 immunolocalization, the capability of the porcine ovary during pregnancy to synthesize androgens.

Burek et al. [1] demonstrated the presence of AR in the fetal testes and ovaries of pigs, suggesting that porcine fetal gonads are the sites of androgen action. Androgens are essential for male development. In pigs, P450c17 was first identified in the cytoplasm of Leydig cells on day 28 post coitum [(p.c.); 20]. Greco and Payne [15] and McCoard et al. [20] reported the absence of steroid production in fetal ovaries due to the lack of steroidogenic enzymes. On the contrary, Goxe et al. [14] showed that both male and female porcine fetuses secrete testosterone. Therefore, the second goal of the study was to determine the presence of P450c17 protein in porcine fetal testes and ovaries.
MATERIALS AND METHODS

Animals
The study was conducted in accordance with the Polish legal requirements, under the licence given by the National Commission of Bioethics at the Jagiellonian and Rzeszow Universities. Naturally cyclic crossbred gilts (Large White×Polish Landrace), exhibiting at least two estrous cycles of normal length (21 days) were observed for estrous behavior twice daily and mated to a fertile boar at the onset of estrus as well as 12 h and 24 h later. The first day of estrus was designated as day 0. Pregnancy was confirmed by the presence of conceptuses in uterine horns. Three pregnant sows per each day of gestation (days 10, 18, 32, 50, 70, and 90 p.c.) were ovarioctomized under anesthesia with thiopental. Fetuses were collected by hysterectomy on days 50, 70 and 90 p.c. from the corresponding animals. Fetal gonads (testes and ovaries) were obtained from 8–10 fetuses per each time point. The days of pregnancy chosen for the experiment are crucial for embryo and fetus development (day 10: before implantation, day 18: implantation, day 32: the beginning of sexual differentiation, days 50–70: midpregnancy, and day 90: late pregnancy).

Tissue collection
Large antral follicles (n=5-8 per ovary) and corpora lutea (n=4-6 per ovary) of pregnant sows as well as fetal gonads (testes and ovaries) were excised. Tissues were washed in phosphate buffered saline (PBS) and immediately fixed in 10% neutral buffered formalin, dehydrated in an increasing gradient of ethanol, embedded in paraplast (Monoject Scientific Division of Scherwood Medical, St Louis, MO, USA) and stored until immunohistochemistry was performed to investigate the localization and distribution of the P450c17.

Immunohistochemistry
Immunohistochemical analysis of P450c17 was performed as described earlier with some modifications [16]. Briefly, the 5 μm-thick sections were mounted on 3’3’-aminopropyl-triethoxysaline (APES, Sigma-Aldrich, St Louis, MO, USA) coated slides. Then, they were deparaffinized in xylene, rehydrated gradually using a series of ethanol dilutions and rinsed in water. Next, the sections immersed in 0.01 M citric acid buffer (pH 6.0) were
placed in the microwave oven (3×for 4 min, 600 W) to retrieve antigenicity. Endogenous peroxidase activity was blocked by incubation with 0.3% H$_2$O$_2$ in TBS (Tris-buffered saline, pH 7.4), and non-specific binding was prevented by 5% normal goat serum (Sigma-Adrich, St Louis, MO, USA). The primary rabbit polyclonal anti-porcine P450c17 antibody (provided by prof. Dale B. Hales from Southern Illinois University, Carbondale, USA) was applied to the sample at a dilution 1:100 and incubated overnight at 4°C. The antigen was visualized using biotinylated secondary antibody: goat anti-rabbit IgG (1:300, 1.5 h at room temperature, RT; Vector Laboratories, Burlingame CA, USA), avidin-biotin-peroxidase complex (1:100, 40 min at RT; StreptABComplex-HRP, Dako), and 3, 3’-diaminobenzidine (DAB, Sigma-Aldrich; USA) as a chromogen staining substrate. The sections were dehydrated and mounted in DPX (Fluka Chemie GmbH, Buchs). Negative control reaction included sections incubated with non-immune rabbit IgG instead of primary antibody and were processed as above. Selected sections were photographed using the Nikon Eclipse E600 microscope and the Coolpix P6000 digital camera (Nikon, Japan) with corresponding software.

RESULTS

P450c17 immunohistochemical staining was observed in the cytoplasm of cells forming the examined gonadal structures collected from pregnant pigs and fetuses. Negative controls did not exhibit positive staining (fig. 1b). On all days of pregnancy, the presence of P450c17 in the porcine ovarian follicles was restricted only to theca interna cells (fig. 1a, TI). It should be noted that not all theca cells showed a positive immunoreaction. Regardless of pregnancy day, no P450c17 protein was detected in granulosa (fig. 1a, G) and theca externa (fig. 1a, TE) cells of any follicles. The pattern of P450c17 staining was the same on each investigated day of pregnancy. Thus, fig. 1a is a representative for all examined follicles.

In porcine luteal cells, P450c17 immunostaining was not demonstrated by day 32 p.c. (fig. 1c). However, on days 50 p.c. (fig. 1d), 70 p.c. (fig. 1e) and 90 p.c. (fig. 1f), the P450c17 protein was detected only in small luteal cells.

In the male fetuses, an intense P450c17 immunostaining was observed in the fetal Leydig cells on each day of the prenatal period (fig. 2a-c). The fetal porcine ovarian cortex contained oocytes surrounded by stromal cells (oocyte nests; fig. 2d, e inset; ON) and numerous primary follicles with partial or single layer of granulosa cells on days 70 and 90 p.c. (fig. 2e, f).
The P450c17 staining was observed in oocyte nests (fig. 2d, e inset) and granulosa cells of primary follicles (fig. 2e, f).

Figure 1. Representative paraffin sections of P450c17 immunoexpression in ovarian follicles (a, b: day 70) and corpora lutea (days 32: c, 50: d, 70: e, 90: f) collected from pregnant sows. In ovarian follicles of pregnancy, P450c17 protein was observed only in the theca interna cells (a, small arrows). In corpora lutea of pregnancy, P450c17 immunoexpression was expressed in small luteal cells (arrows) on days 50 (d), 70 (e) and 90 (f). P450c17 protein was not demonstrated in corpus luteum before day 50 of pregnancy (c). P450c17 immunostaining was not observed when the primary antibody was substituted by non-immune rabbit IgG (b). G: granulosa cells; TI: theca interna cells; TE: theca externa cells. Bar = 50 µm.
Figure 2. Representative paraffin sections of P450c17 immunoexpression in fetal porcine testes (a-c) and ovaries (d-f) collected from pregnant sows on days 50 (a, d), 70 (b, e) and 90 (c, f). The P450c17 cytoplasmic immunostaining was observed in fetal Leydig cells (a-c, arrows), oocyte nests (d-e, arrowheads) and granulosa cells of primary ovarian follicles (e-f, short arrows). ON: oocyte nests. Bar = 50 µm
DISCUSSION

In this paper, the immunolocalization of P450c17 in the porcine ovaries of pregnant pigs and fetal gonads is reported. We demonstrated that P450c17 protein was expressed in theca interna cells throughout the entire porcine pregnancy. In luteal tissue, the protein was detected in small luteal cells starting from day 50 p.c. Moreover, in fetal testes, P450c17 was immunolocalized in Leydig cells, and in fetal ovaries, P450c17 was found in oocyte nests and granulosa cells of primary follicles.

Rat [29] and human [33] ovarian follicles produce steroids during pregnancy. Previously, we demonstrated that 3β-HSD is present in theca interna cells of follicles collected from pregnant sows [16]. The present study showed the expression of P450c17 in these cells, indicating their capability to produce steroid hormones. Similarly to 3β-HSD [16], the pattern of P450c17 immunostaining during pregnancy showed functional heterogeneity within porcine theca cells i.e. not all cells were positively stained. Pregnancy affects follicular maturation by reducing estrogenic activity and subsequent blocking ovulation in pigs [34]. It is consistent with the lack of P450 aromatase in follicles of pregnant swine [7]. Previously, we also demonstrated the presence of AR in granulosa cells of porcine follicles during pregnancy [7], which implicates that androgens are important for the regulation of reproductive processes during gestation. Therefore, theca cells are able to synthesize androgens which may be paracrine regulators of porcine follicle development during pregnancy.

In numerous mammalian species, including pigs, luteal tissue is a source of androgens and estrogens [9, 13, 27, 32]. In the current study, P450c17 protein was found in small luteal cells collected from ovaries on days 50, 70, and 90 of pregnancy of pigs. Porcine small luteal cells are steroidogenically active and capable of binding LH, a hormone which increases P450c17 expression [11, 35]. Our data support the hypothesis that LH is a crucial regulator of P450c17 expression in small luteal cells during pregnancy. The P450c17 protein was also demonstrated in small luteal cells of mares [27]. On the other hand, Weng et al. [32] was able to localize this enzyme in all luteal cells during pregnancy in the Shiba goat. It is possible that the observed differences in luteal expression of P450c17 are species-dependent.

In contrast to ovarian follicles, P450 aromatase was localized in porcine CL on day 90 p.c. [7]. This indicates that CL of pregnancy is
able to aromatize androgens. At the same time, the shift of AR from the nuclei to the cytoplasm was observed in porcine luteal cells, a finding indicating lack of AR activation [7]. This suggests that androgens do not act via AR pathway, but serve as a substrate for estrogen synthesis in CL of pregnant pigs. The presence of estrogen receptor β (ERβ) in porcine luteal tissue during gestation period [17] supports this hypothesis. Therefore, we suggest that androgens, in addition to progesterone, are essential for the maintenance of pregnancy in pigs. During pregnancy, androgens may act directly within ovarian follicles and indirectly in CL, after conversion to estrogens.

P450c17 was localized previously in porcine fetal Leydig cells from day 28 to day 50 p.c. [20]. Male sexual differentiation in pigs occurs around day 26 p.c. [19]. Therefore, the early expression of P450c17, an enzyme involved in androgen synthesis, is associated with gonadal differentiation into a male phenotype [24]. In the current study, P450c17 was found in porcine fetal Leydig cells on days 50, 70 and 90 p.c., demonstrating that androgen production also occurs during the second half of pregnancy. On the other hand, P450 aromatase was detected in porcine fetal Leydig cells starting from day 28 p.c. [24], suggesting the possibility of androgen aromatization. The study performed on ERKO mice showed a direct action of estrogens on male germ-cell development and fertility [4]. Moreover, endogenous estrogens during fetal and neonatal life physiologically regulated testicular development in a negative manner by controlling gametogenesis and steroidogenesis [5]. We hypothesize that estrogens, apart from androgens, are important factors affecting fetal male development, especially in view of the presence of AR [1] and ER [18] in male porcine fetuses.

In this paper we demonstrated for the first time the localization of P450c17 protein in the porcine fetal ovary. McCoard et al. [20] reported the absence of this enzyme before day 50 p.c. Our experiment was conducted on fetal ovaries on days 50, 70 and 90 p.c. Since testosterone was detected in plasma of porcine female fetuses during the second half of pregnancy, it is possible that P450c17 is functional in the fetal ovaries [14]. P450c17 activity and androgen synthesis were also shown in human fetal ovary [2, 25]. Although steroids are thought to have a minor role in the early ovarian development, studies on primates [30, 31], sheep [12] and mice [23]
increased the understanding of androgen function in the promotion of early follicular development. The presence of AR in the porcine fetal ovary was demonstrated on different days of gestation [1], which suggests a fetus capacity to respond to androgens.

Our present data together with earlier published results concerning antiandrogen treatment during prenatal period [8] support the hypothesis that androgens are important regulators of pregnancy in pigs by affecting development of ovaries and fetal gonads. In summary, the immunolocalization of P450c17 in ovarian tissue of pregnant pigs and fetal porcine gonads indicates that these organs are crucial for androgen synthesis. It suggests that androgens may be involved in the regulation of maintenance of pregnancy as well as fetal gonad development.

ACKNOWLEDGEMENTS
The authors express sincere acknowledgements to Professor Marek Koziorowski (Department of Physiology and Reproduction of Animals, University of Rzeszow) for a valuable assistance in the preparation of animals and to Dr. Anna Pecio (Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, Krakow, Poland) for providing imaging facilities. This work was supported by K/ZDS/001952. The part of this work was presented at The Society for Reproduction and Fertility Conference, Edinburgh, Scotland, 2008.

REFERENCES


