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Original article

Estrogen production by fetal and infantile rat ovaries

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Summary — The *de novo* production of estrone and estradiol by the fetal and infantile rat ovary and its regulation by cAMP, LH and FSH were investigated.
Basal estrogen secretion was not demonstrable before d 5 after birth. However, the fetal ovary was responsive to cAMP as early as 14 d old. Estrone was the main estrogen secreted. Steroid aromatase inhibitors inhibited cAMP-stimulated estrone secretion. Responsiveness to LH and FSH appeared at 5 d after birth. The proportion of estradiol grew more important with advancing age, becoming predominant at 7 d.

œstrone / estradiol / gonadotrophin / fetal rat ovary

Résumé — Production d'oestrogènes par l'ovaire fœtal et néonatal de rat. *Le présent travail a trait à la production d'œstrone et d'œstradiol par l'ovaire fœtal et néonatal de rat et sa régulation par l'AMPc, LH et FSH.*
Les ovaires ont été cultivés in vitro *sur milieu 199 additionné ou non de gonadotrophines et l'œstrone et l'œstradiol libérés dans le milieu de culture ont été dosés par radio-immunologie. Une secrétion de base n'était pas décelable avant le stade de 5 j après la naissance. Toutefois, en présence d'AMPc, on notait la formation des 2 œstrogènes dès le stade de 14 j de vie fœtale, l'œstrone étant prépondérante. Des inhibiteurs d'aromatisation inhibaient la sécrétion d'œstrogènes stimulée par l'AMPc. Une réponse à LH et FSH apparaissait au stade de 5 j après la naissance. Aux stades néonataux, la sécrétion d'œstradiol gagnait en importance pour devenir prépondérante à 7 j.*

œstrone / œstradiol / gonadotrophines / ovaire fœtal de rat

INTRODUCTION

While castration experiments performed by Jost (1946–1947) in the rabbit fetus had shown that the testis controlled male sexual differentiation in mammals, there was no indication that the ovary played a comparable role in females. Therefore researchers including ourselves were not concerned when they were unable to identify estrogens after incubation of fetal human (Jungmann and Schweppe, 1968), bovine (Weniger *et al*, 1972) or rat ovaries (Schindler and Friedrich, 1975) with [^{14}C]sodium acetate, [^{14}C]pregnenolone or [^{14}C]testosterone. However, the situation changed when Milewich *et al* (1977), using [^{3}H]testosterone and [^{3}H]androstenedione of high specific activity as precursors, demonstrated the formation of radioactive estradiol and estrone by the ovary of the rabbit fetus. Studies in man (George and Wilson, 1978), guinea pig (Sholl and Goy, 1978) and mouse (Terada *et al*, 1984) confirmed the existence of aromatase activity in the fetal ovary of these species.

In the rat fetus, ovaries cultured in the presence of either [^{3}H]testosterone, [^{3}H]androstenedione or [^{3}H]dehydroepiandrosterone almost exclusively formed estrone (Weniger *et al*, 1984, 1985; Weniger and Zeis, 1985). However, after birth synthesis of estradiol increased in importance and by d 14 had supplanted that of estrone (Picon *et al*, 1985; Weniger and Zeis, 1986, 1987). Aromatase activity could be stimulated by cAMP in the fetal ovary as early as 17-d-old (Weniger and Zeis, 1988a, 1988b). Stimulation of aromatase activity by FSH was not noted in ovaries earlier than 2 d after birth (Weniger and Zeis, 1990). LH was without effect.

The aim of the present investigation was to study the *de novo* production of estrogens by the fetal and infantile rat ovary and its regulation by gonadotrophins.

MATERIALS AND METHODS

Animals

Rats of the Wistar strain were used. The day following the night of cohabitation was considered to be d 0 of gestation.

Organ culture

The ovaries were removed aseptically from 14- to 21-d-old fetuses and from 1- to 7-d-old infantile rats. They were cultured *in vitro*, with 1 ovary per culture dish, in 0.1 ml Medium 199 alone or with addition of various substances in a Nunc plastic Petri dish, either whole or cut into 2–5 thin pieces according to their size. The Petri dishes were placed in an airtight jar, gassed with 95% O_2–5%CO_2 and incubated at 37°C for various time intervals. To provide the best culture conditions, care was taken not to immerse the explants, so that their upper surface was exposed to the atmosphere. When the culture time was over, the Petri dishes were inspected for infection and condition of the explants. Infected media and media of necrotic explants were discarded. Healthy explants were rinsed with 1 ml water. The diluted culture media were kept at –20 °C until radioimmunoassay.

Radioimmunoassay

Direct radioimmunoassay of either estrone or estradiol was not possible. Extraction of the culture media with 2 x 2 ml of isooctane–ethyl acetate 7:3 was an obligatory step in the procedure. The dry extract was taken up in 1 ml of gelatin-containing phosphate-buffered saline (pH 7.4), which also served as the assay buffer. Twenty to 300-μl aliquots were used in the assay. The volume of the reaction mixture was 1 ml. [2,4,6,7-^{3}H]Estrone (105 Ci/mmol) and [2,4,6,7-^{3}H]17β-estradiol (100 Ci/mmol) came from Du Pont de Nemours. Anti-estrone and anti-estradiol antisera were gifts from Roussel–Uclaf Romainville); they were directed toward 7-carboxymethyloxime estrone–bovine serum albumin and 7-carboxymethyloxime estradiol–bovine serum albumin, respectively, and used at

a final working dilution of 1/450 000 and 1/500 000, respectively. Cross-reaction of the anti-estrone antiserum with estradiol was 1.9%, and that of the anti-estradiol antiserum with estrone was 0.7%. Standards ranged from 3–240 pg/tube. Free estrogen was absorbed on a charcoal–dextran mixture. Samples were assayed in duplicate. All samples of a series were run in the same assay to avoid interassay variations.

Substances tested

Bovine LH (NIH-LH-B9) and ovine FSH (NI-ADDK-oFSH-17), gifts from the National Hormone and Pituitary Program (NIH, Bethesda), were used at a 10 μg/ml concentration and (Bu)2 cAMP (Sigma) at a 0.25 mM concentration. 4-Hydroxyandrostenedione, a gift from Ciba–Geigy (Basel), and 1-methyl-1,4-androstadiene-3,17-dione, donated by Schering (Berlin), were used in the range of 0.1–10 μM. In order to determine possible deleterious effects of these substances that might have escaped macroscopic observation, several explants exposed to the highest concentration, *ie* 10 μM, were examined histologically.

Statistical analysis

Results are shown as means ± SD, with *n* given in parentheses. Differences between group means were analyzed by 1-way analysis of variance, followed by the Tukey test or Newman–Keuls test if differences were significant. A P value of < 0.05 was considered significant. Regression analysis and Student's paired *t*-test were also employed (Zar, 1984). Data shown are representative of at least 2 experiments of the same kind.

RESULTS

Validation of estrone and estradiol radioimmunoassay

Total binding represented ≈ 50% of the radioactivity added. Non-specific binding was 3–4%. The sensitivity of the assay, defined as the amount of estrogen that lowers the initial binding by 5%, was 1.4 ± 0.7 pg (n = 10) for estrone and 2.1 ± 0.5 pg (n = 10) for estradiol. The accuracy of the assay was evaluated by determining the recovery of known amounts of estrogen added to the culture media before the extraction step and by the linearity test. The recoveries of 12, 24 and 48 pg estrone were, respectively, 13.8 ± 0.7 (n = 5), 26.7 ± 2.6 (n = 5) and 44.2 ± 3.8 (n = 4) and of 12.5, 20 and 25 pg estradiol, 13.0 ± 1.4 (n = 4), 22.4 ± 3.6 (n = 12) and 26.0 ± 2.3 pg (n = 4), respectively. The differences between the theoretical and the determined values were evaluated by the paired-sample *t*-test and were found to be not significant. The linearity test for estrone, performed on 6 samples at 3 different volumes (20, 50 and 100 μl), showed that the amount of estrone was proportional to the volume of extract (r = 0.9978). The test of linearity for estradiol test was also conclusive (25, 50, 100 and 200 μl; n = 10, r = 0.9898). The intra- and inter-assay coefficients of variation were, respectively, 6.7% (n = 10) and 8.5% (n = 10) for estrone and 7.3% (n = 10) and 10.5% (n = 10) for estradiol. Net sample values were obtained after subtracting the extraction blank.

The cross-reactions of 1-methyl-1,4-androstadiene-3,17-dione and 4-hydroxyandrostenedione with the anti-estrone antiserum were determined to be 0.0010% and 0.0013%, respectively. At the concentrations used, the contribution of these substances in the estrone radioimmunoassay was negligible.

Effect of LH, FSH zand $(Bu)_2$ cAMP on estrogen secretion by the 21-d-old fetal rat ovary

In the pilot experiment, neither estrone nor estradiol were detectable in the culture me-

dia of 21-d-old fetal rat ovaries cultured for 3 d in Medium 199 alone (n = 4) or with the addition of 10 µg/ml LH (n = 4) or FSH (n = 4). On the contrary, after culture in the presence of 0.25 mM $(Bu)_2$cAMP for 3 d, the quantity of estrone in the medium rose to 930 ± 520 pg (n = 3) and the quantity of estradiol to 120 ± 60 pg. So when the ovary is taken from the fetus at 21 d, *ie* just before birth, and cultured for 3 d under cAMP stimulation, it secretes much more estrone than estradiol 'E_1/E_2 = 7.8 ± 0.5).

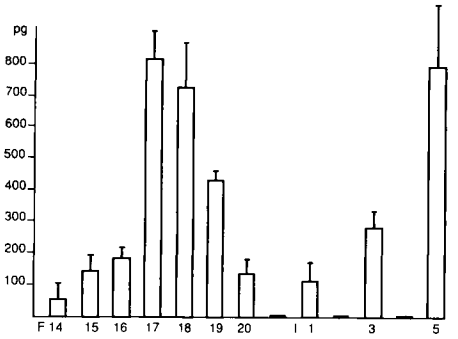

Fig 1. Total estrogen secretion of 14- to 20-d-old fetal (F) and 1- to 5-d-old infantile (I) rat ovarie during a 24-h culture period in the presence of 0.25 mM $(Bu)_2$cAMP. Three to 4 determinations were made at each stage. Bars: SD.

Early responsiveness to $(BU)_2$cAMP

The fetal rat ovary responds to $(Bu)_2$cAMP as early as 14 d old, *ie* soon after morphological sexual differentiation of the gonadal primordia at 13.5 d (table I). cAMP-stimulated estrogen secretion is highest at fetal age 17–18 d and lowest at the perinatal stages (fig 1).

Influence of culture time

Figure 2 shows total estrogen secretion of 17-d-old fetal rat ovaries cultured for 12, 24, 48 and 72 h in the presence of 0.25 mM $(Bu)_2$cAMP. Regression analysis supported the linearity hypothesis.

Onset of basal estrogen secretion and responsiveness to LH and FSH after birth

Neither estrone nor estradiol were detectable in the media of ovaries from 1-d-old ne-

Table I. Secretion of estrone and estradiol by fetal (F) and infantile (I) rat ovaries during a 24-h culture period in the presence of 0.25 mM $(Bu)_2$cAMP.

	F14 d (n = 4)	*F15 d* (n = 4)	*F16 d* (n = 4)	*F17 d* (n = 4)	*F18 d* (n = 4)	*F19 d* (n = 3)	*F20 d* (n = 8)	*I1 d* (n = 8)	*I3 d* (n = 4)	*I5 d* (n = 4)
Estrone	70±60	215±93	120± 9	730±81	683±124	373±32	134±47	111±59	229±37	533±101
Estradiol	42±46	79±25	66±30	86±17	46± 21	55±4	ND	ND	52±24	255±108
E_1/E_2	1.7	2.7	1.8	8.5	14.8	6.8	–	–	4.4	2.1

Means (pg) ± SD; ND: not detectable; at fetal stages 14 and 15 d, both ovaries of a fetus were cultured in the same dish; at all other stages, only 1 ovary was cultured per dish.

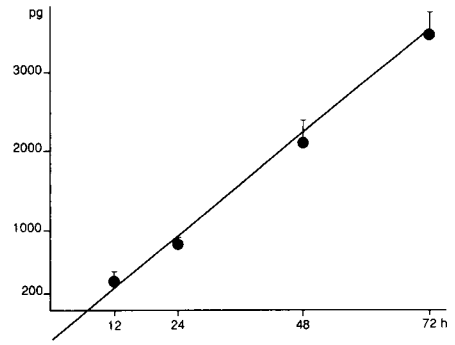

Fig 2. Time-course study of total estrogen secretion of 17-d-old fetal rat ovaries cultured in the presence of 0.25 mM $(Bu)_2$cAMP.

onates cultured for 3 d in Medium 199 alone (n = 4) or with the addition of 10 μg/ml LH (n = 4) or FSH (n = 4). Ovaries from 3-d-old neonates gave the same result. Estrone and estradiol were first present in the media of 5-d-old infantile rat ovaries cultured for 24 h under basal conditions. LH and FSH greatly enhanced the release of estrogen (table II). Therefore basal estrogen secretion and responsiveness to LH and FSH appear in the rat ovary at the same time, *ie* at ≈ 5 d after birth. The secretion of estrone, which was still predominant at 5 d, drops behind that of estradiol at 7 d. A higher intracellular concentration of cAMP leading to a higher estrogen production seems to be brought about through LH or FSH rather than by passive penetration of $(Bu)_2$cAMP into the cell.

Effect of aromatase inhibitors

The presence in the culture medium of an aromatase inhibitor besides $(Bu)_2$cAMP resulted in a dose-related decrease in estrone release as shown in figure 3. Explants exposed to the highest concentrations of inhibitor were found to be in a healthy condition. This experiment demonstrates the stimulatory action of cAMP upon aromatase.

DISCUSSION

The *de novo* production of estrogens by the fetal ovary has already been investigated in several mammalian species. The fetal sheep ovary secretes large amounts of

Table II. Secretion of estrone and estradiol by 5- and 7-d-old infantile rat ovaries cultured for 24 h under basal conditions or in the presence of LH (10 μg/ml), FSH (10 μg/ml) or $(Bu)_2$cAMP (0.25 mM).

		–	*LH*	*FSH*	*$(Bu)_2$cAMP*
5 d (n = 4)	E_1	77 ± 78	535 ± 44	900 ± 23	533 ± 101
	E2	28 ± 22	210 ± 63	550 ± 165	255 ± 108
	E_1/E_2	2.8	2.5	1.6	2.1
7 d (n = 5)	E_1	176 ± 38	462 ± 232	356 ± 130	276 ± 53
	E2	480 ± 98	1 388 ± 654	1 242 ± 461	630 ± 148
	E_1/E_2	0.4	0.3	0.3	0.4

Means (pg) ± SD.

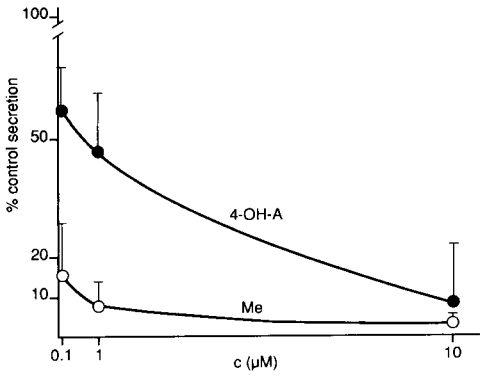

Fig 3. Inhibition of $(Bu)_2$cAMP-stimulated (0.25 mM) estrone secretion of 19-d-old fetal rat ovaries cultured for 24 h in the presence of 0.1–10 μM 4-hydroxyandrostenedione or for 48 h in the presence of 0.1–10 μM 1-methyl-1,4-androstadiene-3,17-dione. Each point represents the mean of 4 determinations. Bars: SD.

estradiol in culture around the time of sexual differentiation (d 31). This capacity is lost at ≈ 62 d (Mauléon *et al,* 1977). A similar transient phase during which the ovary secretes estradiol also exists in the bovine fetus. It corresponds to the stage of 5–8-cm crown-rump length. LH and testosterone increased estradiol secretion 3- and 4-fold, respectively (Shemesh, 1980). In the rabbit fetus, ovaries as early as 16-d-old secrete estradiol in culture (George and Wilson, 1980).

In the rat fetus, estradiol secretion by the ovary could not be detected unless $(Bu)_2$cAMP was added to the culture medium (Benhaïm *et al,* 1983). This has been confirmed in the present study. Under cAMP stimulation, estrogen production occurs in the ovary as early as fetal age 14 d. During the whole fetal period, the main estrogen secreted is estrone, *ie* the weaker estrogen. It is only at the end of the first week after birth that estrone is supplanted by estradiol, the main estrogen in adult life. It should be noted that cAMP-stimulated fetal ovarian aromatase activity is inhibited by anti-Müllerian hormone (Vigier *et al,* 1989; Di Clemente *et al,* 1992).

Basal estrogen secretion and responsiveness to LH and FSH appear in the rat ovary at ≈ 5 d after birth, which is also the time of the appearance of LH and FSH receptors (Peluso *et al,* 1976; Smith White and Ojeda, Sokka and Huhtaniemi, 1990). LH may enhance the availability of androgen precursor, while FSH may stimulate aromatase activity. When aromatase activity was measured by the conversion of 19-hydroxyandrostenedione into estrone and estradiol, a stimulating effect of FSH but not of LH was demonstrated (Weniger and Zeis, 1990).

The notion of basal estrogen secretion may be misleading. What we term basal secretion has probably been elicited by endogenous LH and FSH (Ojeda and Ramirez, 1972) after appearance of their receptors. This is supported by the fact that estrogen was present in media of 5-d-old ovaries cultured for 24 h in the absence of ganodotrophin, and not in media of 3-d-old ovaries cultured for 3 d. Steroidogenesis in the ovary cannot begin before cholesterol side-chain cleavage activity has been initiated by LH (Rouiller *et al,* 1990). So the sequence of events leading to estrogen secretion by the rat ovary may be the following: secretion of LH and FSH from fetal stages (Chowdhury and Steinberger, 1976), appearance of LH and FSH receptors in the ovary at ≈ 5 d after birth, induction or activation of the cholesterol side-chain cleavage enzyme, and autonomous or FSH-stimulated aromatization of androgen precursor. However, this scheme may not be applicable to other species in which estrogen is secreted during fetal life.

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