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CONVERSION OF ANDROGEN TO ESTROGEN BY THE RAT FETAL AND NEONATAL FEMALE GONAD: EFFECTS OF dcAMP AND FSH

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Summary—Female gonads of fetal (on days 14.5, 16.5, 18.5 and 20.5 postcoitum) and neonatal rats (on days 4.5 and 8.5 postpartum) were cultured in Medium 199 in the presence of [^{3}H]testosterone and the conversion into [^{3}H]estrone and [^{3}H]estradiol was estimated. Formation of both estrogens was found in all fetal and neonatal ovaries explanted in control medium. Dibutyryl cAMP (1 mM) had a clear-cut stimulatory effect as early as 16.5 days postcoitum, but had little or no effect at 8.5 days postpartum. In contrast, ovine or rat FSH (0.3 or 1 μg/ml, respectively) increased the aromatase activity only from 20.5 days postcoitum. The effects of FSH and dibutyryl cAMP were more obvious after preculture for 48 h in control medium. These results indicate that: (1) a biochemical sex differentiation, revealed by the difference in aromatase activity levels between ovaries and testes or other tissues occurs in female gonads as early as 14.5 days postcoitum; (2) aromatase activity in the ovaries increases markedly after birth; (3) functional FSH receptors are absent before 20.5 days postcoitum in the ovaries.

INTRODUCTION

During fetal development, the differentiation of the female phenotype does not require the presence of gonads [1] although the existence of steroidogenic activity in fetal ovaries has not been excluded. Actually, in some species, a faint or transitory synthesis of estrogens has been described [2–4]. In the rat, the onset of estrogen synthesis seems to occur a few days after birth [5]. However, we noticed that fetal rat ovaries treated with dibutyryl cAMP (dcAMP) started to produce estradiol-17β *in vitro* from 14.5 days [6]. Several steps of steroidogenesis, one of which is aromatization [7], can constitute a target for the nucleotide.

The aim of this work was to investigate the aromatase activity both in control and FSH- and dcAMP-treated ovaries through the fetal and neonatal period of sex differentiation. We found that aromatization of an androgen can be demonstrated very early in the female fetus and can constitute a target step for dcAMP.

EXPERIMENTAL

Rats

Female rats of the Wistar strain were caged with males at 6 p.m. and separated the next morning at 9 a.m. The estimated time of ovulation was 2 a.m. and the day following an overnight mating was counting as day 0.5 of gestation. Pregnancy was diagnosed by palpation 13 days later. Delivery took place on day 22 of gestation. The day of birth was designated as day 0.5 after birth.

Chemicals

[7-^{3}H(*N*)]testosterone (25 Ci/mmol) was obtained from New England Nuclear (Boston, Mass., U.S.A.). [4-^{14}C]estradiol-17β (55 mCi/mmol) and [4-^{14}C]-estrone (55 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). Purity was verified by silica gel chromatography. Unlabeled steroids and $N^6,O^{2'}$-dibutyryl adenosine 3′ : 5′-cyclic monophosphoric acid, monosodium salt (dcAMP) were obtained from Sigma (St Louis, Mo., U.S.A). Organic solvents and silica gel plates 60 F 254 were provided by Merck (Darmstadt, F.R.G.). AG 1-X2 200–400 mesh Cl resin was purchased from Bio-Rad Labs (Richmond, Wash., U.S.A.). Medium 199 (Earle's salts) was obtained from Eurobio (Paris, France) and tissue culture dishes were purchased from Falcon Plastics (Oxnard, Calif., U.S.A.). Ovine FSH (NIH-FSH-S11, 1.15 U/mg) and rat FSH (NIAMD-rat FSH-B-1, 3.7 U/mg) were kindly supplied by Dr P. G. Condliffe (NIAMDD, Bethesda, Md, U.S.A). LH contamination was estimated to be less than 0.010 NIH-LH-S1 U/mg for ovine FSH and less than 0.0045 NIH-LH-S1 U/mg for rat FSH.

Estimation of ovarian aromatase activity by organ culture

At sacrifice, the pregnant females were anesthetized with ether and fetuses were delivered by Cesarean section: the neonates were killed by decapitation. The ovaries of the fetal and neonatal rats were removed aseptically. Between 1 and 40 ovaries were immersed in 0.5 ml of Medium 199 supplemented with glutamine (0.35%), gentallin (40 μg/ml), specillin G

(100 U/ml) and [^{3}H]testosterone (80 nM) in the presence or absence of dcAMP (1 mM) or various concentrations of FSH. Stock solutions of hormones were [^{3}H]testosterone, 3 μmol/ml in benzene–ethanol (9 : 1, v/v) and FSH 1 mg/ml in 0.004% (w/v) BSA. The Petri dishes were placed in an airtight jar, gassed with O_2–CO_2 (95 : 5, v/v) and incubated at 37°C.

After culture, 50μg of estradiol-17β and estrone were added to the medium as carriers and [4-^{14}C]estradiol-17β (2500 dpm) and [4-^{14}C]estrone (2500 dpm) as internal recovery standards and the medium was collected and steroids were extracted twice with 3 ml of dichloromethane. The organic phase was taken to dryness under a stream of nitrogen and resuspended in 0.5 ml of 100% methanol for identification of steroids. The extracts were first subjected to column chromatography to separate estrogens from androgens as described by Järvenpää *et al.* [8]. Microcolumns (0.5 × 5 cm) were filled with AG 1-X2 resin suspended in distilled water. The resin was washed as follows: 3 ml of $NaHCO_3$ (1 M); verification of the pH (8.5); 4 ml of H_2O; 25 ml of 100% methanol. The extract was applied to the column with 0.5 ml of methanol. The androgens were first eluted with 7 ml of 100% methanol and the estrogens were recovered with 9 ml of 70% methanol. The 70% methanol fraction was evaporated, resuspended in 0.5 ml and submitted to a second chromatography by the same procedure. The yield of estrogens was 75.6 ± 1.6% ($n = 80$). Contamination by androgens was evaluated in medium without ovaries for each series of culture. These values (0.13 ± 0.02%, $n = 22$) were considered as blanks and subtracted for each series of experiments from the values obtained in medium with organs and referred to as total estrogens. The amount of total estrogens produced was proportional to the number of ovaries (Fig. 1).

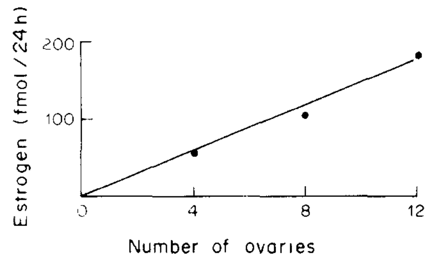

Fig. 1. Estrogen production from testosterone by various numbers of female gonads of 20-day-old rat fetuses. Between 4 and 12 ovaries were cultured for 24 h in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM). After culture, the amount of estrogen in the medium was estimated.

The phenolic fraction was further separated into estradiol-17β and estrone by silica gel TLC in a chloroform–ethyl acetate system (5 : 1, v/v). The radioactive metabolites were detected using a Panax XY radiochromatogram scanner. The resulting metabolite peaks were eluted with 3 ml of methanol and aliquots were counted in 10 ml of EP scintillation fluid (Beckmann, Glenrothes, U.K.) in an intertechnique liquid scintillation spectrometer. ^{3}H- and ^{14}C-windows were set so as to eliminate ^{3}H-interference in the ^{14}C-channel. Fourteen percent of the ^{14}C passed into the ^{3}H-channel and were subtracted from the ^{3}H-value. Counting efficiency was about 34% for ^{3}H and 84% for ^{14}C. The mean percentages of recovery of [^{14}C]estrone and [^{14}C]estradiol during

Table 1. Recrystallization to constant isotopic ratio of estradiol-17β and estrone isolated from the incubation of rat ovaries with [^{3}H]testosterone

	^{3}H/^{14}C ratio				
Estradiol-17β:	A	B	C	D	E
Mother liquor	3.47	4.02	3.10	2.67	—
1. Liquor	3.54	4.49	3.25	3.26	—
Crystals	3.28	3.28	3.06	1.43	—
2. Liquor	3.49	4.39	3.41	3.19	—
Crystals	3.24	3.72	3.25	1.89	—
3. Liquor	3.65	4.52	3.39	3.06	—
Crystals	3.33	4.36	3.14	1.89	—
4. Liquor	3.88	4.19	3.47	3.72	—
Crystals	2.86	3.72	2.95	1.62	—
Estrone:	A	B	C	D	E
Mother liquor	—	3.36	3.52	3.96	2.64
1. Liquor	—	3.60	3.27	3.58	2.73
Crystals	—	2.90	*	*	2.37
2. Liquor	—	3.39	3.57	4.06	2.79
Crystals	—	2.90	3.20	2.72	2.66
3. Liquor	—	3.40	3.35	4.08	2.68
Crystals	—	3.09	2.97	3.04	2.60
4. Liquor	—	3.89	3.82	3.50	2.81
Crystals	—	2.97	2.86	2.47	2.73

[4-^{14}C]estrogen was added to the [^{3}H]estrogen isolated from the medium together with 30 mg of authentic estrogen carrier in order to obtain a ^{3}H/^{14}C ratio equal to 3–4. Then four successive recrystallizations from methanol–water were performed: A, authentic [^{3}H]estradiol; B and C, [^{3}H]estrogen formed in medium containing [^{3}H]testosterone with ovaries from 8.5-day-old neonates; D and E, [^{3}H]estrogen formed in medium containing [^{3}H]testosterone and dcAMP (1 mM) with ovaries from 16.5-day-old fetuses. Incubation: 24 h.

*Sample lost.

Table 2. Estradiol-17β and estrone in the medium after 14.5-day-old fetal rat gonads were cultured with testosterone

		No. of gonads	Estrogen (fmol/gonad per 3 h)		
			Total estrogen	Estradiol-17β	Estrone
Ovaries		31	3.23	1.22	1.75
		22	2.07	0.95	1.11
		34	3.60	1.17	1.32
Testes		26	0.79	0.21	0.11
		14	0.95	ND	0.26
		32	1.22	0.21	0.16
Cardiac tissue	(a)	—	ND	—	—
	(b)	—	ND	—	—

Ovaries, testes and cardiac tissue from 14.5-day-old fetuses were incubated for 3 h in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM). Cardiac tissue was incubated with (a) or without (b) dcAMP (1 mM).
ND = not detectable.

the whole procedure were, respectively, 48.0 ± 2.6 and 47.9 ± 4.5 ($n = 12$).

Identification of the metabolites

The mobilities of the ^{3}H-labeled metabolites on thin-layer chromatograms were identical to those of the authentic standards. The chemical behavior of the metabolites and the respective standards were mutually identical after acetylation, hydrolysis and reduction. The radioactive metabolites (E_1 and E_2) were, respectively, diluted with the corresponding authentic radioinert estrogen and with ^{14}C-labeled estrogen and crystallized to a constant isotopic ratio (Table 1).

Statistical analysis

All mean values are expressed with SEM. The significance of the differences between the mean values was evaluated by Student's t-test with paired evaluation for some data.

RESULTS

Aromatase activity in ovaries of fetal and neonatal rats in control medium

14.5-Day-old fetuses. Gonads from male and female fetuses of the same litters were explanted with [^{3}H]testosterone in simultaneous incubations. Cardiac tissue from female fetuses was chosen as a control. In order to avoid any effect of endogenous testosterone whose synthesis starts punctually on day 15 in male gonads, the culture time was limited to 3 h (Table 2). Ovaries from 14.5-day-old fetuses produced detectable amounts of estradiol-17β and estrone, while production of estrogens by testes was absent or very faint and probably never significant. It should be noted that the total estrogens counted before separation corresponded to the sum of estradiol-17β and estrone in female media, while the total estrogens in male media were not found again

Table 3. Estradiol-17β and estrone in the medium after fetal and neonatal rat ovaries were cultured with testosterone

Age	No. of ovaries	Estrogen (fmol/ovary per 24 h)		
		Total estrogen	Estradiol-17β	Estrone
16.5 days postcoitum	8	5	1	7
	8	20	3	19
18.5 days postcoitum	40	29	2	24
8.5 days postpartum	5	2152	549	1169
	5	1707	950	1072
	6	2202	943	1083

Ovaries from 16.5- and 18.5-day-old fetuses and from 8.5-day-old neonates were cultured for 24 h in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM). After culture, estrogens in the medium were estimated before (total estrogen) and after separation (estradiol-17β and estrone).

Table 4. Estradiol-17β and estrone in the medium after 16.5-day-old fetal rat ovaries were cultured with testosterone in the presence of dcAMP

	No. of ovaries	Estrogen (fmol/ovary)		
		Total estrogen	Estradiol-17β	Estrone
A	14	261	52	185
	12	174	41	139
	46	108	8	132
B	18	51	17	51
C	9	183	29	169
	7	273	32	245

Ovaries from 16.5-day-old rat fetuses were precultured for 48 h in 0.5 ml of Medium 199 containing dcAMP (1 mM) and then cultured with [^{3}H]testosterone (80 nM) and dcAMP (1 mM) for 24 h (A) or 3 h (B). No pretreatment with dcAMP was performed in the third series of experiments (C); in this case, the culture lasted 24 h.

after separation and probably do not represent aromatase activity. Control tissue was devoid of any aromatase activity in the presence or absence of dcAMP.

16.5 And 18.5-day-old fetuses. Ovaries from 16.5- and 18.5-day-old fetuses incubated for 24 h with [^{3}H]testosterone in control medium showed aromatase activity; the conversion was in favor of estrone (Table 3). At the same time no aromatase activity was detectable in medium where renal metanephric anlage from 16.5-day-old female fetus was incubated.

8.5-Day-old neonates. Ovaries of neonates produced both estradiol-17β and estrone from testosterone to about the same extent (Table 3). The sum of the two estrogens was not significantly different from the total estrogens counted before separation.

Effects of dcAMP and FSH on aromatase activity in ovaries of fetal and neonatal rats

First, experiments were performed on 16.5-day-old fetal ovaries with and without preculture with dcAMP for 2 days before assay of aromatase activity in the presence of dcAMP (Table 4). Synthesis of both estradiol-17β and estrone could be detected in all experiments; but estrone was the major product. The sum of the two estrogens was not significantly different from the total estrogens counted before separation (paired test).

In the presence of dcAMP, the aromatase activity was greatly enhanced at all stages studied, except 8.5 days after birth where a stimulating effect could be observed only when the control values were comparatively low (Figs 2 and 3A).

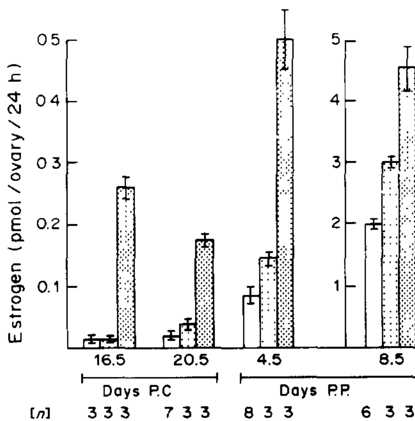

Fig. 2. Estrogen production from testosterone by female gonads of fetal and neonatal rats in the presence or absence of FSH or dcAMP. Ovaries were cultured for 24 h in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM) in the presence of oFSH (300 ng/ml) or dcAMP (1 mM). After culture, the amount of estrogen in the medium was estimated. oFSH ⊡; dcAMP ■; control □; n = number of determinations. Note the difference in scale for day 8.5 postpartum oFSH (300 ng/ml) efficiency was not statistically different from rFSH (1 μg/ml) on day 4.5: respectively, 145 ± 13 and 179 ± 11 pmol/ovary per 24 h.

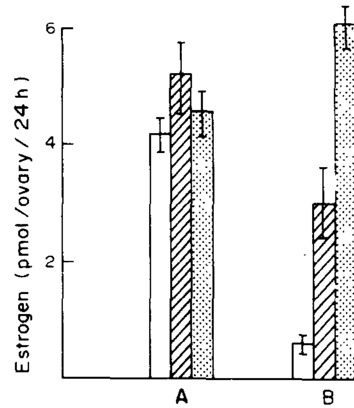

Fig. 3. Estrogen production from testosterone by female gonads of 8.5-day-old neonatal rats in the presence or absence of FSH or dcAMP. Ovaries were cultured for 24 h in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM) in the presence or absence of rFSH (1 μg/ml) or dcAMP (1 mM). After culture, the amount of estrogen was estimated. rFSH ▨; dcAMP ■; control □. A, Ovaries were incubated with [^{3}H]testosterone without delay. B, Ovaries were precultured for 48 h in control medium before incubation with [^{3}H]testosterone. Each determination was made in triplicate.

No stimulation of aromatase activity was detected with FSH at day 16.5 of gestation. The stimulating effect of FSH was detectable at day 20.5 of gestation and increased after birth. But, when the control values were high on day 8.5, no clear-cut effect of FSH was noticeable (Figs 2 and 3A). However, when ovaries from 8.5-day-old neonates were precultured for 48 h in control medium, the aromatase activity decreased considerably and, in this case, both dcAMP and FSH had a very important stimulatory effect (Fig. 3B). On the other hand, when the aromatase activity of 20.5-day-old fetal ovaries was followed *in vitro*, the amount of estrogens produced was observed to increased almost linearly with culture time up to 72 h (Fig. 4). These observations could imply an endogenous stimulation from the hypophyses when the ovaries were collected from 8.5-day-old neonates.

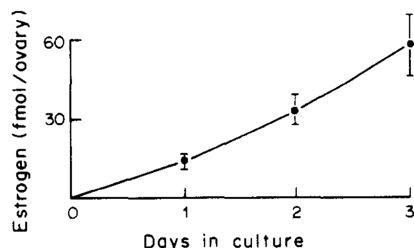

Fig. 4. Time-course of estrogen production from testosterone by female gonads of 20-day-old fetuses. Ovaries were cultured for 3 days in 0.5 ml of Medium 199 containing [^{3}H]testosterone (80 nM). The medium was changed every 24 h and the amount of estrogen was estimated. The results expressed were cumulative. Each point represents the mean ± SEM of three separate determinations.

DISCUSSION

The present study demonstrates that aromatization of testosterone to estrogen occurs in cultured fetal and neonatal rat ovaries. Moreover, the aromatase activity differentiates female from male gonads before testosterone production starts in male gonads. At the same time, no aromatization was observed in other tissues such as kidney or cardiac tissue. In the adult, a variety of tissues other than ovaries has been described to exhibit an aromatase activity [9]. Our negative results could be explained by a very low and undetectable level which will increase in the course of development. The difference in aromatase activity noted between male and female gonads on day 14.5 of gestation can be compared with previous observations on the capacity of gonads to convert DHA into testosterone which was significantly higher in male gonads than in female ones as early as 13.5 days and nil in other tissues such as the brain [10]. Furthermore, at the same time, estradiol-17β was detectable only in medium with female gonads [11], indicating the probable existence of aromatase activity at their level. The possibility of a biochemical difference between male and female gonads before morphologic differentiation has been recently pointed out by a two-dimensional gel electrophoresis investigation [12].

The aromatase activity present in fetal and neonatal rat female gonads shows the formation of both estradiol-17β and estrone. Although the conversion was in favor of estrone before birth, the yield of estradiol-17β was not negligible. This result does not agree with other data which failed to identify estradiol-17β in incubations of 19-day-old fetal rat ovaries with testosterone [13]. The treatment of the semi-solid medium which precedes the extraction and identification could be responsible for this discrepancy. Indeed, in other species where an aromatase activity has been described in the fetal ovaries, both estrogens have been identified with the predominance of either estradiol-17β [14–16] or estrone [17].

dcAMP was observed to enhance aromatization in female gonads, to a great extent, in all stages studied. This effect could account in part for the *de novo* production of estrogen revealed by radioimmunoassay when fetal ovaries were treated with dcAMP [6]. On the other hand, no effect of FSH was observed until day 20 of gestation. The presence of FSH receptors has been demonstrated in male rat gonads from day 17.5 of gestation [18] and an effect of FSH on the Sertoli cell population was noted on day 18.5 [19]. No results have been mentioned for female fetal rat gonads. In mice, an aromatase activity could be demonstrated only in postnatal ovaries, but in the presence of FSH, activity was revealed as soon as day 18 of gestation [20]. In other species, the effect of FSH on fetal ovaries was not mentioned even in studies which have shown that female gonads produce estrogen *de novo* [2–4] or exhibit an aromatase activity [14–17].

The physiological meaning of an aromatase activity in the fetal ovary at the time of sexual differentiation of the gonads remains to be determined. Enzymatic capacity for estrogen formation from testosterone does not imply that *de novo* estrogen synthesis actually takes place in the female gonad. On the other hand, testosterone in female fetal plasma is not negligible in the course of gestation [21]. Determination of the gonadal cells which exhibit aromatase activity would contribute to bring information on the significance of this early sex differentiation.

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