J. Steroid Biochem. Molec. Biol. Vol. 39, No. 6, pp. 953–957, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

RESPONSIVENESS OF RAT OVARIES TO dcAMP IN THE PERINATAL PERIOD: EVIDENCE FOR AN INHIBITORY INFLUENCE *IN VIVO*

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(Received 11 February 1991)

Summary—The progesterone production by rat ovaries from 18-day-old fetuses to 6-day-old neonates was measured *in vitro* in the presence of dibutyryl cAMP (dcAMP, 1 mM). A pronounced decline was observed at the end of fetal life. The 5α-reductase activity did not seem sufficient to explain this decrease. Preculture of the ovaries for 48 h in the basal medium enhanced responsiveness to the nucleotide. Addition of spironolactone, an inhibitor of 17α-hydroxylase to dcAMP did not modify this evolution.

3β-hydroxysteroid dehydrogenase activity, detectable in fetal ovaries in the absence of dcAMP was also increased after preculture. In the presence of spironolactone and trilostane, the pregnenolone production showed the same evolution as progesterone and was also enhanced after culture. These results suggest the existence of inhibitory factor(s) present *in vivo* at the end of fetal life.

INTRODUCTION

Although rat ovaries produce no steroids during fetal life [1, 2], progesterone synthesis can be induced *in vitro* with dibutyryl cAMP(dcAMP) [3]. However, near term, the nucleotide effect was markedly impaired. This unexpected result could be either due to an extensive metabolism of progesterone or to the presence of inhibitory factor which influences the steroidogenesis *in vivo*.

The purpose of this study was therefore to clarify the mechanism(s) by which the steroidogenesis is blunted in response to dcAMP near term. We assumed that *in vivo*, environmental factor(s) were likely to play a role in this process.

EXPERIMENTAL

Animals

Female rats of the Wistar strain weighing about 200 g were caged with males at 6 p.m. and separated the next morning at 9 a.m. Pregnancy was confirmed by palpation 13 days later. Delivery occurred on day 22 of gestation.

Materials

[4,7-^{3}H]pregnenolone (10 Ci/mmol), [4-^{14}C]-progesterone (56 mCi/mmol), [1,2,6,7-^{3}H]progesterone (82 Ci/mmol), [1,2-^{3}H]testosterone (53 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). Unlabeled steroids, N^6,O^2-dibutyryl adenosine 3′:5′ cyclic monophosphoric acid, monosodium salt (dcAMP) and spironolactone were obtained from Sigma (St Louis, MO, U.S.A.). 4α,5-epoxy-17β-hydroxy-3-keto-5α androstan-2α-carbonitrile (trilostane) was a gift from Sterling–Winthrop Research Institute (Rensselear, NY, U.S.A.). The 5α-reductase inhibitor: *N*,*N*-diethyl-4-methyl-3-oxo-4-azaandrostane-17β-carboxamide (L-636028-000J) was a gift from Merck, Sharp and Dohme Research Laboratories (NJ, U.S.A.). Organic solvents and silica gel plates LK6DF were provided by Whatman (Maidstone, England). Medium 199 (Earle's Salts) was obtained from Eurobio (Paris, France). The progesterone antiserum was a gift from INRA (France). The pregnenolone antiserum was obtained from Steranti Research Ltd (St. Albans, U.K.)

Organotypic cultures

Ovaries removed aseptically from fetuses and neonates were immersed in organ culture dishes

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(Falcon Plastics, Oxnard, CA, U.S.A.) in jars which were gassed with a mixture of 5% CO_2–95% O_2. The culture medium M199 supplemented with glutamine (0.35%), penicillin (100 U/ml) and gentamycin (40 μg/ml) was devoid of serum. DcAMP (1 mM), spironolactone (10 μM) and trilostane (30 μM) were added to the culture medium as indicated in results. Progesterone and pregnenolone produced in the media were measured by radioimmunoassay according to a technique previously described [3].

Measurement of 3β-hydroxysteroid dehydrogenase activity

The ovaries were homogenized in ice-cold 0.05 M potassium buffer pH 7.4 and the assays were performed in duplicate in glass tubes as previously described [4]. In brief, incubations were carried out in the presence of [^{3}H]pregnenolone (0.2 μCi) unlabelled pregnenolone (0.2 nmol) and spironolactone (10 μM) at 37°C for 10 min in a shaking water bath. After extraction, steroids were separated by silica gel TLC, extracted and counted. The mean percentage of recovery of [^{14}C]progesterone added to each tube was 70 ± 2 (n = 52).

Measurement of 5α-reductase activity

The 5α-reductase activity was determined by measuring the conversion of [^{3}H]testosterone to 5α-reduced ^{3}H-metabolites: 17β-hydroxy-5α-androstan-3-one (5α-DHT), 5α-androstan-3β, 17β-diol (5α-diol) and 5α-androstan-3,17-dione (5α-dione).

The ovaries were incubated with [^{3}H]testosterone (5 μCi/2 ml) for 18 h. After incubation, the steroids were extracted twice with 5 ml of ethyl acetate/cyclohexane (1:1). The organic phase was removed after centrifugation (2000 g for 15 min), evaporated to dryness under a stream of nitrogen and redissolved in 70 μl ethylacetate. Unlabelled testosterone, androstenedione, 5α-DHT, 5α-diol and 5α-dione were added as carriers. The metabolites were separated by silica gel TLC in the system dichloromethane, ethyl acetate, methanol; (85:15:3), which resolves the major 5α-reduced metabolites from 5β-metabolites, estradiol and estrone [5]. Testosterone and androstenedione (4-ene-A) were visualized under u.v. light and 5α-diol, 5α-dione with iodine vapor. The metabolites were scraped off, extracted with 1 ml of methanol overnight and counted in 8 ml of EP scintillation fluid.

Protein determination

Protein content of homogenates was determined according to the technique described by Bradford [6].

Statistical analysis

All values are means ± SEM. Data were analyzed by the Student's t-test, by the one-way analysis of variance (ANOVA) (Scheffé F-test) or by the Mann–Whitney test for small samples. A paired statistical evaluation was used to compare results from both ovaries of the same fetuses and neonates.

RESULTS AND DISCUSSION

The diminution of progesterone production previously observed in ovaries treated with dcAMP in the perinatal period was confirmed (Table 1). The addition of spironolactone, an inhibitor of 17α-hydroxylase [7], increased the amount of progesterone in all cases, indicating the existence of a 17α-hydroxylase activity; but the same evolution of responsiveness to dcAMP was observed: after 24 h of culture, a pronounced decline of progesterone production was noted in ovaries from 20-day-old fetuses

Table 1. Responsiveness of fetal and neonatal ovaries to dcAMP

	Progesterone pmol/ovary/24 h					Pregnenolone pmol/ovary/24 h	
	dcAMP		dcAMP + spironolactone			dcAMP + spironolactone + trilostane	
		(a)		(a)	(b)		(a)
16 days p.c. (4)	2.55 ± 0.31	NS	5.06 ± 0.41	NS	*	5.84 ± 0.38	NS
18 days p.c. (6)	2.09 ± 0.32	—	4.70 ± 0.84	—	*	6.39 ± 1.28	—
20 days p.c. (6)	0.28 ± 0.02	**	0.88 ± 0.06		***	2.03 ± 0.19	*
22 days p.c. (5)	0.26 ± 0.06	***	0.37 ± 0.04	***	NS	0.88 ± 0.29	**
4 days p.p. (5)	0.45 ± 0.06	*	1.37 ± 0.29	**	*	5.35 ± 1.88	NS
6 days p.p. (6)	2.09 ± 0.47	NS	2.83 ± 0.16	NS	NS	—	

Values are means ± SEM. (a) Significance of differences with 18 days p.c. as calculated by analysis of variance. (b) Significance of differences between data with dcAMP and with dcAMP + spironolactone by paired t-test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS not significant. In parentheses, number of cases for each experimental condition.

Table 2. Percentage formation of ^{3}H-steroids from [^{3}H]testosterone by rat ovaries *in vitro*

	Testosterone (unchanged)	5α-reduced metabolites			
		4-ene-A	5α-DHT	5α-diol	5α-dione
No addition					
Ovaries 18 d.p.c. (3)	53 ± 9	39 ± 7	4.1 ± 1.2	2.5 ± 0.6	5.2 ± 0.9
Ovaries 20 d.p.c. (4)	46 ± 8	43 ± 7	4.8 ± 0.7	3.2 ± 0.5	4.8 ± 1.3
Cardiac tissue (2)	98	0.3	0.9	0.5	1.0
L-636028-000J					
Ovaries 18 d.p.c. (1)	61	36	0.9	1.5	0.8
Ovaries 20 d.p.c. (1)	47	48	2.0	0.8	1.7
Cardiac tissue (1)	96	1.7	1.8	0.4	0.4

A pool of 24 ovaries and cardiac tissue as control were used for each experiment. In parentheses, number of experiments. Assays were performed in duplicate.

compared to 18-day-old ones. The metabolism of progesterone through other pathways cannot be ruled out since in immature ovaries, an important 5α-reductase activity has been described [8].

Thus, an attempt to evaluate the 5α-reductase activity present in 18- and 20-day-old ovaries was performed by measuring the conversion of [^{3}H]testosterone to ^{3}H-5α-reduced metabolites after 24 h of culture in the presence of dcAMP and spironolactone (Table 2). The predominant metabolite formed in the media was androstenedione, confirming that fetal ovaries contain an important 17β-hydroxysteroid dehydrogenase (17β-DSH) activity [9]. The conversion of [^{3}H]testosterone to ^{3}H-5α-reduced metabolites was proportional to the number of ovaries incubated (not shown) and very low when referred per ovary (0.36% for 18-day-old fetuses and 0.40% for 20-day-old fetuses) after substraction of the blank (cardiac tissue). L-636028-000J, a 5α-reductase inhibitor was observed to reduce the formation of the 5α-reduced metabolites by 90%. No effect was observed on the 17β-DSH activity. So, the diminution of responsiveness to dcAMP in the perinatal period does not appear to be related to an increase of the 5α-reductase activity.

In order to evaluate whether the diminution of responsiveness to dcAMP on day 20 of gestation was related to environmental conditions, ovaries from 18-day-old fetuses were precultured for 2 days in the basal medium and the progesterone production was then measured 24 h later in the presence of dcAMP or dcAMP and spironolactone. Ovaries without preculture from 18- and 20-day-old fetuses were tested at the same time (Fig. 1). Preculture in the basal medium prevented the inhibition observed in 20-day-old ovaries ($P < 0.001$ in all cases). Moreover progesterone production was higher than in 18-day-old controls ($P < 0.01$ with dcAMP, NS with dcAMP + spironolactone).

These results suggest the presence of an inhibitory factor at the end of fetal life. It could be produced by adjacent tissues. To test this hypothesis, ovaries from 18-day-old fetuses were precultured with mesonephros and Müllerian ducts for 2 days; then the ovaries were cut off and treated with dcAMP. Progesterone production was higher than in controls and no significant difference was observed between ovaries precultured with and without mesonephros (Fig. 1). These results suggest that the mesonephros does not exert any inhibitory effect and could even enhance the progesterone production to a certain extent, possibly with a cellular contribution. The role of mesonephros in the development of ovaries of rats [10] and mice [11] has been already suggested from histological observations.

Then, the impact of environmental conditions was evaluated on the two steps of progesterone biosynthesis. First, conversion of radiolabeled pregnenolone into progesterone was measured

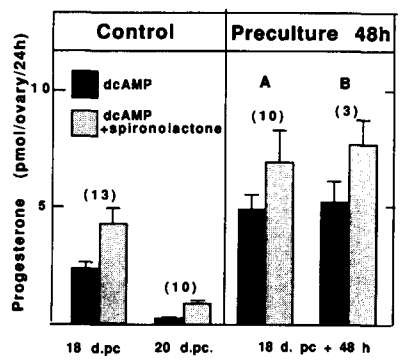

Fig. 1. Effect of preculture on the ovarian responsiveness to dcAMP. Progesterone production was measured in the presence of dcAMP with or without spironolactone. Control ovaries from 18- and 20-day-old fetuses were immediately treated with dcAMP (left panel). Ovaries from 18-day-old fetuses were precultured for 48 h in M 199 before being treated with dcAMP (right panel). Preculture was performed in the absence (A) or in the presence (B) of mesonephros. Number of experiments in parentheses. Values are means ± SEM.

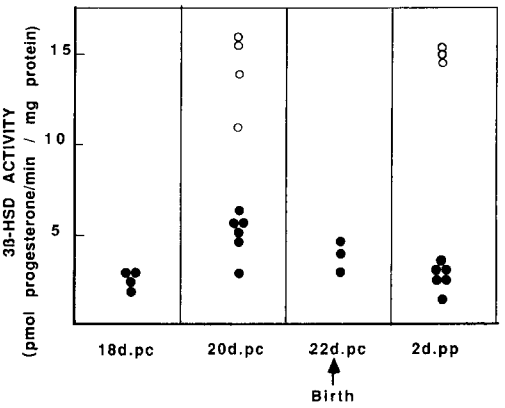

Fig. 2. Effect of preculture of the 3β-HSD activity in fetal and neonatal ovaries. The 3β-HSD activity was evaluated in control ovaries (●) and in ovaries precultured for 48 h in M 199 (○). In these cases, final age was considered. Homogenates of ovaries were incubated with [^{3}H]pregnenolone and conversion into [^{3}H]progesterone was evaluated after 10 min. For details, see Experimental. 3β-HSD activity was significantly higher ($P < 0.001$) in ovaries precultured for 48 h versus control ovaries, whatever stages were compared.

in ovaries from 18-, 20- and 22-day-old fetuses and from 2-day-old neonates. In late gestation and in neonates as well, the enzymatic activity was constant. In contrast, preculture of ovaries from 18- and 22-day-old fetuses in the basal medium for 48 h markedly increased the 3β-HSD activity (Fig. 2). Secondly, the production of pregnenolone in the presence of dcAMP, spironolactone and trilostane was followed during the fetal and perinatal period (Table 1). Pregnenolone production decreased on day 20 of gestation and the same evolution of responsiveness to dcAMP was observed. In the same way, the preculture of ovaries from 18-day-old fetuses in the basal medium, prevented this decline (Fig. 3).

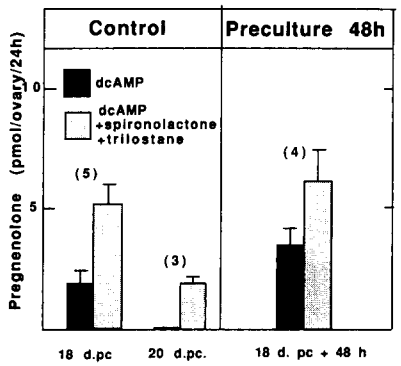

Fig. 3. Effect of preculture on the ovarian responsiveness to dcAMP. Pregnenolone production was measured in the presence of dcAMP with or without spironolactone and trilostane. Control ovaries from 18- and 20-day-old fetuses were immediately treated with dcAMP (left panel). Ovaries from 18-day-old fetuses were precultured for 48 h in M 199 before being treated with dcAMP (right panel). Number of experiments in parentheses. Values are means ± SEM.

In male fetuses, a pronounced decline was shown in the total steroid concentration and content per interstitial cell during the last days of intrauterine life [12]. The reason for this abrupt decline is not clear. The dependence of fetal testes on pituitary gonadotropins present *in vivo* [13] makes it difficult to show the presence of an inhibitory factor on steroidogenesis at the same time. Although comparisons between male and female gonads remain speculative, our data indirectly indicate a similar evolution in fetal ovaries. Moreover our *in vitro* experiments strongly suggest that *in vivo*, a putative factor could have an inhibitory effect on steroidogenesis.

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