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Steroidogenesis of the Fetal Adrenal Gland *in Vitro*: Influence of Metopirone Applied *in Vivo* and *in Vitro*[1] (39013)

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Recently Milković *et al.* (1) have shown that injection of fetuses with Metopirone prevents the maintenance of normal plasma corticosterone concentration in their adrenalectomized mothers during the last days of pregnancy to the same extent as the removal of fetuses or simultaneous removal of fetuses, placenta and uterus (2). In their experiments possible inhibition by Metopirone of corticosterone synthesis in the placenta was not completely ruled out.

Metopirone is known to inhibit 11β-steroid hydroxylase and 18-steroid hydroxylase activity of the adrenal cortex in *in vivo* and *in vitro* experiments (3–6) and the activity of these enzymes in rat fetuses in *in vivo* experiments (7–9).

The results of this study have shown that fetal adrenal glands produce all corticoids on the last day of intrauterine development and that in *in vitro* experiments Metopirone inhibits 11 β- and 18-steroid hydroxylase activity, which is characteristic for the glands of adult rats.

Materials and methods. Pregnant albino rats of the Fischer strain, raised in an air-conditioned animal room at 22–24° on a daily regimen of 10 hr of light and 14 hr of darkness, were used throughout the experiments. Standard laboratory food and water were continuously available. The females were placed with males overnight for mating, and the appearance of sperm in the vaginal smear was considered as the first day of pregnancy.

In the first series of experiments, on the 22nd day of pregnancy the females were adrenalectomized under ether anesthesia and immediately thereafter laparotomized. Half of their fetuses were injected sc through the uterine wall with 3 mg of Metopirone (Su4885, Ciba Geigy, Switzerland) in 0.1 ml of saline and the other half with saline only. Two hours later the animals were killed, their fetuses were removed, the fetal adrenal glands dissected and cleaned of adhering tissue under a stereomicroscope of tenfold magnification and weighed on an analytical balance. Each pair of fetal adrenal glands was incubated in 2 ml of Krebs–Ringer bicarbonate buffer to which 200 mg of glucose/100 ml and 68,000 cpm of 4-^{14}C-progesterone (The Chemical Centre, Amersham, Great Britain; specific activity, 61.0 mCi/mmole) was added. Incubation of samples was performed in a Dubnoff-type metabolic shaker in an $O_2 + CO_2$ (95 + 5%) atmosphere for 1 or 3 hr at 37°.

In the second series of experiments, the females were killed on the 22nd day of pregnancy and the adrenal glands of their fetuses, prepared as described previously, were preincubated for 30 min in 1.9 ml of Krebs–Ringer bicarbonate buffer to which 3 mg of Metopirone in 0.1 ml of saline or saline alone was added. After preincubation the fetal adrenal glands were transferred into the incubation medium containing 68,000 cpm of 4-^{14}C-progesterone. To one-third of the samples, 1.9 ml of Krebs-Ringer bicarbonate buffer with 3 mg of Su4885 in 0.1 ml of saline and to the rest of the samples the same volume of buffer with 0.1 ml of saline was added. Fetal adrenal glands were incubated for 1 or 2 hr in an $O_2 + CO_2$ (95 + 5%) atmosphere at 37°. The incubation medium was extracted with 15 ml of a mixture of chloroform and methanol (2:1), evaporated in a stream of N_2 and transferred quantitatively to thin-layer chromatography plates.

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The extracted steroids were separated by two-dimensional chromatography on a thin layer of silica-gel GF_{254} (Merck, Darmstadt, West Germany) using the dichlormethane–*n*-heptane–methanol (15:4:1, v/v/v) and benzene – acetone – *n* - heptane – dichlormethane (4:4:2:1, v/v/v/v) solvent systems. The radioactive steroids were located following radiography by placing an X-ray film on the chromatogram and identified by precrystallization to constant specific activity according to the method of Axelrod *et al.* (10).

After the adrenal glands, the placentas of mothers with untreated fetuses were analyzed. The placentas were washed in Krebs–Ringer bicarbonate buffer and 5–30-mg samples were transferred into the incubation medium to which 68,000 cpm of 4-^{14}C-progesterone were added. Incubation of samples was performed in an $O_2 + CO_2$ (95 + 5%) atmosphere for 1 or 2 hr at 37°. The incubation medium was extracted with a 15-ml mixture of chloroform and methanol (2:1), evaporated in a stream of N_2 and transferred to thin-layer chromatography plates. Following two-dimensional chromatography, the radioactive steroids were located by radiography on an X-ray film.

Radioactivity of the samples was measured by a Mark II (Nuclear Chicago) liquid scintillation counter. Single steroid hormones were scraped off the thin layer into test tubes into which 10 ml of toluol containing 0.5% PPO and 0.1% POPOP was added. The values obtained were expressed in manograms per milligram of fetal adrenal and in percent of radioactivity. In the first series of experiments, the differences between these two values were calculated by Student's *t* test, and in the second series, after the analyses of variances and the test of homogeneity, Student's *t* test or Kramer's test were used to calculate the differences among the mean values obtained.

Results. In *in vitro* experiments fetal adrenal glands hydroxylate 4-^{14}C-progesterone into 11-deoxycorticosterone (DOC), corticosterone (B), 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), 18-hydroxycorticosterone (18-OH-B), aldosterone, 11 β-hydroxyprogesterone (11-OH-P) and 20 α-hydroxyprogesterone (20-OH-P). After a 1-hr incubation, the adrenal glands of control fetuses hydroxylated progesterone into corticosterone, DOC and 18-OH-DOC to the greatest extent. In the adrenal glands of fetuses pretreated with Su4885 *in vitro*, hydroxylation of 4-^{14}C-progesterone into corticosterone, 18-OH-DOC and 18-OH-B decreased and hydroxylation into DOC was enhanced (Table I). Under these conditions of incubation fetal adrenal glands did not synthesize aldosterone.

The adrenal glands of control and Metopirone-treated fetuses incubated with 4-^{14}C-progesterone for 3 hr synthesized equal amounts of corticosterone and 18-OH-DOC; i.e., here inhibition was reversible, which is characteristic of competitive antagonism. In the adrenal glands of fetuses injected with Metopirone, hydroxylation of progesterone into aldosterone decreased and the synthesis of DOC increased (Table I).

The adrenal glands of fetuses preincubated with Su4885 and incubated for 1 hr with 4-^{14}C-progesterone synthesized less corticosterone than the adrenal glands of control fetuses. In the adrenal glands preincubated with Su4885, hydroxylation of progesterone into DOC and 20-OH-P increased. Addition of Metopirone into the preincubation and incubation media caused an even greater hydroxylation of progesterone into DOC and decreased corticosterone synthesis by the fetal adrenal glands (Table II). This ratio of steroid synthesis remained unchanged after a 2-hr incubation with 4-^{14}C-progesterone. The only difference noted after the 2-hr incubation was the appearance in the control fetuses of 18-OH-B and aldosterone, whose synthesis decreased following addition of Su4885 into the incubation medium (Table II).

Rat placentas incubated with 4-^{14}C-progesterone did not synthesize adrenal corticosteroids. Only a small amount of progesterone was metabolized into 17 α-hydroxyprogesterone.

Discussion. The results obtained in this study have shown that the placenta, incubated with 4-^{14}C-progesterone under identical conditions as the fetal adrenal glands, does not synthesize corticosteroids. This finding has clearly demonstrated that the placenta cannot be the source of maternal

TABLE I. CORTICOSTEROID SYNTHESIS BY THE ADRENAL GLANDS OF 22-DAY-OLD FETUSES, INJECTED WITH 3 MG OF METOPIRONE *IN UTERO*, FROM 4-^{14}C-PROGESTERONE AFTER 1- OR 3-HR INCUBATION.

Treatment	18-OH-B	Aldosterone	18-OH-DOC	B	11β-OH-P	20α-OH-P	DOC	Progesterone (residual substrate)
				Ng/mg gland, 1-hr incubation period				
NaCl	1.23±0.08[a]	1.11(1)	6.29±0.34	9.67±0.36	1.12±0.18(5)	1.80±0.20(5)	7.94±0.50	66.72±0.95
NaCl + metopirone	0.83±0.06[c](4)	1.18(1)	2.89±0.46[b]	6.07±0.73[b]	1.06±0.18(4)	1.90±0.21	11.98±1.03[c]	64.66±1.32
				Percent dpm				
NaCl	0.30±0.01	0.04(1)	1.79±0.07	2.96±0.09	0.23±0.06(5)	0.41±0.04(5)	2.05±0.12	91.15±0.51
NaCl + metopirone	0.14±0.05[b](4)	0.29(1)	1.22±0.18[c]	1.81±0.23[b]	0.19±0.07(4)	0.51±0.07	3.17±0.38[c]	90.60±1.15
				Ng/mg gland, 3-hr incubation period				
NaCl	5.32±0.32	3.42±0.30(11)	21.23±1.22	37.89±0.83	2.63±0.38(8)	2.36±0.16	16.60±1.20	48.72±1.31
NaCl + metopirone	4.20±0.24[b]	2.40±0.33[b](6)	21.34±0.65	35.58±0.83	2.59±0.22(9)	2.49±0.18	22.22±1.22[c]	45.01±1.00[c]
				Percent dpm				
NaCl	1.38±0.07	0.89±0.05(11)	5.92±0.28	10.28±0.32	0.50±0.12(8)	0.67±0.04	4.72±0.30	73.07±1.13
NaCl + metopirone	1.14±0.08[c]	0.67±0.07[b](6)	6.22±0.17	10.23±0.21	0.62±0.09(9)	0.76±0.04	6.40±0.33	72.26±1.07

[a] Values are means ± SE; 12 fetuses per sample if not otherwise indicated in parentheses.
[b] $P < 0.01$.
[c] $P < 0.05$.

TABLE II. CORTICOSTEROID SYNTHESIS BY THE ADRENAL GLANDS OF 22-DAY-OLD FETUSES INCUBATED WITH 4-^{14}C-PROGESTERONE AND METOPIRONE FOR 1 OR 2 HR.

Treatment[a]	18-OH-B	Aldosterone	18-OH-DOC	B	11β-OH-P	20α-OH-P	DOC	Progesterone (residual substrate)
				Ng/mg gland, 1-hr incubation period				
1	—	—	4.56±0.19[b]	8.85±0.36	0.94±0.07	1.81±0.05	7.96±0.66	62.07±0.85
2	—	—	3.17±0.38(3)	6.57±0.38[c]	1.49±0.21	2.45±0.20	17.35±0.97[c]	59.24±1.41
3	—	—	2.96±(1)	3.89±0.27[c,e]	2.77±0.23[c,e]	2.52±0.18	20.24±1.80[c]	60.12±0.93
				Percent dpm				
1	—	—	1.16±0.03	2.57±0.14	0.26±0.01	0.50±0.02	2.58±0.31	90.63±0.70
2	—	—	0.96±0.03[d](3)	1.83±0.06[c]	0.43±0.06	0.69±0.05[d]	5.01±0.24[c]	88.49±1.04
3	—	—	0.18[c](1)	1.05±0.07[c,e]	0.68±0.03[c]	0.72±0.04[d]	5.76±0.32[c]	89.06±0.74
				Ng/mg gland, 2-hr incubation period				
1	2.34±0.17	1.19±0.07(4)	12.46±0.55	19.22±1.33	1.34±0.24	2.27±0.09	12.52±1.17	59.83±1.30
2	1.91±0.03	—	8.83±0.60[c]	13.67±0.45[c]	1.07±0.09	1.00±0.09[c]	19.72±0.76[c]	59.46±0.42
3	1.72±0.10[a]	—	1.70±0.03[c,e]	5.19±0.23[c,e]	3.64±0.32[c,e]	5.06±0.24[c,e]	32.42±0.41[c,e]	55.50±0.94
				Percent dpm				
1	0.53±0.03	0.28±0.03(4)	3.16±0.09	4.96±0.26	0.35±0.06	0.69±0.005	3.57±0.23	84.56±0.69
2	0.47±0.01	—	2.34±0.17[c]	3.60±0.11[c]	0.29±0.001	0.25±0.01[c]	5.29±0.21[c]	85.45±0.21
3	0.44±0.01	—	0.46±0.01[c,e]	1.42±0.04[c,e]	1.02±0.06[c,e]	1.49±0.06[c,e]	9.39±0.01[c,e]	83.70±0.23

[a] 1, Preincubation for 30 min in 1.9 ml of Krebs–Ringer bicarbonate buffer plus 0.1 ml of saline and incubation with 4-^{14}C-progesterone in 2 ml of Krebs–Ringer bicarbonate buffer for 1 or 2 hr. 2, Preincubation for 90 min in 1.9 ml of Krebs–Ringer bicarbonate buffer plus 0.1 ml of saline containing 3 mg of metopirone and incubation with 4-^{14}C-progesterone in 2 ml of Krebs–Ringer bicarbonate buffer for 1 or 2 hr. 3, Preincubation for 30 min in 1.9 ml of Krebs–Ringer bicarbonate buffer plus 0.1 ml of saline containing 3 mg metopirone and incubation with 4-^{14}C-progesterone in 1.9 ml of Krebs–Ringer bicarbonate buffer plus 0.1 ml saline containing 3 mg of metopirone for 1 or 2 hr.

[b] Values are means ± SE; six fetuses per sample unless otherwise indicated in parentheses.

[c] $P < 0.01$ (vs 1).

[d] $P < 0.05$ (vs 1).

[e] $P < 0.01$ (vs 2).

plasma corticosterone during the last days of pregnancy following adrenalectomy (1, 2). Unlike the placenta, the adrenal glands synthesize all corticosteroids from 4-^{14}C-progesterone added to the incubation medium.

This study has also established that the effect of Metopirone on the *in vitro* synthesis of fetal adrenal corticoids is the same as that found by experiments with fetal adrenal glands conducted *in vivo*. Moreover, it has been demonstrated that Metopirone inhibits 18-hydroxylase in the fetal adrenal gland, which has been noted by other authors in experiments with incubation of Metopirone with the adrenal glands of adult rats (4, 11, 12). On the basis of these findings it is concluded that the fetal adrenal glands are capable of secreting corticoids and that the pathways of corticoid synthesis in the fetal adrenal glands are partially and, perhaps, also completely identical to those existing in the adrenal glands of adult rats.

Summary. In vitro conversion of 4-^{14}C-progesterone into corticosteroids in the adrenal glands of rat fetuses treated with Metopirone (Su 4885) on the last day of intrauterine development was studied. After a 1-hr incubation of the adrenal glands of fetuses injected with Metopirone, hydroxylation of progesterone into corticosterone (B), 18-hydroxycorticosterone (18-OH-B) and 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) decreased and the synthesis of 11-deoxycorticosterone increased.

Following preincubation of the fetal adrenal glands and 1-hr incubation with Metopirone, hydroxylation of progesterone into DOC increased and the synthesis of B decreased. Preincubation and a 2-hr incubation with Metopirone caused a decrease in the synthesis of B, 18-OH-B and 18-OH-DOC and an increase in DOC.

The results constitute direct evidence of the ability of the fetal adrenal glands to synthesize all corticoids and indicate that most probably corticoids are synthesized by the fetal adrenal glands in the same way as in the adrenals of adult animals.

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