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4-HYDROXYANDROSTENEDIONE IS NOT AN AROMATASE INHIBITOR IN THE NEONATAL GUINEA PIG

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Abstract—One-day-old newborn guinea pigs were treated with 4-hydroxyandrostenedione (50 mg/kg body weight/day) for 5 or 12 consecutive days. This compound did not decrease unconjugated or sulfo-conjugated estradiol and estrone levels in the plasma or in the uterine tissue itself. It also did not have any effect on uterine wet weight or the estrogen and progesterone receptor concentrations in the uterus. Moreover, progesterone receptor synthesis which is maintained when neonatal uteri are placed in organ culture conditions for 2 days was not affected by the 4-hydroxyandrostenedione treatment.

The search for more specific adjuvant agents which either antagonize the action of estrogens or inhibit their synthesis has become of great interest in recent years in the treatment of estrogen-dependent tumors, particularly in breast cancers. One approach has been to develop inhibitors of estrogen synthesis by the ovaries and by peripheral tissues as demonstrated in humans and in some animal species such as the rat and rhesus monkey [1]. One compound which shows promise is 4-hydroxyandrostene-3,17-dione (4-OH-A) [2], which inactivates aromatase *in vitro* and decreases ovarian estrogen secretion and peripheral aromatization *in vivo* [1, 3, 4].

The potent anti-aromatase properties of 4-OH-A are also potentially useful in other experimental systems which require the suppression of estrogen biosynthesis. Previous studies in our laboratory have shown that estrogens can induce the progesterone receptor in the fetal guinea-pig uterus when administered *in vivo* [5] but when uteri are taken from previously untreated fetuses and placed in organ culture in estrogen-free medium, progesterone receptor rises spontaneously [6]. We suggested that this might be due to the endogenous estrogens sequestered in the fetal uterine tissue so that a compound which would decrease the endogenous estrogen levels would thus be extremely useful. Since no data were available on the effect of 4-OH-A on aromatization in the guinea pig, preliminary studies were first undertaken using neonatal guinea pigs. The effects of 4-OH-A administration on the concentration of unconjugated and sulfo-conjugated estrogens in plasma and uteri of newborn guinea pigs and estrogen and progesterone receptor levels are reported in this paper.

MATERIALS AND METHODS

Biological material. One-day-old newborn female guinea pigs of a local tri-colored breed received 50 mg/kg body weight of 4-OH-A per day in the morning in 0.3 ml 40% ethanol–60% propanediol s.c. for 5 or 12 days. The animals were sacrificed the afternoon of the last injection. Blood was collected by cardiac puncture and uteri were excised and weighed. The uteri were divided into three portions: (1) for processing for receptor assays, (2) for organ culture in Medium 199 for 2 days as previously described [6], and (3) for the radioimmunoassay of estrogens (stored at −20° along with the plasmas).

Chemicals. [6,7-^{3}H]-Estradiol (s.a. 47.4 Ci/mmol), [6,7-^{3}H]-estrone (s.a. 47 Ci/mmol), [17α-methyl-^{3}H]-R5020 (17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) (s.a. 87 Ci/mmol) and non-radioactive R5020 were obtained from New England Nuclear France (Paris, France). All other non-radioactive steroids were purchased from Steraloids (Touzart et Matignon, Vitry-sur-Seine, France). The 4-OH-A was generously provided by Dr A. M. H. Brodie (Baltimore, MD).

Test of the anti-aromatase activity of 4-OH-A in vitro. The inhibitory effect on aromatase activity of the 4-OH-A administered to the newborn guinea pigs was verified *in vitro* using a term human placenta. A 1200 *g* supernatant of human placenta was incubated with [1,2-^{3}H]-testosterone (s.a. 52 Ci/mmol, New England Nuclear France) using the method described by Johnston *et al.* [7] in the presence and absence of 2 μM 4-OH-A. The tritium released as ^3H_2O was quantitated in the incubation medium. The equivalent of 100 mg of placenta used as the source of aromatase activity converted 9.5–10.1% of the testosterone to estrogen after 30 min at 37°, and this conversion was inhibited by 97–97.4% by 4-OH-A.

Radioimmunoassay of estrogens. 0.5–1.0 ml of plasma and 50–100 mg of uterus were used to determine the concentrations of estrogens (unconjugated and sulfates). The concentrations of unconjugated estrogens (estradiol and estrone) were evaluated by radioimmunoassay according to the method previously described [8]. 4-OH-A at quantities up to 10 μg per assay tube showed negligible cross-reaction with the antibodies used. The unconjugated estrone and estradiol were extracted with 10 vol. of ethyl

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acetate:hexane (3:2, v/v). The organic phase was evaporated to dryness and redissolved in 0.1 M phosphate buffer for the assay. Estrone sulfate and estradiol sulfate were solvolysed prior to radioimmunoassay. For the solvolysis, the procedure described by Nunez *et al.* [9] was used. The estrogens liberated after solvolysis were determined by radioimmunoassay as the unconjugated estrogens.

Estrogen and progesterone receptor assays. Uteri before culture or explants from a pool of 8 organ culture dishes were homogenized in a Teflon–glass Potter–Elvehjem homogenizer in 0.01 M Tris–HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol (pH 7.4) buffer. The 200,000 *g* supernatant and a 0.6 M KCl extract of the crude nuclear-myofibrillar pellet were prepared as previously described [10].

Estrogen receptor binding was determined in protamine sulfate precipitates of the 200,000 *g* supernatant fraction and the 0.6 M KCl crude nuclear extract after incubation with 1×10^{-8} M [^{3}H]-estradiol with and without a 100-fold molar excess of diethylstilbestrol under conditions which measure available and estrogen-occupied receptor sites [10].

Progesterone receptor binding was measured by incubation with 5×10^{-9} M [^{3}H]-R5020 with and without a 100-fold molar excess of non-radioactive R5020 [5]. Bound and unbound steroids were separated by adding 1 vol. of dextran-coated charcoal (0.5% charcoal/0.05% dextran).

Specific binding was considered to be the difference between total binding and non-saturable binding. The receptor values represent the sum of binding in the 200,000 *g* supernatant and the 0.6 M KCl nuclear extract.

RESULTS

Effect of 4-OH-A treatment of 1-day-old neonates on endogenous estrogen concentrations

One-day-old female guinea pigs were treated with 4-OH-A (50 mg/kg/day) for either 5 or 12 consecutive days and the endogenous estradiol and estrone (both the unconjugated and the sulfo-conjugated) were measured in plasma and in the uterine tissue itself. Table 1 shows that 4-OH-A did not decrease the total unconjugated and sulfo-conjugated estrogen concentrations in either the plasma or the uterus even after 12 days of treatment. If anything, the unconjugated estrogens increased in both the plasma and the uterus, especially after the 12-day-treatment.

Effect of 4-OH-A treatment on uterine wet weight and estrogen and progesterone receptors

Since we had expected 4-OH-A to diminish considerably the endogenous estrogen concentrations, we also looked at the eventual repercussion of this decrease on estrogen receptor concentrations and on two parameters which could possibly depend on the endogenous estrogen concentrations: (1) the progesterone receptor and (2) the uterine wet weight. Moreover, since previous studies had shown that endogenous estrogens in the uterine tissue could be responsible for the maintenance of progesterone receptor synthesis in neonatal uterine explants in organ culture in synthetic, estrogen-free medium [6], progesterone receptor was also measured after organ culture. Table 2 shows that 4-OH-A had no clear effect on uterine wet weight. Five days of treatment with 4-OH-A had no effect on either the estrogen or the progesterone receptors either before or after organ culture as compared to the untreated control uteri. After the 12-day treatment, both the estrogen and progesterone receptor concentrations before culture were somewhat decreased and after culture the progesterone receptor levels did not surpass the concentrations already present before culture. Estradiol added to the culture medium had no inductive effect at any time (2.51–4.94 pmol/mg DNA).

DISCUSSION

The results of the present study unexpectedly revealed that 4-OH-A, a potent aromatase inhibitor in some other animal species, did not decrease the unconjugated or sulfo-conjugated estradiol and estrone concentrations in either the plasma or the uterine tissue of the neonatal guinea pig. Comp-

Table 1. Effect of 4-OH-A treatments on the concentrations of unconjugated and sulfo-conjugated estrogens in the plasma and uterus of 1-day-old newborn guinea pigs

Treatment	Plasma (pg/ml)					Uterus (pg/g)				
	Unconjugated		Sulfates		Total	Unconjugated		Sulfates		Total
	E_2	E_1	E_2	E_1		E_2	E_1	E_2	E_1	
5 Days										
Untreated	77	136	781	515	1509	3678	334	2282	1560	7,854
Treated	121	53	476	450	1100	4641	941	2793	2082	10,457
12 Days										
Untreated	120	91	241	127	579	1854	414	7000	2200	11,468
Treated	247	218	447	116	1028	6660	2341	3497	3080	15,578

One-day-old newborn guinea pigs were injected with 50 mg/kg/day of 4-OH-A for 5 or 12 consecutive days. Estradiol (E_2) and estrone (E_1) concentrations were evaluated by radioimmunoassay. The values represent the average of 2–3 animals for each group.

Table 2. Effect of 4-OH-A treatment on uterine wet weight and estrogen and progesterone receptor concentrations

Treatment	Uterine wet weight (mg)	Estrogen receptor (pmol/mg DNA)	Progesterone receptor (pmol/mg DNA)
5 Days			
Untreated	127		
Before Culture		6.60	3.46
After Culture		0.50	4.50
Treated	123		
Before Culture		7.82	4.19
After Culture		0.35	3.81
12 Days			
Untreated	116		
Before Culture		8.56	5.45
After Culture		0.81	4.55
Treated	103		
Before Culture		6.62	2.78
After Culture		0.80	3.46

Uterine tissues were taken from the same experiments described in Table 1. Receptor assays were performed on portions of tissue either before or after 2 days of organ culture in Medium 199 which was carried out in duplicate. The values represent the average of 2–3 animals for each group.

lementary studies on estrogen and progesterone receptors and wet weight of the uteri also indicated no difference as compared to untreated control uteri. Furthermore, the maintenance of progesterone receptor synthesis by uterine explants in organ culture was also unaffected.

These data are in contrast to those observed in the rat where twice daily injections s.c. for 2 weeks of 4-OH-A (50 mg/kg/day) led to a 60–70% decrease in ovarian venous estradiol concentrations in normal cyclic rats or rats with DMBA-induced mammary tumors [11] or in women with breast cancer who had been treated with 500 mg of 4-OH-A i.m. once weekly whose mean levels of plasma estradiol during days 1–6 after treatment was 46 ± 3 (SD)% of the baseline level [1]. Also, in male rhesus monkeys, peripheral aromatization was reduced by up to 97% of control values [4] so that both ovarian as well as extra-ovarian aromatization is blocked by 4-OH-A in other animal species.

It is interesting to note that in rat neonates another compound, 1,4,6-androstatriene-3,17-dione, inhibits the conversion of androgens to estrogens *in vivo* in neonatal female rat brain [12] and prevents central nervous system defeminization in neonatal male rats [13].

Why estrogen biosynthesis by aromatization was not inhibited by 4-OH-A in the neonatal guinea pig could be due to several as yet unexplored aspects which could regulate the action of 4-OH-A in the animal such as: (1) its metabolism and half-life, (2) its compartmentalization and penetration, and (3) its possible binding to proteins in the plasma or in the target tissues. Differences in these parameters could lead to hitherto unsuspected species-differences in the action of this otherwise very potent aromatase inhibitor.

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