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Induction of Pituitary and Mammary Tumors in Male, "Fale" and Female Rats by Either DMBA, Estradiol Implant or Combined Treatment (40438)

K. J. KAO AND V. D. RAMIREZ

Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

Induction of mammary tumors by 7,12-dimethyl benz(a)anthracene (DMBA) in castrated female rats requires ovarian hormones (1–3). Previous research has indicated that the incidence of DMBA-induced mammary tumors is substantially greater in female as opposed to male rats (1, 4). The present investigation represented an attempt to study the endocrinological basis of this sex difference. Experiment I consisted of injecting DMBA into neonatally orchidectomized male rats to produce a feminization condition in a genetic male (5), the so-called "fale" (6) by Gorski, and female rats ovariectomized (ovx) at 25 days of age. Both "fale" and ovx animals were subsequently treated with empty or estradiol benzoate (E_2) silastic capsules to maintain low but continuous levels of blood estrogen. It was expected that this treatment would eliminate previously observed sex differences in the incidence of DMBA-induced mammary tumors. Surprisingly, upon autopsy, pituitary tumors were found in almost every rat bearing E_2 implant. Although the tumorigenic effect of estrogen on anterior pituitary gland has long been known, it was believed that pituitary tumors could only be induced by long term administration of highly potent estrogen analogs or high doses of estrogen (7, 8). This explanation does not seem applicable to the present study since E_2 implants maintained only moderately elevated serum E_2 levels (9, 10). In an attempt to explain this unexpected result, Experiment II was initiated to examine the relationship between serum estrogen level, plasma levels of prolactin, LH, FSH, and estrogen-induced pituitary tumors in rats.

Materials and methods. Animals. Laboratory raised second generation Sprague-Dawley rats (Laboratory Supply, Indianapolis, Indiana) served as subjects in the experiments. Animals were maintained on *ad libitum* food and water in an air conditioned room with a 14L-10D light cycle (lights on between 0500 and 1900).

Experimental design. Experiment I. Neonatal female and male rats were divided into eight groups. Male rats, thermally immobilized (0°), were orchidectomized ("fale") on day 1 by bilateral inguinal incision. Female rats were ovariectomized under light ether anesthesia at 25 days of age with a single median lumbar incision. At 45 days of age, one group of intact rats and one group of castrated rats of both sexes received β-estradiol-3-benzoate (Sigma Co.) crystals silastic implants (5 mm in length, 1.57 mm i.d., 3.18 mm o.d., Dow Corning Corp.) subcutaneously in the back of the neck. The average weight of the crystals in the capsule was 5.06 $\pm$ 0.26 mg (n = 5). Control animals were implanted with empty silastic capsules. The silastic implants were prepared following the method described by Legan *et al.* (9). According to these authors, serum estradiol could be maintained at levels around 100 pg/ml after implantation. At 50 days of age, all rats were bled through the external jugular vein under light ether anesthesia (0.5 ml/rat) for prolactin determination prior to DMBA administration (1 ml of DMBA emulsion (5 mg/ml, Upjohn Co.)). Subsequently, all rats were palpated every 6 days, and sizes of the mammary tumors were measured by caliper until they were sacrificed by decapitation at 195 days of age. Blood was collected from the trunk and serum prepared for estrogen determination. Wet weight of the anterior pituitary gland of each rat was also recorded.

Experiment II. Since all the rats bearing estrogen implants and treated with DMBA in Experiment I developed pituitary tumors, it was of interest to study the effect of only chronic low levels of estrogen on pituitary tumor induction and on the response of these pituitary tumors to a well-known inhibitor of prolactin secretion (α-bromoergocriptine-

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CB-153, Sandoz, Co.). Nine female rats received E_2 implants at 45 days of age. Fifteen days later serum estrogen levels were determined for three consecutive days in these nine rats. Ninety-six days after E_2 implantation, these animals were divided into two groups (4 and 5 rats respectively). The control group (4 rats) was injected subcutaneously with 0.4 ml ethanol–saline vehicle (96% normal saline, 4% absolute ethanol) at 10:00 a.m. The experimental group (5 rats) was injected with 400 μg of CB-154 suspended in ethanol–saline. Two ml of blood were collected from the external jugular vein prior to CB-154 injection and one ml of blood was sampled at 2, 4, 8, and 24 h after the injection in animals anesthetized lightly with ether. All blood samples were assayed for prolactin concentration. FSH and LH concentrations were also determined in the blood samples collected prior to CB-154 injection. All animals were sacrificed at 100 days after E_2 implantation, and their anterior pituitary glands were collected and weighed. An identical procedure was employed for intact female rats bearing an E_2 implant for only 3 days. The E_2 capsules were implanted to mimic the previous experimental condition but in animals without pituitary tumors.

Serum preparation. All blood samples collected from the external jugular vein or from the trunk after decapitation were allowed to clot at 4°. Sera were separated by centrifugation and stored at −20° until assayed for hormones.

Radioimmunoassays of prolactin, FSH, and LH. The serum concentration of these three hormones was assayed using the NIAMD radioimmunoassay kits with RP-1 reference preparations as standards (prolactin 11 IU/mg, FSH 2.1 × NIH-FSH-S1, LH 0.03 × NIH-LH-S1). All samples were assayed in duplicate.

Estrogen assay. Serum estrogen levels were measured by a single antibody radioimmunoassay according to Hotchkiss *et al.* (10). Tritiated estradiol-17β (New England Nuclear Co.) and Caldewell's 029 antiestradiol-17-β were used for the assay. All serum samples (0.8 ml) were extracted twice with 5 ml of anesthetic grade ether and were assayed in duplicate. The recovery of extraction was 95%.

Results. Estrogen and DMBA-induced mammary tumors. The incidence of carcinogen-induced mammary tumors in the different experimental groups of rats is presented in Table I. The results indicate that mammary tumors could not be induced in castrated female (group 5) or in "fale" rats (group 9). However, when E_2 capsules were implanted 5 days before DMBA injection, the incidence of tumor induction increased from 0% to 44.4% (group 6) and 30% (group 10) respectively. Incidence of tumors failed to increase significantly in intact female and male rats following E_2 implantation (group 3 vs. group 4; group 7 vs. group 8). However, the incidence in female rats was considerably greater than that in male rats (groups 3 and 4 vs. groups 7 and 8). Plasma prolactin levels at the time of DMBA injection revealed that high levels of prolactin could be induced by E_2 implant in both female and male rats, but the incidence of mammary and pituitary tumors does not appear to be related to these initial levels of prolactin. Furthermore, prolactin levels determined in some animals at later intervals did not reveal any correlation with mammary tumor induction (data not shown).

Serum estrogen level and pituitary tumor induction. Total serum estrogen levels of normal female rats at different stages of the estrus cycle were determined (group 1, Table I). Values for three consecutive days' serum estrogen concentration in female rats bearing E_2 implants (group 2, Table I) indicated that serum estrogen levels remained constant following E_2 implantation, confirming similar results reported by Legan *et al.* (9).

In the present experiment it was found that serum estrogen concentrations in the different groups ranged between 119.3 pg/ml to 194.1 pg/ml after E_2 implantation. The variation in the total estrogen levels might be due to sex difference and castration. There was no significant difference among serum estrogen levels of normal cycling female rats at proestrus (group 1), of female rats with E_2 implants (group 2), or of female rats with E_2 implants (group 4) bearing mammary tumors. However, the serum estrogen levels of "fale" (group 10) and male (group 8) with E_2 implants were lower than that of proestrus female rats. Interestingly, pituitary tumors were

TABLE I. INDUCTION OF MAMMARY TUMORS AND PITUITARY TUMORS BY EITHER DMBA ALONE, ESTROGEN IMPLANT OR COMBINED TREATMENT.

Group No.	Experimental condition	No. of rats	DMBA	Serum PRL at time of DMBA injection (ng/ml)	Incidence of rats with mammary tumors Tumor/Total (%)	Average no. of tumors per rat	Serum estrogen (pg/ml)	Pituitary wt. (mg)	% of rats with pituitary tumors (above 30 mg)
1	Female	12	–		0	0	D1 66.6 ± 6.3 D2 69.3 ± 6.9 P 136.3 ± 7.6 (12)[c] E 65.9 ± 2.6	13.1 ± 0.6[b]	0
2	Female[a] + E_2	9	–		0	0	178.0 ± 18.8[d] 188.0 ± 21.6 (9) 183.4 ± 13.0	53.6 ± 6.5	100
3	Female	12	+	66.6 ± 9.0[b]	6/12 (50)	1.6	147.8 ± 40.9 (8)	13.9 ± 0.3	0
4	Female + E_2	9	+	169.2 ± 11.6	6/9 (66.6)	1.4	194.1 ± 33.1 (7)	125.5 ± 24.0	100
5	Ovx	8	+	47.3 ± 3.5	0/8 (0)	0	60.1 ± 9.9 (7)	13.7 ± 1.0	0
6	Ovx + E_2	18	+	138.0 ± 9.2	8/18 (44.4)	2.9	145.2 ± 18.6 (12)	98.3 ± 10.4	100
7	Male	12	+	44.8 ± 4.9	2/12 (16.7)	1	48.5 ± 3.6 (11)	9.6 ± 0.5	0
8	Male + E_2	9	+	164.3 ± 15.2	2/9 (22.2)	1.4	118.3 ± 13.6 (8)	66.6 ± 9.7	66.6
9	"Fale"	7	+	44.6 ± 2.9	0/7 (0)	0	55.3 ± 4.8 (7)	12.5 ± 0.8	0
10	"Fale" + E_2	10	+	133.1 ± 10.5	3/10 (30)	1.3	119.3 ± 7.6 (10)	90.8 ± 14.4	100

D1: early diestrus; D2: late diestrus; P: proestrus; E: estrus (3 animals per stage).

[a] Group 2 received E_2 implants for 100 days before sacrifice; the other groups received the E_2 for 150 days. Mean estrogen level of group 3 was from animals at different stages of estrus cycle. Analysis of variance indicated no significant difference among estrogen levels in group 1, 2, and 4. ($P > 0.05$).

[b] Mean ± SEM.

[c] No. of rats for serum estrogen and pituitary weight determinations. Since some animals died during the course of the experiment, there is a discrepancy between the initial number and the final number of animals indicated in this column.

[d] Serum estrogen levels were determined for three consecutive days, 15 days after implantation.

found in almost all rats receiving E_2 implants (Table I). No pituitary tumors were found in groups of rats without E_2 implants. Employing the criterion of Clifton and Meyers (8), pituitary tumors were defined by weights of the anterior pituitary glands greater than 30 mg. Visually, these anterior pituitary glands were enlarged and protruded dorsally to compress the basal hypothalamus. Some tumors were hemorrhagic and cystic.

Prolactin, LH and FSH levels in rats with E_2-induced pituitary tumors. The serum levels of prolactin, FSH, and LH in nine pituitary tumor-bearing rats of group 2 were 5620 ± 911 ng/ml, 151.29 ± 9.7 ng/ml, and 23.7 ± 3.4 ng/ml respectively. The serum prolactin concentration was increased substantially, but FSH and LH levels were about normal or subnormal in these rats as compared to intact controls. Normal control values (N=5) of prolactin, FSH, and LH were 66.9 ± 9.0 ng/ml, 150 ± 10 ng/ml, and 60 ± 5 ng/ml, respectively. The huge increase in serum prolactin levels in pituitary tumor-bearing rats could be reduced effectively by the injection of 400 μg CB-154 subcutaneously (Table II and Fig. 1). The maximal inhibitory effect on prolactin secretion occurred about 4 hr after injection; however, inhibition was still evident by 24 hr. Normal female rats with 3 days E_2 implant responded similarly to CB-154 treatment (Table II). Seven pituitary tumors from group 2 (Table I) were thoroughly homogenized in PBS pH 7.5 and the buffer extracts subjected to prolactin determination. The prolactin concentration of pituitary tumor tissue was 2.85 ± 0.23 μg/mg of pituitary tumor tissue. These values were within normal range as reported previously in normal female rats (3.9 ± 0.6 μg/mg) (11) and female rats with DMBA-induced mammary tumors (2.0 ± 0.2 μg/mg) (12).

Discussion. The present results confirm the observation that estrogen is required for the induction of mammary tumors by DMBA in castrated female rats (1–3). The data also demonstrate that DMBA injections in castrated female or in "fale" rats did not induce

TABLE II. BLOOD PROLACTIN LEVELS AFTER A SINGLE INJECTION OF 400 μg α-BROMOERGOCRIPTINE.[a]

Group	Treatment	Time after injection (hr)				
		0 hr	2 hr	4 hr	8 hr	24 hr
Female rats with E_2 implants for 3 days	Vehicle (5)*	272.0 ± 115.6**	348.0 ± 147.1	370.0 ± 58.6	532.5 ± 171.7	543.0 ± 189.2
	CB 154 (5)	208.8 ± 59.6	66.8 ± 34.2	43.9 ± 18.6	66.6 ± 18.6	96.5 ± 40.6
Female rats with E_2 induced pituitary tumors	Vehicle (4)	5622 ± 1259	5285 ± 1748	5122 ± 1986	6260 ± 2067	6152 ± 476
	CB 154 (5)	5618 ± 1320	337.8 ± 69.0	234.8 ± 19.7	422.3 ± 76.7	1297.4 ± 284.7

[a] Analysis of variance of the data indicated significant difference for both groups of rats after CB154 injection ($P < 0.05$). No significant difference in both control groups after vehicle injection.

* ( ) No. of animals.

** Mean ± SEM: Values expressed as ng/ml of serum.

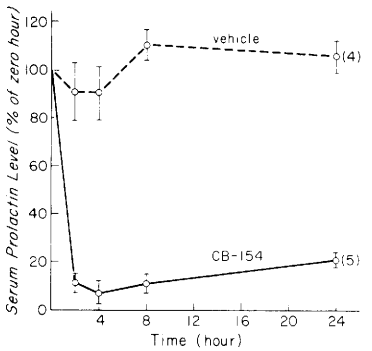

FIG. 1. Changes in prolactin levels to an injection of CB-154 in E_2 induced pituitary tumor rats. Prolactin levels were expressed as % of zero hour value of each rat. Each point is the mean ± SEM. Dashed line represents the changes of prolactin level after vehicle injection. Solid line represents the changes after CB-154 injection. See Table II also.

mammary tumors, though low levels of immunoreactive estrogens were still present in the serum. Castrated female and "fale" rats implanted with E_2, were susceptible to the carcinogenic effects of DMBA, and the mammary tumor incidence increased from 0% to 44.4% and 30% respectively. However, this potentiating effect of estrogen in the induction of mammary tumors by DMBA was not noted in intact male rats. The low tumor incidence was still detected in both intact male (17%) and in intact male with E_2 implants (22%), though these later animals had high initial blood levels of prolactin, comparable to those in female rats bearing E_2 implants. These results suggest that a certain level of estrogen is required for the induction of mammary tumors by DMBA, and that this effect in male rats can be partially inhibited by the presence of an unknown testicular factor(s). It is known that androgenic steroids can inhibit the growth of mammary tumors in rats (13, 14) and man (15). The increased serum estrogen levels provided by the implant in these animals could raise plasma prolactin levels and induce a pituitary tumor but could not change the low incidence of DMBA-induced mammary tumors in males. An alternative hypothesis for the lower mammary tumor incidence in estrogen treated males and fales is perhaps the presence of fewer target mammary cells at the time of carcinogen administration. The role of estrogen in the induction of mammary tumor by DMBA is not clear at this moment. Whether the estrogen is required for the activation of DMBA (procarcinogen) into ultimate carcinogen (16–18), or is required for the action of activated ultimate carcinogen in the target cells needs to be studied further. Also, the nature by which the testes inhibit the potentiation action of estrogen in DMBA-induced mammary tumors warrants further investigation. The fact that neonatally feminized genetic males bearing estradiol implants as adults show an intermediate susceptibility (30%) between intact males (22%) and ovx females (44%) bearing similar E_2 implants to the effect of DMBA on mammary tumor induction suggests that some kind of biochemical differentiation in the mammary gland might occur early in development, making the female breast more susceptible than the male gland to the carcinogenic action of DMBA. Indeed, previous work has demonstrated that male fetuses feminized by cyproterone acetate given to the mothers had mammary glands similar in histological appearance to those of normal female rats (19). Perhaps earlier feminization in genetic males would be required to obtain a full expression of the female mammary gland susceptibility to the carcinogenic action of DMBA.

The tumorigenic effect of estrogen on the anterior pituitary glands has been known since 1936 (20, 21). It has been well established that long-term treatment and high doses of estrogen can induce anterior pituitary tumors. Therefore, classic methods typically employed to induce anterior pituitary tumors are: (a) implantation of 8–15 mg diethylstilbestrol (DES) pellet subcutaneously for more than 170 days (8, 22, 23), (b) implantation of 200 μg of estrone pellet subcutaneously plus drinking water containing 2 mg per liter of estrone every day for approximately one year (24), or (c) a single injection of 20 mg DES dipropionate subcutaneously with 20% of the rats developing tumors 406 days later (25). To the best of the authors' knowledge, few experiments have been performed in the rat to clarify the quantitative relationship between blood estrogen level and pituitary tumor induction (26).

In the present investigation, estradiol capsules were implanted to provide a stable source of serum estrogen in ovariectomized rats treated with DMBA. The serum estrogen level chosen was thought to be close to normal level in proestrus rats. However, to our surprise, prolactin secreting pituitary tumors occurred in almost all rats receiving E_2 implants. Our data demonstrated that the pituitary tumors could be induced simply by maintaining blood estrogen levels at the proestrus level of normal cycling rat or even lower. These results implicate the high risk of long-term, low-dose administration of estradiol in pituitary tumor induction or pituitary malfunction.

In the present study, the prolactin concentration in serum was very high in pituitary tumor rats, but FSH and LH concentrations were around normal or subnormal range. These changes in blood gonadotropin concentrations detected in rats with estrogen-induced pituitary tumors are similar to previous reports (25, 27, 28). Usually, the tumors are predominantly prolactin secreting, but occasionally growth hormone is also secreted concomitantly. This hormonal secretion pattern is also similar to that found in human prolactin secreting pituitary tumors (29). The mechanism of high prolactin secretion might be due to the induced proliferation of prolactin secreting cells by estrogen (8), or to an increase in PRL turnover in the tumor cells (23). The pituitary prolactin concentration of rats with E_2 induced pituitary tumor was the same as that of normal animals. This proves that the increase in mass of the pituitary gland was due to an abnormal proliferation of the prolactin secreting cells in the anterior pituitary gland.

The inhibition of prolactin secretion from pituitary tumors by administration of L-dopa or dopamine has been reported both in rats (24) and humans (30, 31). A dopamine agonist, α-bromoergocriptine (32, 33), which acts on dopamine receptors (34), is also capable of inhibiting prolactin secretion effectively in humans with pituitary tumors (29, 35). However, it was reported in rats that α-bromoergocriptine was not effective in inhibiting the growth and prolactin secretion of transplantable prolactin secreting pituitary tumors (36, 37). In the present experiment the huge blood levels of prolactin detected in the E_2 induced pituitary tumor rats could be lowered markedly by subcutaneous injection of 400 μg α-bromoergocriptine. This response coincided with the observation of Child *et al.* in pituitary tumors (29), and of Dickey *et al.* in postoral contraception galactorrhea-amenorrhea patients (38). Although the similarities of hormonal blood levels (high levels of PRL, normal levels of LH-FSH) and of PRL response to α-bromoergocriptine administration detected in rats and in humans with pituitary tumors probably do not represent the same pathogenic process, our results suggest a possible risk of pituitary tumor induction by long-term, low-dose administration of estrogenic substance in humans. Furthermore, it seems clear that chronic maintenance of constant levels of estrogen by silastic implants in blood does not mimic physiological levels of these steroids.

Summary. Implanting silastic capsules containing estradiol (E_2) into castrated female and "fale" rats 5 days before 7,12-dimethyl benz(a)anthracene (DMBA) injection increased the incidence of mammary tumors from 0% to 44.4% and 30% respectively. However, this E_2 potentiating effect on DMBA-induced mammary tumors was not detected in intact female or male rats, but the incidence of mammary tumors was much lower in intact male rats than in intact female rats

with or without E_2 implants. Most rats receiving E_2 implants developed prolactin secreting pituitary tumors within 100 days after implantation. Serum estrogen level of rats bearing E_2 implants in different groups was maintained between 118.3 pg/ml and 194.1 pg/ml. These estrogen levels were not significantly different from that of normal cycling rats at proestrus. The pituitary tumors induced by E_2 implants secreted huge amounts of prolactin as revealed by enormous levels of PRL in blood, but normal levels of blood FSH and LH were determined. A clear-cut decrement of blood prolactin levels was measured when α-bromoergocriptine was given to female rats with or without pituitary tumors. An unexpected induction of prolactin secreting pituitary tumors in rats by maintaining with silastic implant constant blood proestrus estrogen concentrations was clearly demonstrated.

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