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Estrogens with Reduced Catechol-Forming Capacity Fail to Induce Implantation in the Rat (42243)

S. K. DEY, D. C. JOHNSON, P. L. PAKRASI, AND J. G. LIEHR*

*Departments of Obstetrics & Gynecology and Physiology, University of Kansas Medical Center, Ralph L. Smith Research Center, Kansas City, Kansas 66103, and *Department of Pharmacology, University of Texas Health Science Center, Houston, Texas 77025*

Abstract. Catechol estradiol can induce implantation of the embryo in a progesterone-primed uterus, but we do not know whether conversion of estrogen to a catechol is essential for implantation. The present study examined the ability of fluorinated estradiols that have a reduced capability of catechol formation to induce implantation. Delayed implantation in rats that were hypophysectomized on the third day postcoitum was maintained by daily injection of progesterone. On the fifth day of progesterone treatment they were injected intravenously with estradiol-17β (E2), or various doses of 2-fluoroestradiol-17β (2-Fl-E2) or 4-fluoroestradiol-17β (4-Fl-E2) and examined 24 hr later for evidence of initiation of implantation. All animals treated with 25 ng of E2 showed normal numbers of implantation sites, as did those receiving 60 ng of 4-Fl-E2. In contrast, 2-Fl-E2 failed to initiate implantation with doses as high as 300 ng per animal; there were only single sites in two of eight rats treated with 500 ng. Initiation of implantation was not correlated with lack of uterotropic estrogenicity. The results suggest that formation of catechol estrogen may be an important step in mediating estrogen function for implantation of the embryo.

Recently we have proposed that catechol estrogens mediate some important aspects of estrogenic action, possibly by stimulation of prostaglandin synthesis, essential for the process of embryo implantation (1, 2). The following observations form the bases for our view: (i) Systemic administration of catechol estradiol can initiate implantation in the uteri of mice or rats treated with progesterone (3, 4); (ii) catechol estrogens are more potent stimulators of uterine prostaglandin synthesis than are their phenolic counterparts (5–7). This has importance because considerable evidence has accumulated associating prostaglandin synthesis with capillary permeability changes that are prerequisite for implantation (8, 9). (iii) Stimulation of prostaglandin synthesis by estrogens *in vivo* is not inhibited by antiestrogens or inhibitors of protein or RNA synthesis, suggesting that their action is not mediated by the classical estrogen receptor (10).

More direct evidence for the role of catechol estrogens in implantation could be obtained by use of either specific and potent inhibitors of estrogen 2/4-hydroxylase or by use of estrogens that do not readily form catechols. The former are not available but fluorinated estradiols can be useful for the latter approach. 2-Fluroestradiol (2-Fl-E2) and 4-fluroestradiol (4-Fl-E2) are potent estrogens in terms of classical cytosolic-nuclear receptor binding, uterotrophic effects, and other estrogenic responses (11–13), but they are poorer substrates than estradiol for catechol estrogen formation (13). Specifically, fluorination at the 2 position impedes 2-hydroxylation while fluorination at the 4 position appears to impede 4-hydroxylation. In the present study we have compared the ability of these fluorinated estrogens with that of estradiol in inducing implantation in the rat. The results are consistent with the view that impeded production of catechol estrogen is correlated with failure of implantation.

Materials and Methods. *Steroids.* Progesterone and estradiol-17β (E2) were obtained from Sigma Chemical Company (St. Louis, Mo.), and 4-hydroxyestradiol (4-OH-E2) was purchased from Steraloids (Wilton, N.H.). The latter steroid was free from E2 as determined by high-performance liquid chromatography. Fluroestradiols (2-Fl-E2 and 4-Fl-E2) were synthesized by the method of Utne *et al.* (14). Both steroids were examined for purity by direct inlet mass spectrometry and by gas chromatography–mass spectrometry. E2 could not

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be detected in the preparations but the 2-Fl-E2 contained approximately 2% of another fluorinated estradiol, possibly 4-Fl-E2.

The delayed implantation model was used to compare implantation-inducing potency of the fluorinated estradiols with that of E2. Adult (250–275 g) female rats (Holtzman Co., Madison, Wis.) were hypophysectomized on the third day of pregnancy (Day 1 = morning of finding spermatozoa in the vagina). The animals were injected (sc) at the time of operation and daily thereafter for 5 days with 2 mg of progesterone dissolved in 0.1 ml of sesame seed oil. On the fifth day of progesterone treatment (Day 8 of pregnancy) animals were injected (iv) either with saline, 25 ng of E2, or various amounts of 2-Fl-E2 or 4-Fl-E2 dissolved in saline, and killed 24 hr later.

To determine if the high doses of 2-Fl-E2 used in some experiments were toxic to the embryos the following study was carried out. Rats with delayed implantation were injected, on Day 8 of pregnancy, with 150 ng of 2-FL-E2 and infused, via an osmotic minipump (Alza Corp. Palo Alto, Calif.), for 24 hr with 4-OH-E2 (50 ng/μl) and then killed. This dose of 4-OH-E2 had been established as effective in inducing implantation (4).

Initiation of implantation was evaluated by injecting 1 ml of a 2% solution of Chicago Blue B (Sigma) dissolved in normal saline 15 min before killing with an overdose of ether. Implantation sites were identified as discrete blue areas because of the increased uterine capillary permeability at the location of the blastocysts (15). Uteri were flushed with saline, the blastocysts counted and the uteri weighed. Occassionally sperm-positive animals were without sites or blastocysts and were excluded from the study.

To eliminate the effect of the implanting embryos on uterine weight and to compare the uterotropic effect of 2-Fl-E2 with that of E2 in the progesterone-primed uterus, adult female rats were hypophysectomized without regard to the stage of the estrous cycle. Seven days later daily treatment with 2 mg of progesterone was begun and continued for 4 days. On the last day of progesterone treatment the animals were injected (iv) with either saline, 30 ng of E2 or 150 ng of 2-Fl-E2 dissolved in saline. The animals were killed 24 hr later and their uterine weights determined.

Results. Progesterone alone did not initiate implantation, but a single injection of 25 ng of E2 into the progesterone-primed rats was effective in all of the animals. In contrast, only single implantation sites were found in each of two of eight rats injected with 500 ng of 2-Fl-E2; all lower doses were completely ineffective. The rats infused with 4-OH-E2 following an injection of 150 ng of 2-Fl-E2 had a full complement of implantation sites (10.9 $\pm$ 0.8, n = 7). The uterine wet weight increased with increasing doses of 2-Fl-E2 even though there was no evidence of initiation of implantation (Table I). 4-Fl-E2 was much more effective than the 2-fluorinated compound but somewhat less effective than E2 at initiating implantation. Implantation sites were found in 57% of the animals given a single injection of 30 ng of 4-Fl-E2. The number of blastocysts recovered in these animals was higher than the number of blue sites indicating some blastocysts failed to implant. An injection of 60 ng of this fluoroestrogen initiated implantation of the normal number of blastocysts in all of the animals (Table I).

The increase in uterine wet weight (mg/100 g body wt, n = 5) in hypophysectomized progesterone-treated rats was comparable after injection of 30 ng of E2 (91.0 $\pm$ 4.9) or 150 ng of 2-Fl-E2 (101.6 $\pm$ 2.0); controls receiving only progesterone had weights of 69.9 $\pm$ 2.5.

Discussion. The results of the present investigation are consistent with the view that conversion of phenolic estrogens to their catechol metabolites is an important requirement for the initiation of implantation. Which catechol is the more potent for inducing implantation has not been established but the present results suggest that 2-OH-estradiol is more important than 4-OH-estradiol. 4-FL-E2, which was a relatively potent inducer of implantation, could be hydroxylated at the 2-position. On the other hand, 2-Fl-E2 could be hydroxylated at the 4 position, but either this did not occur, or the amount produced was insufficient to induce implantation. Another factor for consideration is that dehalogenation of halogenated estrogens and then formation of catechol metabolites by hamster liver microsomes has been reported (16): these catechol metabolites are not likely to reach the target organs because of the high degree of *O*-methylation of catechols by the liver. Whether

TABLE I. THE EFFECT OF ESTROGENS UPON INITIATION OF IMPLANTATION IN THE DELAYED IMPLANTING RAT

Treatment	Dose (μg)	Rats with sites / Total No. rats	Number of sites/rat	Number of blastocysts recovered/rat	Uterine wet wt mg/100 g body wt
Saline	—	0/8	0	9.8 ± 0.3	76.8 ± 3.9
Estradiol	25	10/10	8.6 ± 1.0	7.8 ± 1.2	105.6 ± 7.9*
2-Fluoroestradiol	80	0/7	0	7.6 ± 1.1	77.3 ± 5.7
	125	0/8	0	11.0 ± 1.0	82.5 ± 3.8
	150	0/8	0	8.0 ± 1.1	85.8 ± 0.4
	200	0/5	0	7.0 ± 1.6	92.0 ± 3.0*
	250	0/5	0	9.6 ± 1.3	99.7 ± 3.1*
	300	0/6	0	11.5 ± 1.2	104.2 ± 7.9*
	500	2/8	1	9.0 ± 1.5	96.9 ± 4.0*
4-Fluoroestradiol	30	4/7	5.8 ± 0.6	6.7 ± 1.4	108.3 ± 5.2*
	60	5/5	8.8 ± 0.4	7.4 ± 0.9	102.7 ± 7.4*

Note. Values are means ± SEM. Animals were hypophysectomized on the third day postcoitum and given progesterone daily. Five days later they were injected (iv) with the estrogens dissolved in saline and killed 24 hr later, 15 min after injection (iv) of Chicago Blue B. The number of blue stained implantation sites were counted, the uteri flushed with 0.5 ml of saline, and the number of blastocysts recorded. Mean number of sites represent only animals with sites. Mean uterine weights with the same superscript are not significantly different from each other ($P < 0.05$).

this can occur in other tissues and in other species remains to be determined.

Lack of correlation between uterotrophic and implantation inducing action of the fluorinated estrogens suggests that some of their effects required for implantation are mediated through mechanisms that do not involve the classical estrogen receptor. Previous studies have implicated increased uterine vascular permeability, secondary to histamine release and uterine esosinophilia, with a nongenomic action of estrogen (17). This does not mean, however, that the function of the classical estrogen receptor is unimportant for implantation. Probably both genomic and nongenomic actions are involved. Some indication of this is revealed by the finding that neither 2-Fl-E2 nor histamine alone induce implantation but a combination of 75 ng of 2-Fl-E2 and histamine is effective (unpublished data). Because catechol estrogens have the capability of acting via both genomic and nongenomic mechanisms they would be appropriate agents for induction of implantation (18–22).

The mechanism by which catechol estrogens could participate in implantation remains to be defined. One possibility is that these steroids, formed locally in the blastocyst and/or the endometrium at the implantation site, stimulate prostaglandin synthesis required for the vascular changes which initiate implantation. Whether the rat blastocyst or uterus has the capacity to form catechol estrogens has not been established. However, both the pig and hamster blastocysts have been shown to have the capacity to form catechol estrogens, in particular 2-hydroxyestradiol (2, 23). The fact that 2-Fl-E2 was so much less effective than 4-Fl-E2 suggests that in the rat 2-hydroxylation also predominates. Stimulation of prostaglandin synthesis is one of several functions of catechol estrogens (18–22, 24) that requires consideration in determining the mechanism by which these estrogens participate in implantation.

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