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Inhibition of Ovulation in Rats with Epostane, an Inhibitor of 3β-Hydroxysteroid Dehydrogenase (41865)

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Abstract. Epostane (Win 32729), an inhibitor of the 3β-hydroxysteroid dehydrogenase enzyme system, inhibited both spontaneous and pregnant mare's serum/human chorionic gonadotropin-induced ovulation in rats. When administered on the morning of proestrus, the drug blocked pregnancy in females that were inseminated that evening. The blockage of pregnancy occurred at a dose of 200 mg/kg but not at 50 mg/kg. Similarly, when administered on the morning of proestrus at a dose of 200 mg/kg, epostane inhibited the appearance of ova in the oviducts the next day. In gonadotropin-primed immature rats, epostane inhibited ovulation in a dose-related fashion with an ED_{50} between 25 and 50 mg/kg. The drug also decreased plasma progesterone levels in these animals. The inhibitory effect of epostane on gonadotropin-stimulated ovulation was reversed by injections of progesterone at a total daily dose of 6.25 mg/rat or greater. These results support the contention that steroidogenesis, specifically progesterone synthesis, is a prerequisite to ovulation.

A preovulatory rise in progesterone levels has been observed during the proestrous phase of the rat estrous cycle (1) and several lines of evidence indicate that this rise is necessary for the ensuing ovulation that occurs that night. For example, Lipner and Greep (2) showed that aminoglutethimide and cyanoketone, drugs that block the synthetic pathway to progesterone, prevented ovulation in immature rats injected with gonadotropins. Further evidence in support of a role for progesterone in the ovulatory process comes from the experiments of Mori *et al.* (3) who demonstrated that injection of an antiserum directed against progesterone inhibited the induction of ovulation in immature rats. Thus, agents that block either the synthesis or action of progesterone inhibit ovulation in rats.

Luteinizing hormone (LH) is also known to play a role in the ovulatory process. Rondell (4, 5) demonstrated that follicular wall tissue underwent increases in distensibility immediately before rupture of the follicle and this effect could be duplicated in follicular strips maintained in tissue culture by treatment with either LH or progesterone; cyanoketone was shown to inhibit the increased distensibility induced by LH but not by progesterone. Takahashi *et al.* (6) presented evidence for the occurrence of a two-step process on the afternoon of proestrus that leads to ovulation; the first step requires a short exposure to LH and the second an exposure to progesterone. These studies are consistent with the concept that the ovulatory surge of LH stimulates progesterone production which, in turn, activates the ovulatory process.

In contrast, Bullock and Kappauf (7) claimed to have dissociated the preovulatory rise of progesterone from ovulation by administration of cyanoketone or aminoglutethimide in doses that blocked one of these physiological events but not the other. These authors concluded that follicular changes leading to ovulation were not initiated by steroidogenesis.

In this paper, we examine the ability of a new inhibitor of the 3β-hydroxysteroid dehydrogenase enzyme system, epostane [Win 32729, (4α,5α,17β)-4,5-epoxy-3,17-dihydroxy-4,17-dimethylandrost-2-ene-2-carbonitrile], to block ovulation in mature rats and gonadotropin-stimulated immature rats. The structure of epostane is shown in Fig. 1. The compound has recently been shown to lower plasma progesterone levels and terminate pregnancy in rats and rhesus monkeys (8).

Materials and Methods. *Animal procedures.* Rats used in these experiments were of the Sprague–Dawley strain obtained from Taconic Farm Breeding Laboratories, Inc., Germantown, New York. They were housed in an air-conditioned room with a 12-hr light:dark

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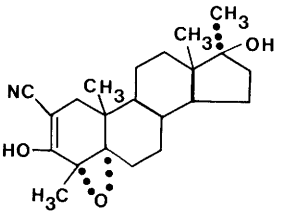

FIG. 1. Structure of epostane.

schedule. Rat chow and water were provided *ad libitum.*

Mature rats to be used for mating were subjected to daily vaginal smears. Animals with two consecutive 4-day cycles were administered vehicle or epostane, orally, on the morning of proestrus of the third cycle. After medication, each rat was caged overnight with a proven breeding male. The following day (expected day of estrus), the female rats were separated from the males and vaginal smears were made and examined microscopically for the presence of sperm. Nine days after mating, the animals were killed with an overdose of sodium pentobarbital and their uteri excised and examined for evidence of implantation.

Ovulation was assessed in regularly cycling mature female rats that were treated with vehicle or drug by sacrificing the animals on the expected day of ovulation and excising their oviducts for inspection. The oviducts were gently pressed between two glass slides and the ova were visualized and counted with the aid of a dissecting microscope (40×).

Immature female rats approximately 22 days of age were induced to ovulate with a subcutaneous injection of 12.5 IU pregnant mare's serum (PMS) followed 48 hr later by an intraperitoneal injection of 20 IU human chorionic gonadotropin (HCG). Oral administration of epostane or vehicle occurred at the time of HCG medication. In one experiment progesterone was injected subcutaneously in equally divided doses 6 hr prior to and at the time of HCG and drug administration. The total daily dose of progesterone administered in these experiments ranged from 1.56 to 50 mg/rat.

Blood samples for plasma progesterone determinations were obtained by cardiac puncture from unanesthetized rats. Progesterone levels were measured by radioimmunoassay as supplied by Pantex Inc. (Method 037). The sensitivity of the assay was 0.2 ng/ml and the coefficient of variation was 6.8% within an assay and 11.6% between assays. The cross-reactivities of related steroids to the progesterone antibody were (progesterone equals 100%): desoxycorticosterone, 1.5%; 17α-hydroxyprogesterone, 0.18%; pregnenolone, 17-hydroxypregnenolone, and corticosterone, <0.01%. The assay of increasing volumes of serum remained parallel to the standard.

Solutions. PMS and HCG (Sigma Chemical Co., St. Louis, Mo.) were diluted with 1% bovine serum albumin (Sigma) in saline to an injectable volume of 0.1 ml/rat. Epostane was prepared as a solution/suspension in absolute ethanol:cottonseed oil (1:9 v/v) and administered orally (po) at a volume of 0.5 ml/rat in immature rats and 1.0 ml/100 g in mature rats. Progesterone (Sigma) was prepared as a solution/suspension in absolute ethanol:-cottonseed oil (1:9 v/v) and administered subcutaneously (sc) at a volume of 0.2 ml/rat.

Statistical evaluation. Comparisons between treatment and control values were made using Fisher's exact test except for the data in Table V where Student's *t* test was used.

Results. Table I illustrates the effect of epostane when administered orally to regularly cycling female rats on the morning of proestrus. One hundred percent of the mated animals treated with either vehicle alone or the low dose of epostane (50 mg/kg) were pregnant when autopsied 9 days later. In contrast, none of the mated animals treated with 200 mg/kg of epostane had implantation or resorption sites 9 days after mating. Epostane did not adversely affect mating success since the percentage of females that mated did not differ between the high drug dose (200 mg/kg) group and the vehicle control group.

TABLE I. EFFECT OF EPOSTANE ON PREGNANCY IN MATURE RATS

Epostane (mg/kg po)	Proportion mating (%)	No. pregnant at autopsy	Sites/ uterus[a]
0	15/26 (58)	15/15	11.9 ± 0.3
50	11/13 (85)	11/11	10.6 ± 0.8
200	6/14 (43)	0/6[b]	0

[a] Mean ± SE.
[b] $P < 0.01$ compared to the vehicle control.

To investigate the possibility that epostane may be inhibiting a postovulatory event (e.g., implantation), a direct measurement of ovulation was employed. The oviducts of mature regularly cycling rats were excised and examined for the presence of tubal ova 24 hr after proestrus treatment with epostane or vehicle. Epostane did not alter the proportion of animals having cornified vaginal smears on the expected day of estrus. Ova were not observed in females treated with epostane (200 mg/kg), whereas 9 of 14 vehicle-treated animals ovulated (Table II).

An experiment was performed to determine whether the ovulation inhibition effect of epostane was due to an inhibition of gonadotropin secretion. Immature female rats were induced to ovulate with injections of PMS followed 24 hr later by concomitant administration of HCG and either epostane or vehicle. The results of this experiment, shown in Table III, illustrate that epostane inhibited the number of rats ovulating in a dose-related fashion. A dose of 50 mg/kg significantly reduced the number of rats that ovulated while the 100 and 200 mg/kg doses of epostane inhibited ovulation in all the animals treated. Doses of less than 50 mg/kg were not effective in inhibiting ovulation.

Progesterone was administered to determine whether it could reverse the inhibition of ovulation observed after treatment with epostane. Progesterone was injected subcutaneously at various doses into gonadotropin-primed immature female rats given an oral medication of epostane at 50 mg/kg. Progesterone injected twice on the same day as oral administration of epostane overcame the inhibition of ovulation in a dose-related fashion (Table IV). Reversal of epostane inhibition of ovulation occurred at a total progesterone dose of 6.25 mg/rat or greater, whereas a lower dose of 1.56 mg/rat was ineffective in blocking the epostane effect.

TABLE II. EFFECT OF EPOSTANE ON SPONTANEOUS OVULATION IN THE MATURE RAT

Epostane (mg/kg po)	Proportion ovulating	Ova/rat[a]
0	9/14	9.0 ± 0.8
200	0/17[b]	0

[a] Mean ± SE.
[b] $P < 0.001$ compared to vehicle control.

TABLE III. DOSE-RELATED EFFECT OF EPOSTANE ON OVULATION IN THE GONADOTROPIN-PRIMED IMMATURE RAT

Epostane (mg/kg po)	Proportion ovulating	No. of ova/ ovulatory rat[a]
0	18/20	13.8 ± 2.3
12.5	10/10	9.8 ± 1.8
25.0	10/10	19.4 ± 3.4
50.0	2/20[b]	2.0 ± 1.0
100.0	0/10	—
200.0	0/10	—

[a] Mean ± SE.
[b] $P < 0.001$ compared to vehicle control.

The effect of epostane on circulating progesterone levels is presented in Table V. Plasma samples for progesterone determination were obtained 4 hr after oral administration of epostane. Dose levels of drug as low as 12.5 mg/kg lowered plasma progesterone significantly below vehicle control levels even though ovulation was not inhibited at this dose. Epostane at doses of 25 mg/kg and greater inhibited ovulation as well as decreased plasma progesterone concentrations.

Discussion. This study supports the concept that steroidogenesis is a prerequisite for the process of ovulation since an agent that interfered with the synthesis of steroids also blocked ovulation. Epostane blocked ovulation in cycling rats as evidenced by inhibition

TABLE IV. EFFECT OF EPOSTANE ON OVULATION IN GONADOTROPIN-PRIMED AND PROGESTERONE-SUPPLEMENTED IMMATURE RATS

Treatment			
Epostane (mg/kg po)	Total daily progesterone (mg/rat sc)	Proportion ovulating	Ova/ ovulatory rat[a]
0	0	16/20	27.9 ± 3.0
50	0	0/20	0
50	1.56	0/10	0
50	6.25	8/10	13.4 ± 3.7
50	12.5	8/10	16.3 ± 4.4
50	25	7/10	12.3 ± 3.8
50	50	19/20	28.4 ± 3.3
0	50	7/9	25.9 ± 4.6

[a] Mean ± SE.

TABLE V. EFFECT OF EPOSTANE ON OVULATION AND PROGESTERONE PLASMA CONCENTRATION IN THE GONADOTROPIN-PRIMED IMMATURE RAT

Epostane (mg/kg po)	Proportion ovulating (%)	Ova/ovulating rat (mean ± SE)	Plasma progesterone[a] (ng/ml)	n^b
0	24/25 (96)	27.8 ± 2.7	33.3 ± 2.4	13
12.5	9/10 (90)	36.0 ± 4.0	4.6 ± 0.8[c]	6
25	5/24 (21)	11.4 ± 3.5	2.8 ± 0.3	14
50	2/10 (20)	13.0 ± 12.0	1.6 ± 0.2	6
100	0/15 (0)	—	1.5 ± 0.2	8
200	0/15 (0)	—	1.4 ± 0.2	7

[a] Mean ± SE.
[b] No. of rats randomly chosen for progesterone determination.
[c] $P < 0.001$ compared to the vehicle control.

of pregnancy in mated animals and more specifically by lack of ova in the oviducts on the morning of estrus. A direct effect of epostane on the ovary is likely since the drug also inhibited the induction of ovulation in immature animals treated with PMS and HCG.

Epostane has been shown to inhibit the 3β-hydroxysteroid dehydrogenase enzyme system. *In vitro* preparations of this enzyme from rat adrenal (8) and human placenta (9) were competitively inhibited by epostane. When administered orally, epostane terminated pregnancy in rats and rhesus monkeys and this effect was overcome by injections of progesterone (8). In rats epostane was equipotent as an inhibitor of adrenal and ovarian steroidogenesis, but in rhesus monkeys the drug interrupted pregnancy at doses that did not inhibit adrenal steroidogenesis (8). Epostane has no apparent hormonal activity in a number of standard test systems.

In this study, epostane sharply reduced circulating progesterone levels in rats injected with PMS and HCG and, at the same time, inhibited ovulation. Progesterone injections reversed the inhibitory effects of epostane on ovulation. Lipner and Greep (2) used cyanoketone to inhibit ovulation but were unable to override this inhibition with a single injection of 4 mg progesterone. The failure to overcome the inhibitory effects of cyanoketone on ovulation may have been due to administration of insufficient amounts of progesterone since in the present study, two successive injections of 3.1 mg of progesterone were required to restore the proportion of ovulating animals to normal. Even higher doses were required to return the number of ova shed per rat to the control levels. Perhaps high doses of exogenous progesterone are required to raise intrafollicular concentrations to the levels that are needed for ovulation.

Bullock and Kappauf (7) claimed to have disassociated the effects of cyanoketone and aminoglutethimide on ovulation and steroidogenesis. By administering the appropriate dose, they showed that aminoglutethimide decreased HCG-elevated progesterone levels without inhibiting ovulation. In contrast, cyanoketone was shown to partially block ovulation without having a significant effect on progesterone levels. In this study, we observed that low doses of epostane also lowered circulating progesterone levels without blocking ovulation (see Table V). However, we do not believe that these observations preclude the involvement of progesterone in the ovulatory process. One possibility is that progesterone levels within the follicle may differ significantly from levels measured peripherally. Second, progesterone levels in this study, as well as the Bullock and Kappauf (7) study, were measured at only a single time point after drug administration and it is possible that, at a later critical time point, progesterone levels had increased sufficiently to facilitate ovulation.

A preovulatory increase in progesterone has been observed in primates but the significance of this rise to ovulation is not clear. Plasma progesterone levels were shown to rise abruptly on the day prior to ovulation in rhesus monkeys (10, 11) and about 12 hr before the midcycle LH surge in humans (12, 13). The preovulatory progesterone rise in women has been shown to increase the duration of the midcycle LH surge (14), an effect that could facilitate

ovulation. It is not known whether the preovulatory rise of progesterone in primates facilitates ovulation by a direct effect on the ovary. These observations suggest that studies are warranted to determine whether epostane will inhibit ovulation in primates.

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