Enclomiphene induces luteolysis in the nonpregnant guinea pig

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Summary. The effect of two antiestrogens, enclomiphene and tamoxifen, on luteal function in the guinea pig was compared to that of estradiol, a known luteolysin. Enclomiphene caused premature luteolysis when administered during the early or mid-luteal phase of the cycle, but was not as potent as estradiol. Tamoxifen had no effect. The luteolytic effect of enclomiphene was mediated by the uterus, as has been shown for estradiol.
Key words. Enclomiphene; luteolysis; guinea pig.

Prostaglandin $F_{2\alpha}$ (PGF) produced by the uterus is responsible for spontaneous luteolysis during nonfertile estrus cycles in the guinea pig[1]. Exogenous estrogen is also luteolytic in this species[2] and estradiol-17β (E2), in the presence of luteal phase concentrations of progesterone (P), stimulates uterine PGF secretion[3]. Utero-ovarian vein concentrations of E2 increase concurrently with PGF concentrations prior to the onset of luteolysis[4] and Poyser[1] suggested that during nonfertile estrus cycles a rise in ovarian E2 secretion stimulates PGF production by the P-primed uterus and the increasing titers of PGF inhibit luteal P production. The original purpose of the present investigation was to determine the role of endogenous E2 in spontaneous luteolysis by blocking the effect of E2 with an antiestrogen. Two antiestrogens were employed, enclomiphene [trans-1-(p-β-diethylaminoethoxyphenyl)-1,2-diphenyl-2-chloroethylene] and tamoxifen [trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenyl but-1-ene].

Materials and methods. Animals. Female guinea pigs (*Cavia porcellus*) of the Hartley strain were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed 2–3 per cage under controlled lighting (14 h light:10 h dark) conditions. Guinea pig chow and water supplemented with ascorbic acid (1.14 mmol/l) were provided ad libitum. Animals were checked daily for the presence of the vaginal closure membrane and vaginal lavage was performed when the membrane was absent. The day of maximum cornification prior to the postovulatory leukocytic influx was designated day 1 of the cycle. Only animals exhibiting at least two consecutive estrus cycles of 15–18 days duration were used.

Experiment 1. To determine if an antiestrogen would block the effect of endogenous E2 and prolong luteal function, guinea pigs were treated with enclomiphene citrate (ENC; Merrell Dow Pharmaceuticals, Cincinnati, OH) or tamoxifen citrate (TAM; Sigma, St. Louis, MO). ENC (500 nmol/100 g b.wt/day; n = 7), TAM (same dose; n = 7) or saline:ethanol (95:5) vehicle (n = 6) was injected s.c. once a day between days 7 and 14 of the cycle. This dose of antiestrogen prevents E2-induced luteolysis in primates[5]. Drug administration began prior to the increase in utero-ovarian vein E2 which precedes spontaneous luteolysis[6]. Blood samples were obtained on days 7, 11, 13 and 15 by cardiac puncture of anesthetized (methoxyflurane, Pittman-Moore) animals. All procedures were performed between 08.00 and 10.00 h. The blood was refrigerated overnight and the serum was stored (−20 °C) until assayed for P.

Experiment 2. The results from experiment 1 indicated that ENC-treated animals displayed premature luteolysis rather than prolonged luteal function, so a study to compare the luteolytic effects of E2 and ENC was performed. Animals were injected with one of the following: vehicle (n = 6), E2 benzoate (3 nmol/100 g b.wt/day; n = 5) or ENC (5, 25, or 300 nmol/100 g b.wt/day; n = 6–7 at each dose level). Injections were administered on days 4, 5 and 6 of the cycle. The dose of E2 and time of administration replicated those reported by others[2]. Blood samples were collected on day 4 just prior to the first injection and every other day thereafter for a total of six samples per animal. Serum was stored until assayed for E2 and P.

Experiment 3. The luteolytic effect of E2 requires the uterus[2], so this experiment was designed to determine if the luteolytic effect of ENC was also mediated by the uterus. Seven guinea pigs were hysterectomized on day 6 of the cycle using methoxyflurane anesthesia and aseptic surgical techniques. Four of the animals received ENC (500 nmol/100 g b.wt/day) from day 7 through 14 and the remaining animals were injected with vehicle. Blood samples were obtained on days 9, 11, 13 and 15 and serum was assayed for P.

Radioimmunoassay. Details of the chromatography and assay procedures have been reported[7]. Recovery of steroids from the chromatography columns was 74.7 ± 1.5% (mean ± SEM) for P and 75.9 ± 0.9% for E2. The limits of detection were 9 fmol E2/tube and 64 fmol P/tube and blanks averaged 6.2 ± 0.7 fmol (E2) and 57 ± 8 fmol (P). The within and between assay coefficients of variation for E2 were 9.0% and 10.4% and they were 9.2% and 16.4% for P.

Statistics. Hormone data within each experiment were compared by two factor (treatment and day of cycle) analysis of variance with repeated measures and Dunnett's test. Estrus cycle duration was compared by one factor analysis of variance and Dunnett's test. Differences were considered significant when $p < 0.05$.

Results. Administration of antiestrogens beginning on day 7 of the cycle resulted in premature rupture of the vaginal membrane. Thus, estrus cycles were significantly shorter in ENC-treated (10.9 ± 0.6 days) and TAM-treated (13.6 ± 0.9 days) guinea pigs than in those animals receiving only vehicle (16.7 ± 0.4 days). ENC induced premature luteolysis in 6 of 7 guinea pigs as indicated by significantly lower serum P on

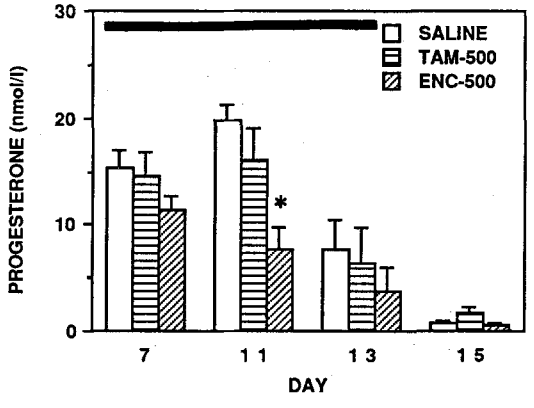

Figure 1. Serum progesterone in adult female guinea pigs treated with enclomiphene (ENC; 500 nmol/100 g b.wt/day; n = 7), tamoxifen (TAM; 500 nmol/100 g b.wt/day; n = 7), or saline (n = 6). The horizontal bar indicates the duration of treatment. Each vertical bar represents the mean and SEM and the asterisk indicates a significant ($p < 0.05$) difference from the saline-treated group.

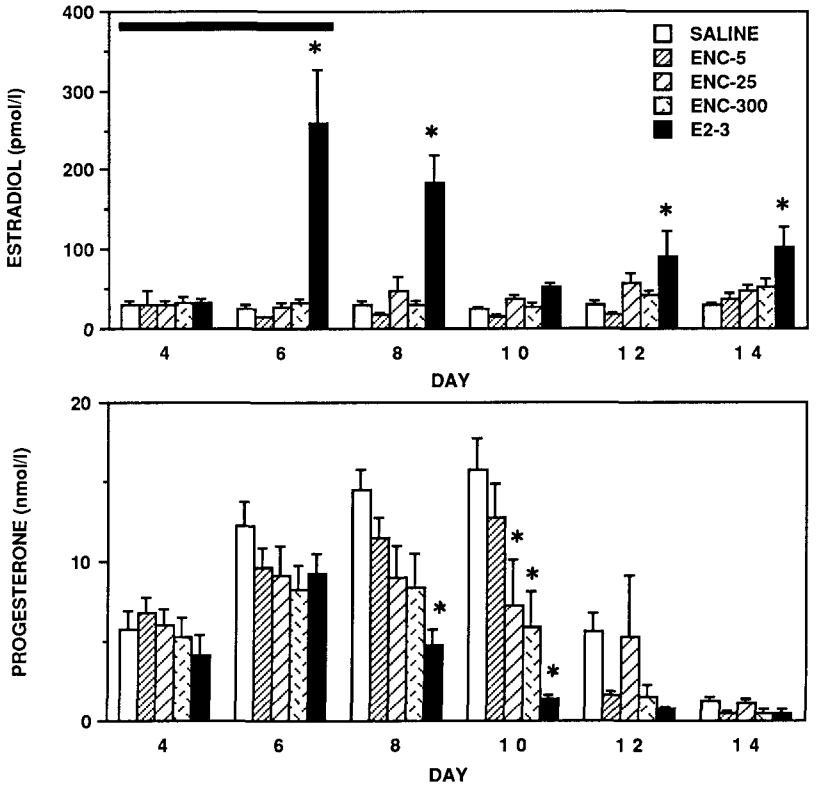

Figure 2. Serum estradiol (top) and progesterone (bottom) in female guinea pigs treated with estradiol benzoate (E2–3; 3 nmol/100 g b.wt/day; n = 5), one of three doses of enclomiphene (ENC; 5, 25, 300 nmol/100 g b.wt/day; n = 6–7), or saline (n = 6). See the legend to figure 1 for further explanation.

day 11 (fig. 1). However, serum P in TAM-treated animals was not significantly different from vehicle-treated animals on any day.

The results of the dose-response study comparing the luteolytic effect of ENC with that of E2 are presented in figure 2. Serum concentrations of E2 were significantly elevated following injection of E2 benzoate on days 4–6 and remained elevated until day 8. Serum E2 rose again on days 12 and 14. The concentration of E2 in ENC-treated animals did not differ significantly from controls on any day. P concentrations increased from days 4–6 in all treatment groups and continued to rise through day 10 in guinea pigs treated with vehicle or the lowest dose (5 nmol) of ENC. However, 3 nmol of E2 administered on days 4–6 resulted in significantly lower P by day 8 and shorter cycles (12.0 ± 0.4 days vs 15.7 ± 0.7 days in controls). Guinea pigs treated with 25 or 300 nmol ENC also exhibited a premature decline in serum P, but this was not observed until day 10. Estrus cycles were also significantly shorter in the 25 nmol ENC group (12.4 ± 1.1 days) and the 300 nmol ENC group (11.1 ± 1.2 days).

Serum P in hysterectomized guinea pigs treated with 500 nmol ENC or vehicle are presented in figure 3. The dose of ENC which caused a premature decline in P in experiment 1 did not affect levels of this hormone in the absence of the uterus. Serum P was maintained at luteal phase levels through day 15 with no significant differences between treatment groups or days.

Discussion. The systemic concentration of E2 achieved with the injection of E2 benzoate was within the range of uteroovarian vein E2 levels just prior to the onset of spontaneous luteolysis[6]. The blood samples collected on day 6 were obtained 24 h after the previous hormone injection and do not reflect the highest level of E2 present in the circulation. However, the observation that this dose of E2 approximates endogenous concentrations of hormone supports the hypothesis that ovarian estrogen may act as a trigger for a series of events culminating in luteolysis.

Antiestrogens are useful tools for studying the role of endogenous estrogen in physiologic events; however, there are also problems associated with the use of these drugs. One of these problems is that these compounds exhibit agonistic as well as antagonistic effects depending on the species, target tissue, prior exposure to estrogen and the biological end point[8]. In the present study, both ENC and TAM exhibited agonistic activity, with the former being the most potent. The dose of TAM employed in Experiment 1 did not induce luteolysis, but was sufficient to cause premature rupture of

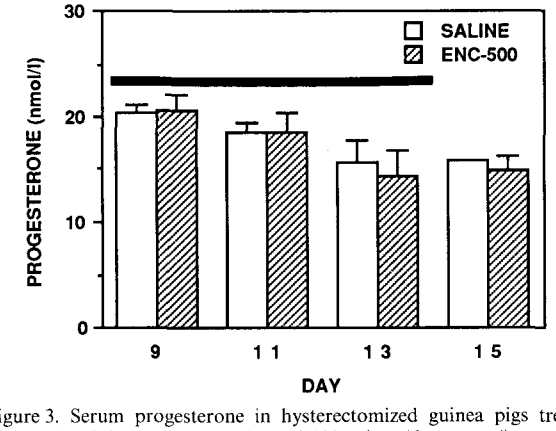

Figure 3. Serum progesterone in hysterectomized guinea pigs treated with enclomiphene (ENC; 500 nmol/100 g b.wt/day; n = 4) or saline (n = 3). See the legend to figure 1 for further explanation.

the vaginal closure membrane. Perforation of the membrane is an estrogen-dependent event[9] and thus TAM acted as an agonist in this target tissue. The dose of TAM used in this study was four times higher (on a body weight basis) than the effective dose in rats[10] but did not prolong luteal function in the guinea pig. TAM is metabolized to 4-monohydroxytamoxifen in vivo and others have shown that this form is more potent[1,10]; however, TAM is also effective when this metabolic conversion is blocked[11]. It is unknown whether TAM or its metabolite was the active agent in the present study.

ENC exerted luteolytic effects whether administered early in the luteal phase or beginning at midcycle. However, ENC was not as potent as E2, requiring approximately ten times the amount of E2 to be effective. In most of the systems studied, ENC (the trans isomer of clomiphene) is a potent estrogen antagonist[12] and clomiphene (a mixture of cis and trans isomers) blocks the luteolytic effect of E2 in primates[5,13]. Agonistic effects of ENC have also been reported[14].

The uterus appears to mediate the luteolytic effect of both ENC and E2, as demonstrated by the absence of luteolysis in hysterectomized guinea pigs treated with either factor (this study and reference 2). Because E2 increases uterine PGF production in guinea pigs, it seems likely that ENC would do the same, but uterine PGF production in response to ENC will have to be measured to verify this. TAM decreases guinea pig uterine PGF production in vitro[15], but did not affect luteal activity in this study. Enclomiphene also affects gonadotropin secretion[16] and luteal steroidogenesis[5], but these potential mechanisms apparently do not play a significant role in the guinea pig because of the absence of any effect of ENC in hysterectomized animals.

In summary, this investigation has demonstrated 1) the luteolytic dose of E2 yields blood levels similar to those measured in the utero-ovarian vein prior to the onset of spontaneous luteolysis, 2) ENC induces premature luteolysis in the guinea pig, and 3) the luteolytic effect of ENC is mediated by the uterus, probably by increasing uterine PGF production.

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1 Poyser, N. L., Adv. Prostagland. Thromb. Res. *2* (1976) 633.
2 Bland, K. P., and Donovan, B. T., J. Endocr. *47* (1970) 225.
3 Blatchley, F. R., and Poyser, N. L., J. Reprod. Fert. *40* (1974) 205.
4 Blatchley, F. R., Maule Walker, F. M., and Poyser, N. L., J. Endocr. *67* (1975) 225.
5 Westfahl, P. K., and Resko, J. A., Biol. Reprod. *29* (1983) 963.
6 Joshi, H. S., Watson, D. J., and Labhsetwar, A. P., J. Reprod. Fert. *35* (1973) 177.
7 Westfahl, P. K., Steroids *51* (1988) in press.
8 Sutherland, R. L., and Murphy, L. C., Molec. cell. Endocr. *25* (1982) 5.
9 Mills, P. G., and Reed, M., J. Endocr. *50* (1971) 329.
10 Jordan, V. C., Dix, C. J., Naylor, K. E., Prestwich, G., and Rowsby, L., J. Toxic. envir. Hlth *4* (1978) 363.
11 Allen, K. E., Clark, E. R., and Jordan, V. C., Br. J. Pharmac. *73* (1980) 83.
12 Harper, M. J. K., and Walpole, A. L., Nature *212* (1966) 87.
13 Westfahl, P. K., and Kling, O. R., Endocrinology *110* (1982) 64.
14 Hsueh, A. J. W., Erickson, G. F., and Yen, S. S. C., Nature *273* (1978) 57.
15 Sharma, S. C., and Pugh, D. M., Eur. J. Pharmac. *42* (1977) 91.
16 Huang, W. S.-R., and Miller, W. L., Endocrinology *112* (1983) 442.

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Ecdysteroid receptors located in the central nervous system of an insect

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Summary. Using thaw-mount autoradiography for steroid hormones, we obtained direct evidence for a nuclear localization of ecdysteroid binding sites in target organs of blowfly (*Calliphora vicina*) larvae. The binding sites revealed properties of ecdysteroid receptors. Endocrine cells of the ring gland were found to be target tissues of ecdysteroids. This observation provides morphological evidence for a network of complex interendocrine regulation. In the central nervous system receptor-containing neurons were identified which include many, if not all, neurosecretory cells of the brain. A map of ecdysteroid sensitive cells of the larval brain is presented.
Key words. Ecdysteroid; steroid hormone receptor; central nervous system; interendocrine regulation; autoradiography; fly.

Ecdysteroids serve as the sole steroid hormone system in arthropods. They elicit a wide variety of effects which range from control of moulting to the induction of vitellogenins[2]. These effects are mediated by hormone receptors. So far there has only been indirect evidence suggesting the localization of ecdysteroid receptors within target cells[3].

Blowfly larvae contain ecdysteroid-binding molecules that fulfill all criteria of steroid hormone receptors: they exhibit an affinity for DNA as well as tight hormone binding (K_d = 30 nM with 20-hydroxyecdysone), show analogue specificity, and a low steroid-binding capacity[4]. The radiolabelled hormone analogue ponasterone A (PoA = 25-deoxy-20-hydroxyecdysone) reveals an increased affinity for the receptor (K_d = 1 nM)[4].

Results and discussion. When tissues, dissected from late third instar larvae (L 3 d 7) of the blowfly (*Calliphora vicina*), were incubated with a low concentration of [^{3}H]PoA the ecdysteroid was taken up and reached a plateau after about 1 h. The final amount of PoA resorbed depended on the age and physiological stage of the larvae from which the tissues were prepared. Higher rates of uptake were obtained with larvae arrested in their development by an additional 7 days