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Inhibition of Gonadotropin by Δ^9-Tetrahydrocannabinol: Mediation by Steroid Receptors?

Abstract. *Competition assays for estradiol receptors in cytosol preparations of uteri from rhesus monkeys and humans showed that Δ^9-tetrahydrocannabinol (THC) does not compete with estradiol for intracellular estrogen receptors. Although isotopically labeled THC bound to macromolecules in uterine cytosol from the rhesus monkey, the binding was not displaced by unlabeled THC, diethylstilbestrol, estradiol, progesterone, cortisol, or 5α-dihydrotestosterone. Scatchard analyses indicated that high-affinity saturable binding of THC to cytosol did not occur. Thus the inhibitory effect of THC on gonadotropin and steroid secretion in primates is not mediated by the interaction of THC with intracellular steroid hormone receptors.*

Long-term use of large amounts of marihuana may adversely affect sexual and reproductive functions (*1*). The mechanism by which marihuana use produces these effects is not known. We showed previously that Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of marihuana, can cause significant decreases in the sex hormones of the adult male and female rhesus monkey (*2*). This inhibitory effect is thought to be produced by a reversible pituitary-hypothalamic action, since both gonadotropins and sex steroids are decreased.

It has also been suggested that marihuana disrupts the reproductive system by acting directly at the cellular level on the reproductive target organs. Studies by Shoemaker and Harmon (*3*) indicate that THC may compete with sex steroids for their receptor proteins in the target organ cells. The binding of THC to these receptors would either antagonize the trophic effects of steroids in these tissues or produce the same trophic effect as the sex steroid. Solomon *et al.* (*4*) demonstrated a positive trophic effect of THC on reproductive tissues in ovariectomized rats injected intraperitoneally with THC. However, their work was criticized by Okey and Bondy (*5*) who claimed that experiments in which THC is administered intraperitoneally give unreliable results and cause inflammation of abdominal organs. Further, Okey and Bondy found that unlike estradiol, THC administration did not elicit a dose-dependent increase in uterine weight and did not compete in vitro for estrogen receptor sites in rodents. In an attempt to settle the dispute, we have studied the binding of THC to estrogen receptors in primates rather than rodents, because Smith *et al.* (*6*) demonstrated that steroid receptors from different animals do not necessarily have the same ligand binding specificity.

We prepared cytosols from the uteri of rhesus monkeys that had been ovariectomized 4 days prior to hysterectomy and from the uteri of humans undergoing voluntary therapeutic hysterectomy. The tissues were minced and homogenized at 4°C in four volumes of buffer containing 10 m*M* tris-HCl (*p*H 7.4), 1.5 m*M* EDTA, 12 m*M* thioglycerol, and 10 percent glycerol. The homogenate was centrifuged at 1000*g* for 15 minutes to remove nuclei, and the resulting supernatant was centrifuged at 105,000*g* for 90 minutes. A fixed concentration of the supernatant was incubated with increasing concentrations of [^{3}H]estradiol (specific activity, 96 Ci/mmole; 0.1 to 4.0 n*M*) for 18 hours at 4°C in the presence and absence of a 250-fold excess of diethylstilbestrol (DES). We used DES to measure the nonspecific binding of [^{3}H]estradiol in cytosol because at these concentrations DES displaces estradiol from its receptor sites rather than from any contaminating serum binding proteins such as sex-steroid binding globulin. After the incubation period, the [^{3}H]estradiol not bound to macromolecules was removed by charcoal adsorption (*7*). Scatchard analyses (*8*) were performed on these data. The specifically bound estradiol was computed by subtracting nonspecific binding from total binding; the specifically bound compound was plotted against the ratio of bound to free estradiol.

Figure 1 shows a typical curve for the rhesus monkey estrogen receptor. The curve is biphasic, similar to that observed previously in Scatchard analyses of data from human uterine cytosol and chick oviduct cytosol (*9*). By using the method described by Rosenthal (*10*) to resolve the two binding components, we found that the receptor having the highest affinity for estradiol had an equilibrium dissociation constant (K_D) of 0.17 ± 0.07 n*M* ($N = 4$). This K_D is similar to that measured for the human uterine cytoplasmic estrogen receptor (*11*). The concentration of binding sites for estradiol in the monkey uterine cytosol was 22 ± 4 pmole per milligram of protein ($N = 4$). From the Scatchard analyses we selected a concentration of [^{3}H]estradiol which preferentially bound to the high-affinity estrogen receptor, and we used this concentration in the subsequent competitive binding studies to confirm that the [^{3}H]estradiol binding was specific for estradiol and DES.

To determine whether THC would compete for estrogen receptor sites, we performed competitive binding assays on cytosol using a fixed concentration of [^{3}H]estradiol (2 n*M*) with increasing concentrations of THC (0 to 3.8 μ*M*); for comparison, we performed similar assays with increasing concentrations of DES (0 to 1.2 μ*M*). The mixtures were incubated for 18 hours at 4°C and the amount of [^{3}H]estradiol bound at each concentration of competitor was determined by charcoal adsorption assay. The amount of [^{3}H]estradiol bound was then plotted against the log of the concentration of the competitor.

Figure 2 shows that whereas DES competes significantly for estrogen receptor sites at a concentration as low as 1.4 n*M* (0.2 ng), THC does not compete even at a concentration of 3.8 μ*M* (600 ng). Table 1 shows results of studies with three additional uterine specimens, together with the results for cytosols from human uteri. The slopes of the DES competition curves from the data in

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Table 1. Competitive binding assays. Cytosols were obtained from uteri of rhesus monkeys that had been ovariectomized 4 days prior to hysterectomy, and from uteri of humans undergoing voluntary therapeutic hysterectomy. The protein concentrations of the cytosols were ~ 5 mg/ml and the percentage displacement of [^{3}H]estradiol (2 nM) was recorded as the mean of the results obtained from three different tissue sources. The assays are described in the text.

Competitor	Source of receptor	Percentage displacement
DES		
0.28 μM	Rhesus monkey and human uteri	100
0.14 nM	Rhesus monkey and human uteri	96 ± 2
28.00 nM	Rhesus monkey uteri	80 ± 6
14.00 nM	Rhesus monkey uteri	48 ± 3
2.80 nM	Rhesus monkey uteri	13 ± 1
THC		
2.80 μM	Rhesus monkey and human uteri	None detected

Table 1 are different from the slope in Fig. 2. This difference is not surprising, because the cytosol protein concentrations were different and the concentration of [^{3}H]estradiol was unchanged at 2 nM. The THC still did not compete with [^{3}H]estradiol for the rhesus monkey or human estrogen receptor. To confirm that equilibration of estrogen binding had been achieved, we measured the specific binding of [^{3}H]estradiol at 3, 14, and 20 hours of incubation at 4°C. No significant differences in binding were observed.

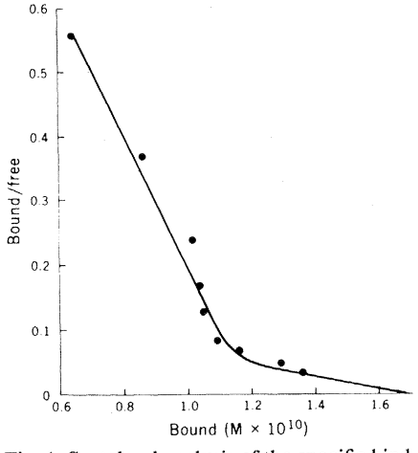

Fig. 1. Scatchard analysis of the specific binding of estradiol in the concentration range 0.1 to 4 nM to uterine cytosol from ovariectomized rhesus monkeys. The cytosol was mixed with 4 to 10 volumes of buffer consisting of 10 mM tris-HCl (pH 7.4), 1.5 mM EDTA, 12 mM thioglycerol, and 10 percent glycerol. The assay tubes were incubated for 18 hours at 4°C and the macromolecular bound [^{3}H]estradiol was separated from unbound estradiol by adsorption on Dextran coated charcoal. Specifically bound [^{3}H]estradiol was determined by subtracting the nonspecific binding, obtained by using similar concentrations of [^{3}H]estradiol incubated in the presence of excess unlabeled DES, from the total binding. The mean dissociation constant (K_D) of four determinations calculated from the reciprocal of the slope with the graphical solution of Rosenthal (*10*) was 0.17 ± 0.07 nM. The concentration of estrogen-specific binding sites was 2.2 ± 0.4 pmole per milligram of protein. Proteins were determined by the method of Lowry *et al.* (*18*).

That THC does not compete for estrogen receptors in the primate uterus implies that it will not compete with estrogen for similar receptors in the hypothalamus and pituitary. Studies in the rat indicate that the cytoplasmic estrogen receptors in estrogen target tissues such as endometrium, myometrium, vagina, anterior pituitary, anterior hypothalamus, and posterior hypothalamus have similar properties (*12*). Similarly, estrogen receptors from calf endometrium, myometrium, and pituitary have similar K_D's and ligand-binding specificities (*13*), as do the progesterone receptors from calf endometrium, myometrium, ovary, hypothalamus, and pituitary (*13*). Thus, although there appear to be differences in receptors from different species, in a particular animal model the hormonal receptors have similar properties. It is generally believed that estrogen controls gonadotropin secretion after interacting with estrogen receptors in the hypothalamus or pituitary, or both. Experiments in vitro (*14*) showed that estradiol alone has no measurable effect on RNA synthesis on chromatin from reproductive tissue, whereas estradiol that had been complexed with its intracellular receptor increased markedly the number of initiation sites for RNA synthesis. Since THC does not interact with the intracellular estrogen receptor, its inhibitory effect on gonadotropin secretion cannot be interpreted as an intracellular estrogenic effect; therefore, an alternative mechanism must be sought. Pietras and Szego (*15*) have shown that estrogen receptors are also present in the cell membranes of target cells, and our studies would not preclude a mechanism in which THC competes with membrane receptors which then could effect the secretion of gonadotrophin-releasing factor or gonadotropins themselves.

To determine whether THC binds to other macromolecules in reproductive tissue, we used cytosols from rhesus monkey uteri. The cytosols were incubated with ^{14}C-labeled THC ($3 \times 10^{-6} M$) in the presence of equal concentrations of unlabeled THC, DES, progesterone, cortisol, estradiol, and dihydrotestosterone. Although there was significant binding of [^{14}C]THC to macromolecules in the cytosol preparations, none of the binding was displaced by either unlabeled THC or by any of the steroids tested. If THC were binding nonspecifically to a progesterone, cortisol, estradiol, or dihydrotestosterone receptor, the isotopically labeled THC would be efficiently displaced from the steroid receptor by the respective unlabeled hormone. With [^{3}H]THC (5 Ci/mmole) in the concentration range 1.7 to 6.8 nM, no specific saturable binding was demonstrable, showing that even at low concentrations of THC no receptor-like binding for THC is measurable in uterine cytosol. These data suggest that the THC binding is nonspecific and that THC is not binding to any of the intracellular receptors for steroid hormones.

Rawitch *et al.* (*16*) reported that THC does compete with estradiol for the rat uterine estrogen receptor. However, it appears that these workers used non-equilibrium conditions. Furthermore, they compared the displacement of [^{3}H]estradiol by THC with displacement by unlabeled estradiol, whereas we used DES because it competes selectively for estrogen receptor sites rather than any serum-binding proteins which may be present as contaminants. Although the

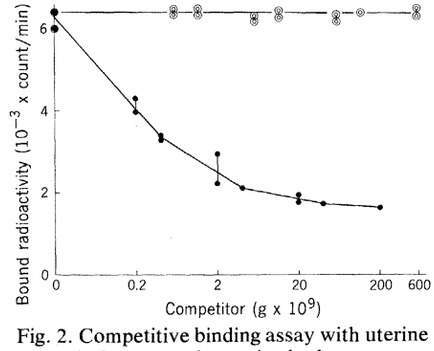

Fig. 2. Competitive binding assay with uterine cytosol from ovariectomized rhesus monkeys. The cytosol was suspended in 10 volumes of buffer consisting of 10 mM tris-HCl (pH 7.4), 1.5 mM EDTA, 12 mM thioglycerol, and 10 percent glycerol (protein concentration, 2 mg/ml). The cytosol was then added to solutions containing 2 nM [^{3}H]estradiol (96 Ci/mmole) with either increasing concentrations of DES, 0 to 1.2 μM (●) or increasing concentrations of THC, 0 to 3.8 μM (○). The solutions were incubated at 4°C for 18 hours before we measured the amount of [^{3}H]estradiol bound to macromolecules using a charcoal adsorption assay. The error bars denote the variation of data within an assay for one animal.

rat does not possess a typical sex-steroid binding globulin, it does contain a similar protein which binds estradiol and estrone with high affinity (*17*). For these reasons we maintain that THC does not compete for cytoplasmic estrogen receptors.

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Genetic Self-Incompatibility in *Oenothera* subsect *Euoenothera*

Abstract. *Although it has been postulated that genetic self-incompatibility was involved in the origin of complex heterozygotes in* Oenothera *subsect* Euoenothera, *it has not been detected in any species of this well-studied group. It is now reported for populations of* Oenothera grandiflora *from west central Alabama, and should be sought in other populations of this species, which has been in cultivation for nearly two centuries.*

Complex heterozygosity in *Oenothera* subsect *Euoenothera* is a well-known evolutionary phenomenon that has been studied extensively since its discovery a half-century ago. Within this group, which we consider to consist of about a dozen species, half are complex heterozygotes. A mechanism for the immediate establishment of such heterozygotes in this group, following hybridization of plants with appropriate chromosome configurations, has been suggested by Steiner (*1*). He based his hypothesis on the presence of self-incompatibility (Si−) alleles in the chromosome complexes of *Oenothera biennis* L. that were normally transmitted via the egg, observations that he later extended to egg complexes of the complex heterozygotes *O. parviflora* L. and *O. villosa* Thunb. [*O. strigosa* (Rydb.) Mack. & Bush] (*2*).

Both Steiner (*1*) and Cleland (*3*) concluded on the basis of their observations that Si alleles were not present in any living populations of *Euoenothera* that were not complex heterozygotes, regardless of whether these populations had the AA, BB, or CC genotype (*4*). Of particular interest in this connection is *Oenothera grandiflora* L'Hér., a species that has been in cultivation for nearly two centuries (*5*), but which is rare and local in nature. It is the only outcrossing species that has the BB genotype from which the egg complex of *O. biennis* was derived. On morphological grounds, it is highly probable that *O. grandiflora* closely resembles one of the parents of *O. biennis* (*6*). It has not been shown, however, to possess Si alleles, and all plants reported have been self-compatible, although outcrossing. This led Cleland (*3*) to postulate that *O. grandiflora* as it exists at present is a remnant of a population (his "population 2") in which genes for self-incompatibility were present. This ancestral population, rather than living *O. grandiflora*, he believed, was the one that furnished the Si alleles to *O. biennis* when it was first formed.

As was pointed out by Steiner (*6*), all populations of *O. grandiflora* that have been available for study until recently came from a restricted area approximately 30 to 65 km north northeast of Mobile, Alabama, and from cultivated plants derived from these populations. In addition to occurring in this area, however, *O. grandiflora* also occurs in Franklin and Marion counties, Tennessee, some 500 km to the north; in Lowndes County, Mississippi, about 240 km north; and in Sumter County, Alabama, 140 km north of the south Alabama localities and 10 km southeast of the one in Mississippi. It probably also occurs as a native plant in still other regions.

We were able to grow two populations of the species from Sumter County, Alabama (*7*). In a series of eight individuals from 2.8 km south of York, grown at Stanford University in 1971, one individual was self-incompatible. In a group of about 70 individuals grown at Düsseldorf in 1977, from seeds collected at Bellamy in 1974, of 21 individuals tested, 20 were self-incompatible. These two localities are about 18 km apart.

This is the first report of genetic self-incompatibility in *Oenothera* subsect *Euoenothera*, in a species that has been in cultivation for two centuries and a group that has been under active genetical study for more than 90 years. In view of it, we may now hypothesize that populations identical to some of those of the living *O. grandiflora* were in fact one of the parents in the original cross that led to the origin of *O. biennis* (*8*), widespread in eastern North America and now a worldwide weed. In an effort to amplify these discoveries, it is necessary to screen as many additional populations of *O. grandiflora* as possible, especially for Si alleles that may be present in low numbers, even in the area from which the plants originally cultivated and studied were derived.

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7. We thank S. B. Jones, Jr., for providing us with these seeds.
8. That is, Biennis I, in which the B (*grandiflora*) complex is transmitted via the egg.
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