Danazol Suppression of Luteinizing Hormone in the Rat: Evidence for Mediation by both Androgen and Estrogen Receptors

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Abstract. **Previous studies had indicated that the effects of danazol on rat peripheral tissues were mediated through both androgen and estrogen receptors. The purpose of this study was to examine the role of androgen and estrogen receptors in danazol suppression of luteinizing hormone (LH) in the rat. The estrogen receptor antagonist, LY 156758, partially antagonized the suppressed levels of LH observed 3–5 hr after administration of danazol to ovariectomized rats. In contrast, the androgen receptor antagonist, flutamide, had no effect on suppressed LH levels 3–5 hr after danazol, but did partially reverse the inhibition of LH 24 hr after danazol administration to ovariectomized rats. Danazol also increased pituitary progesterone receptor levels, an estrogen-sensitive end point. These data indicate that the suppressed levels of LH following danazol treatment resulted from the functional activation of both androgen and estrogen receptors.** [P.S.E.B.M. 1990, Vol 194]

The pharmacologic profile of danazol has been the subject of several studies and varies depending on the species and route of drug administration. In the rat, danazol has been shown to be both androgenic and antigonadotropic (1, 2). A report demonstrating that the luteinizing hormone (LH) inhibitory effects of danazol were partially antagonized or absent in both flutamide-treated male rats and in androgen receptor-deficient rats indicated that the antigonadotropic effect was androgen-receptor mediated (3).

There have been conflicting reports regarding the interaction of danazol with the estrogen receptor. For example, danazol did not bind to the estrogen receptor *in vitro* (4–6) but was reported to increase accumulation of estrogen receptors in pituitary cell nuclei of ovariectomized (OVX) rats (5). The authors of the latter study suggested that danazol interacted with pituitary or hypothalamic estrogen receptors to inhibit gonadotropin production or release. Recently, we reported that danazol induced estrogen-specific responses in the rat but these effects were partially or wholly masked by the inherent androgenic activity of the drug (7). When the androgenic activity was antagonized with an antiandrogen such as flutamide, the estrogenic effects (vaginal keratinization and increased uterine progesterone receptor levels) were fully expressed. We suggested that, while the androgenic and estrogenic effects of danazol are antagonistic in peripheral target tissues, they may be complementary with regard to the inhibition of gonadotropin secretion at the level of the hypothalamus or pituitary (7). The purpose of this study was to examine the role of androgen and estrogen receptors in danazol suppression of LH in the rat.

Materials and Methods

Adult OVX Sprague-Dawley rats weighing 220–240 g were obtained from Taconic Farms, Inc., Germantown, NY, and utilized 7 days after castration. The rats were maintained in temperature- and light-controlled rooms at approximately 24°C and 12 hr of light (lights on at 0600). Animals were provided chow and tap water *ad libitum*. Danazol was administered orally by gavage at a dose of 400 mg/kg. Flutamide was

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administered either orally (40 mg/kg) or subcutaneously (50 mg/kg). The estrogen receptor antagonist, LY 156758 (10 mg/kg), estradiol benzoate (8 μg/kg), and testosterone (25 mg/kg) were injected subcutaneously. All compounds were administered in a vehicle of ethanol:cottonseed oil (1:9 v/v) in a volume of either 10 ml/kg orally or 1 ml/kg subcutaneously.

Danazol and flutamide were synthesized at Sterling Research Group, Rensselaer, NY. LY 156758 (keoxifene) was donated by Eli Lilly Co., Indianapolis, IN. Estradiol benzoate and testosterone were purchased from Sigma Chemical Co., St. Louis, MO.

Blood samples were obtained from rats either via jugular vein puncture under light ether anesthesia or by collection of trunk blood after decapitation. Blood samples were allowed to clot at room temperature, centrifuged (1000*g* for 15 min), and the serum was collected and stored frozen at −20°C until it was assayed for LH content.

LH Radioimmunoassay. Serum LH levels were measured by double antibody radioimmunoassay. Reagents for the rat LH (rLH) radioimmunoassay were obtained from the following sources: rLH reference standard, highly purified rLH for iodination, and rLH antiserum were either provided by Dr. Salvatore Raiti at NIADDK or purchased from Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbour-UCLA Medical Center, Torrence, CA. Rabbit γ-globulin and anti-rabbit γ-globulin (goat) were purchased from Antibodies Incorporated, Davis, CA. Iodination (^{125}I) of highly purified rLH was performed by Dr. George Butterstein, Union College, Schenectady, NY. Serum LH levels are expressed as nanograms of NIADDK rLH-RP-2/ml. Intra- and interassay coefficient of variations ranged from 3.4 to 9.5% for the former and 7.4 to 10.7% for the latter.

Progesterone Receptor Assay. All procedures relating to the quantification of rat pituitary cytosol progesterone receptor (PgR) levels were accomplished at 0–4°C and represent a modification of a previously reported method (7) as described below in brief. Pituitaries were homogenized (approximately 20 mg tissue/ml) in TEMDG buffer (10 m*M* tris(hydroxymethyl) aminomethane, 2 m*M* Na_4-EDTA, 20 m*M* sodium molybdate, 2 m*M* dithiothreitol, 10% glycerol, pH 7.4) using a motor-driven ground glass homogenizer and centrifuged at 50,000*g* for 1 hr. Aliquots of the supernatants (cytosol fraction) were incubated with 10 n*M* [17,*methyl*-^{3}H]R5020 (87 Ci/m*M*; New England Nuclear Corp., Boston, MA) in either the absence (total binding) or presence of a 200-fold molar excess of unlabeled R5020 (nonspecific binding) for 3 hr. Following the incubation period, a suspension of dextran-coated charcoal (1% charcoal, 0.05% Dextran T-70) was added to the ligand-cytosol preparation and incubated for 10 min. The charcoal bound [^{3}H]R5020 was removed by centrifugation (1500*g* for 10 min) and the supernatant (protein-bound [^{3}H]R5020) counted using a Packard model 4640 scintillation counter, Packard Instrument Co., Downers Grove, IL. The data are expressed as fmol [^{3}H]R5020 specific binding (total minus nonspecific) per mg of cytosol protein. Cytosol protein concentration was determined using the Bradford protein assay (8) with bovine γ-globulin as the protein standard.

Statistics. Statistical comparisons between treatment means for LH data were made using either Student's *t* test or one-way analysis of variance. After finding a significant *F* value by analysis of variance, a Newman-Keuls test was applied with the aid of a computer program (9) to determine differences between pairs of means. Treatment means for pituitary PgR content were compared using Dunnett's test.

Results

Preliminary studies demonstrated that danazol suppressed circulating LH levels in OVX rats when administered orally at doses ranging from 25 to 400 mg/kg. The magnitude and duration of LH suppression following a single, oral administration of danazol was dose related. A dose of 400 mg/kg danazol was most consistent in producing a significant inhibition of LH within 3 hr of treatment and having an effect that lasted at least 24 hr (Fig. 1). Hence, only data from rats treated with this dose was included in this report. At both 3 hr and 5 hr after a single dose of danazol, the antiestrogen, LY 156758, partially antagonized the drug-induced

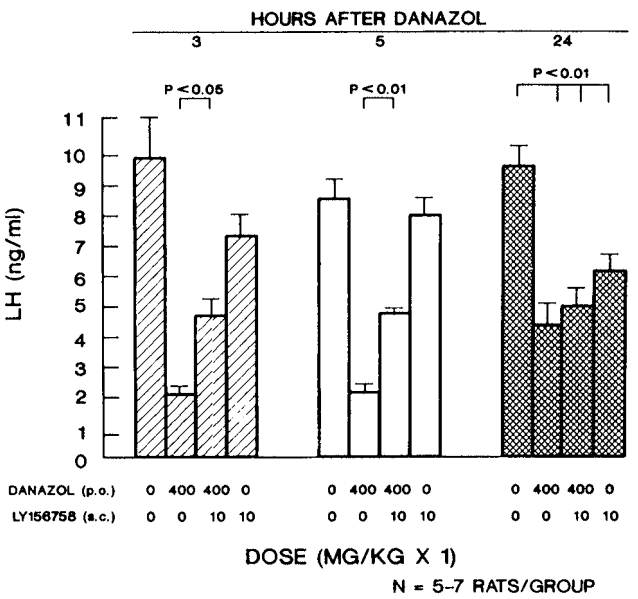

Figure 1. Effect of the antiestrogen, LY 156758, on danazol inhibition of serum LH levels in adult OVX rats. LY 156758 was injected subcutaneously 1 hr prior to a single oral administration of danazol and blood samples were collected 3, 5, and 24 hr later. LY 156758 partially reversed the inhibition of LH 3 and 5 hr after danazol but not 24 hr after danazol.

suppression of LH (Fig. 1); when given alone, LY 156758 had no effect on LH levels 3 and 5 hr later. LY 156758 did not antagonize the reduced levels of LH observed 24 hr after danazol treatment, perhaps because the antiestrogen itself significantly reduced LH levels at this time point.

A study illustrating the effect of flutamide on the suppression of LH after a single dose of danazol is shown in Figure 2. Flutamide (40 mg/kg orally) was administered twice, 18 hr and 1 hr prior to medication with danazol. Flutamide did not antagonize the suppression of LH observed at either 3 hr (Fig. 2) or 5 hr (data not shown) after treatment with danazol (400 mg/kg). However, flutamide did reverse the inhibition of LH seen 24 hr after danazol treatment (Fig. 2). A number of experiments were performed in which flutamide was administered by different routes (oral and subcutaneous), at various doses (40–100 mg/kg), and at various times before or after a single danazol treatment. However, in each case we were unsuccessful in antagonizing the early (3 or 5 hr) suppression of LH by danazol (data not shown).

The effects of daily administration of danazol and flutamide for 9 consecutive days on serum LH are shown in Figure 3. Serum LH levels progressively declined over several days in danazol-treated OVX rats and reached a minimum of less than 1 ng/ml after 3–6 days of treatment. Flutamide (50 mg/kg sc) partially antagonized the LH suppressive effects of danazol. Similar results were obtained when this experiment was performed using castrated adult male rats (data not shown).

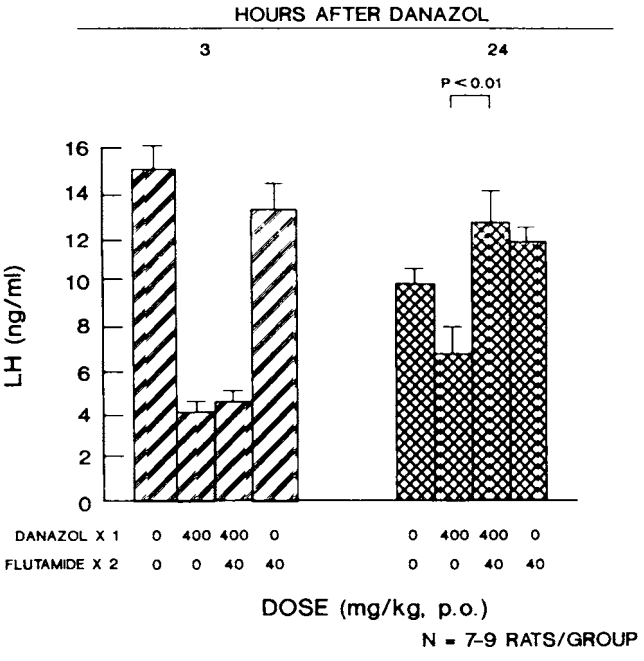

Figure 2. Effect of flutamide on danazol inhibition of serum LH in adult OVX rats. Flutamide was administered orally, 18 hr and 1 hr prior to a single oral dosage of danazol and blood samples were collected 3 and 24 hr later. Flutamide significantly reversed the inhibition of LH 24 hr after danazol but not 3 hr after danazol.

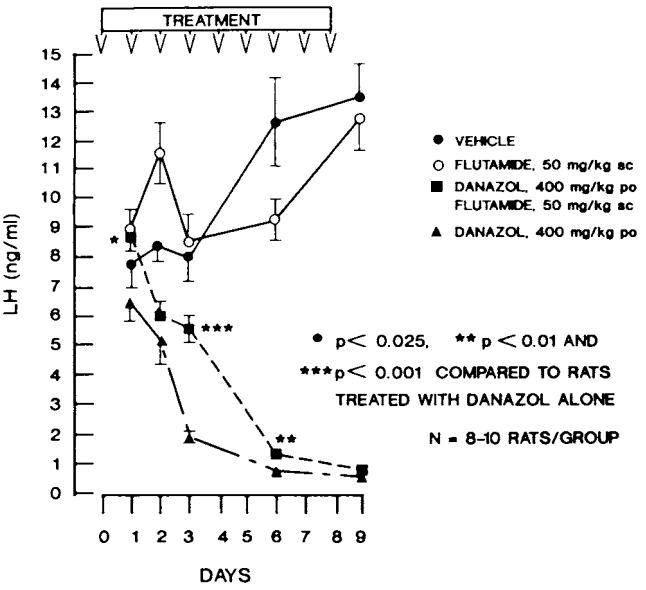

Figure 3. Effect of flutamide on danazol inhibition of serum LH in adult OVX rats. Flutamide, danazol, or vehicle was administered once each day for 9 consecutive days. Danazol-treated animals had progressively lower serum LH levels over the treatment period and concomitant treatment with flutamide partially antagonized this effect. The flutamide reversal was statistically significant 1, 3, and 6 days after treatment commenced.

Table I. Effect of Danazol on Pituitary Cytosol Progesterone Receptor Levels

Treatment	*n*	Progesterone receptor level[a] (fmol/mg cytosol protein)
Vehicle[b]	13	81 ± 3.0
Danazol (400 mg/kg orally)	10	113 ± 6.9[c]
Estradiol benzoate (8 μg/kg sc)	7	159 ± 5.8[c]
Testosterone (25 mg/kg sc)	3	90 ± 6.1

[a] Value represents the mean ± SE for *n* determinations.
[b] Ethanol:cottonseed oil (1:9 v/v).
[c] $P < 0.01$ compared with vehicle control (Dunnett's test).

Pituitary cytosol PgR levels were significantly elevated 24 hr after a single dose of either estradiol benzoate (8 μg/kg sc) or danazol (400 mg/kg orally) (Table I). Testosterone administration (25 mg/kg sc) had no significant effect on pituitary cytosol PgR levels.

Discussion

We recently reported that orally administered danazol activated both androgen and estrogen receptor-mediated effects in the rat (7). Although the intrinsic androgenic activity partially or fully inhibited the expression of the estrogenic effects of danazol in pe-

ripheral target tissues, we suggested that these two components of the drug's activity profile would probably act together to lower circulating LH levels.

The concept of a dual LH inhibitory mechanism is supported by results of the present study showing that LY 156758 partially reversed the early (3- to 5-hr) LH inhibitory effects of a single danazol treatment. However, since the antiestrogen itself reduced LH levels 24 hr after injection, it was not possible to determine whether the inhibition of LH by danazol at this time point was mediated by the estrogen receptor. Thus, these experiments suggest that the early effects of danazol on LH are, at least partially, mediated by the estrogen receptor. The fact that danazol increased pituitary cytosol PgR levels (an estrogen specific effect) in the present study provides further evidence that estrogen receptors are functionally activated following oral administration of danazol.

We found no evidence that the early (3- to 5-hr) inhibitory effects of danazol on LH were androgen receptor mediated. The early suppressive effects of danazol on LH were not reversed, even when the animals (and hence their androgen receptors) were given flutamide twice daily for 4 days prior to danazol treatment (data not shown). At a later time period, however, the androgen receptor appeared to play a partial role in the suppression of LH by danazol. In most experiments, flutamide weakly antagonized the reduced levels of LH observed 24 hr after a single danazol treatment. The effect of flutamide was small and reached statistical significance in most, but not all, experiments. The weak antagonism by flutamide was also evident in OVX rats medicated daily for 9 days. In these experiments, danazol suppressed LH to successively lower levels until an apparent minimum was reached on Day 9. Although flutamide partially reversed the effects of danazol on some of the early days, eventually the antiandrogen had no effect on the complete inhibition of LH by danazol. This result has been observed repeatedly in experiments using both adult OVX rats and castrated adult male rats treated with danazol and flutamide for 9 consecutive days. A partial reversal of danazol-induced suppression of LH by flutamide has also been reported in castrated Stanley-Gumbreck male rats (3). Taken together, the results of these studies suggest that administration of danazol resulted in activation of both androgen and estrogen receptor-mediated events which led to a suppression of LH. It should be noted that these interpretations are based on the assumption that LY 156758 and flutamide are acting solely as estrogen or androgen antagonists and are not affecting the pharmacology or metabolism of danazol in some unknown manner.

Previous studies have suggested that orally administered danazol may activate estrogen receptor-mediated events in the rat. For example, danazol has been reported to increase the accumulation of estrogen receptors in pituitary cell nuclei (5), induce vaginal keratinization in the presence of an antiandrogen (7), and increase uterine cytosol PgR levels (7). Furthermore, LY 156758 was shown to attenuate the uterotropic effect of danazol (7). It is noteworthy that, *in vitro*, danazol does not bind the rat estrogen receptor (4–6). This fact suggests that orally administered danazol may be metabolized to one or more compounds that subsequently activate estrogen receptor-mediated events.

This study demonstrated that an early phase of LH inhibition following oral administration of danazol was partially reversed by the antiestrogen, LY 156758. In contrast, the suppression of LH seen 24 hr after danazol administration was attenuated by flutamide, indicating that this effect was, at least partially, mediated by activation of the androgen receptor. It is likely that, at any given time point, the suppression of LH by danazol was not mediated exclusively by either estrogen or androgen receptors, but rather, a combination of both.

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