Flutamide Blocks the Self-Priming Effect of Luteinizing Hormone-Releasing Hormone in Pubertal Male Rats (43108)

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Abstract. **Castration of pubertal or young adult male rats eliminates the self-priming effect of luteinizing hormone-releasing hormone on luteinizing hormone secretion. Testosterone, dihydrotestosterone, or estradiol will maintain this effect in castrated animals. In order to explore the mechanism by which both dihydrotestosterone and estradiol are capable of maintaining the effect, intact rats as well as castrated animals implanted with testosterone capsules were treated with the antiandrogen Flutamide. In both intact animals and castrated rats bearing testosterone-filled Silastic capsules, Flutamide blocked the self-priming effect. These data suggest that the androgen receptor is of primary importance in the maintenance of the self-priming effect.**
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The pituitaries of pubertal or young adult male rats release more luteinizing hormone (LH) in response to luteinizing hormone-releasing hormone (LHRH) if the animals are pretreated with LHRH than if they are pretreated with saline (1–3). If the animals are castrated 4 days prior to testing, this self-priming effect is no longer present (3, 4). Silastic capsules containing crystalline testosterone implanted at the time of castration will result in the maintenance of the self-priming effect (4). Similar experiments revealed that dihydrotestosterone or estradiol but not androstenedione will also maintain a self-priming effect in castrated male rats (4). These results suggested several possible ways in which testosterone could act to maintain the self-priming effect. Testosterone could be metabolized to either dihydrotestosterone or estradiol with both the activated androgen receptor and the activated estrogen receptor being capable of stimulating the subcellular processes needed to maintain the effect. An alternative possibility was suggested by the observation (5) that 5α-androstane-3β,17β-diol (BDIOL) binds to and activates the estrogen receptor in the male rat pituitary. Since dihydrotestosterone is metabolized to both BDIOL and its isomer 5α-androstane-3α,17β-diol (ADIOL), it seemed possible that only the estrogen receptor was needed to maintain the self-priming effect and that testosterone acted by being metabolized first to dihydrotestosterone and then to ADIOL and BDIOL. However, experiments with these compounds indicated that neither isomer was capable of maintaining the self-priming effect (6).

The experiments reported here were designed to examine the role of the androgen receptor in the maintenance of this effect. The ability of the antiandrogen Flutamide to alter the self-priming effect was determined in both intact rats and castrated animals treated with testosterone.

Materials and Methods

General. Pubertal male rats of a Sprague-Dawley-derived strain were obtained from the Holtzman Co., Madison, WI. They were housed four to five per cage in hanging wire mesh cages under controlled lighting conditions (14-hr light, 10-hr darkness). Food and water were freely available. Capsules were constructed of medical grade Silastic tubing (Dow-Corning Corp., Midland, MI) having an inner diameter of 1.98 mm and an outer diameter of 3.18 mm. This resulted in a surface area of approximately 100 mm^2/cm capsule length. Details of capsule construction have been published elsewhere (7).

Rats were examined for a self-priming effect of LHRH on LH secretion as described previously (1). The animals were anesthetized with ketamine (20 mg/

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100 g body wt) intramuscularly. Supplemental anesthesia was provided as needed. The rats were then injected intravenously (via the right jugular vein) with three priming doses of 10 ng of synthetic LHRH/100 g body wt or saline at half-hour intervals. Thirty minutes after the third priming injection, a blood sample (approximately 1 ml) was obtained by heart puncture followed immediately by a challenge injection of 50 ng of LHRH/100 g body wt via the left jugular. A final blood sample was obtained, also by cardiac puncture, 10 min after the challenge injection. Aliquots of serum were diluted with assay buffer and stored frozen until assayed for LH by radioimmunoassay.

Experimental. The first experiment examined the effect of the antiandrogen Flutamide on the self-priming effect of LHRH in intact animals. Flutamide was obtained from the Schering Corp., Bloomfield, NJ, through the generosity of Drs. R. O. Neri and T. L. Nagabhushan and dissolved in benzyl alcohol. This was then suspended in peanut oil such that the final vehicle consisted of 10% benzyl alcohol and 90% peanut oil. Beginning on Day 50 of life, male rats were injected daily with the vehicle, 10 or 30 mg of Flutamide/kg body wt. On Day 54, they were anesthetized with ketamine at least 2 hr after the final Flutamide injection, and a blood sample (approximately 0.5 ml) was obtained by heart puncture. They were then tested for a self-priming effect as described above. Serum from the initial blood sample was analyzed for testosterone by radioimmunoassay. Secondary sexual organ weights were also obtained.

The next experiment was designed to test the effect of Flutamide on the ability of a testosterone capsule to maintain the self-priming effect. Fifty-day-old male rats were castrated under ether anesthesia and implanted with empty Silastic capsules or a 100-mm^2 testosterone-filled capsule. Sham-operated control rats were implanted with empty capsules. Beginning the next morning, the rats bearing testosterone capsules received daily injections of vehicle, 10 or 30 mg of Flutamide/kg body wt. Sham-operated and castrated controls bearing empty capsules were injected with the vehicle. Four days after surgery, these animals were examined for a self-priming effect beginning at least 2 hr after the last Flutamide injection.

Radioimmunoassays and Statistics. LH concentrations were determined by a radioimmunoassay described previously (1) using antiovine LH serum (#15) obtained from Dr. Gordon Niswender (8) and radioiodinated rat LH. Results were expressed in terms of the NIH rat LH standard RP-2. Testosterone concentrations were determined after extraction with diethyl ether (1), using a double antibody radioimmunoassay that has been described in detail previously (9).

Some data were expressed as the increment in LH after LHRH administration. This was obtained by subtracting the concentration of LH in the sample collected immediately before the injection from the concentration found in the postinjection sample. Expressing the data in this manner allows conclusions to be drawn about the capacity of the anterior pituitary to respond to LHRH regardless of the initial hormone concentration (3). Data were analyzed and significance was determined using Student's t test for comparisons between two groups and Duncan's new multiple range test for multigroup comparisons. In general each subgroup (saline or LHRH primed) contained five to eight rats.

Results

Flutamide and Intact Rats. Flutamide treatment of intact rats resulted in a significant ($P < 0.01$) reduction in relative prostate weight in response to the 30-mg/kg body wt dose. Although lower than vehicle-injected controls, the relative prostate weight in animals treated with 10 mg of Flutamide/kg body wt did not show a statistically significant decline (Fig. 1, lower panel). In contrast, serum concentrations of testosterone, prior to the start of priming, were significantly (P

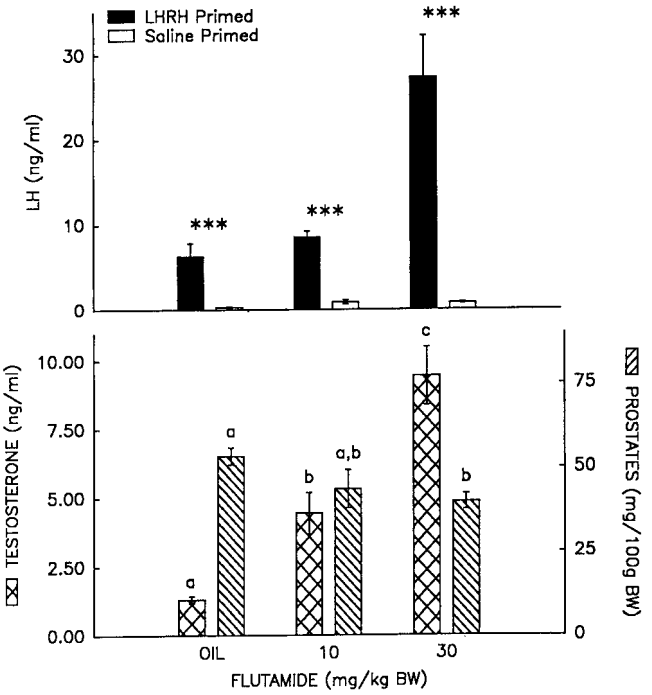

Figure 1. Lower panel, Left axis. Relative prostate weight (mean ± SE) in intact pubertal male rats treated with vehicle, 10 or 30 mg of Flutamide/kg body wt. Right axis, Mean (±SE) serum concentrations of testosterone prior to the start of priming. Groups with different letters (a, b, c) are significantly different ($P < 0.01$). Upper panel, Mean (±SE) serum LH concentrations just prior to the LHRH challenge injection. Animals were pretreated with three half-hourly injections of 10 ng of LHRH/100 g body wt (solid bars) or saline (open bars).

< 0.01) increased by both doses of Flutamide (Fig. 1, lower panel). Rats primed with LHRH had significantly ($P < 0.01$) higher serum LH concentrations just prior to the challenge injection of LHRH, when compared with animals pretreated with saline, in all treatment groups (Fig. 1, upper panel).

In response to the challenge injection of LHRH, the pituitaries of intact male rats treated with the oil vehicle released significantly ($P < 0.001$) more LH when they had been primed with LHRH than when pretreated with saline (Fig. 2). Animals injected with 10 mg of Flutamide/kg body wt also showed a significant ($P < 0.05$) self-priming effect of LHRH on LH secretion. Rats treated with 30 mg of Flutamide/kg body wt did not show a self-priming effect (Fig. 2).

Flutamide and Castrated, Testosterone-Treated Rats. Castration alone resulted in significantly ($P < 0.01$) lower relative prostate weights compared with either sham-operated controls or castrated rats implanted with testosterone-filled Silastic capsules (Fig. 3, lower panel). Flutamide injection resulted in relative prostate weights that were lower than those of either intact ($P < 0.01$) or testosterone-implanted castrated rats ($P < 0.01$), but higher than those of rats that were castrated and implanted with empty capsules ($P < 0.05$). In four of five treatment groups, rats primed with LHRH had significantly ($P < 0.05$–$P < 0.001$) higher serum LH concentrations just prior to the LHRH challenge injection than did saline-primed rats (Fig. 3, upper panel). Serum LH concentrations in the LHRH pretreated rats injected with 10 mg of Flutamide/kg body wt were higher than their saline-primed controls. However, this just missed being a statistically significant increase ($P = 0.052$).

Sham-operated control rats showed a self-priming effect of LHRH on LH secretion. Intact animals pretreated with three doses of 10 ng of LHRH/100 g body wt released significantly ($P < 0.05$) more LH in response to the LHRH challenge than did saline-primed animals (Fig. 4). Castration prevented the appearance of this response. Implantation of a 100-mm^2 testosterone-filled Silastic capsule into castrated rats maintained the response ($P < 0.001$). Both doses of Flutamide eliminated the self-priming effect (Fig. 4).

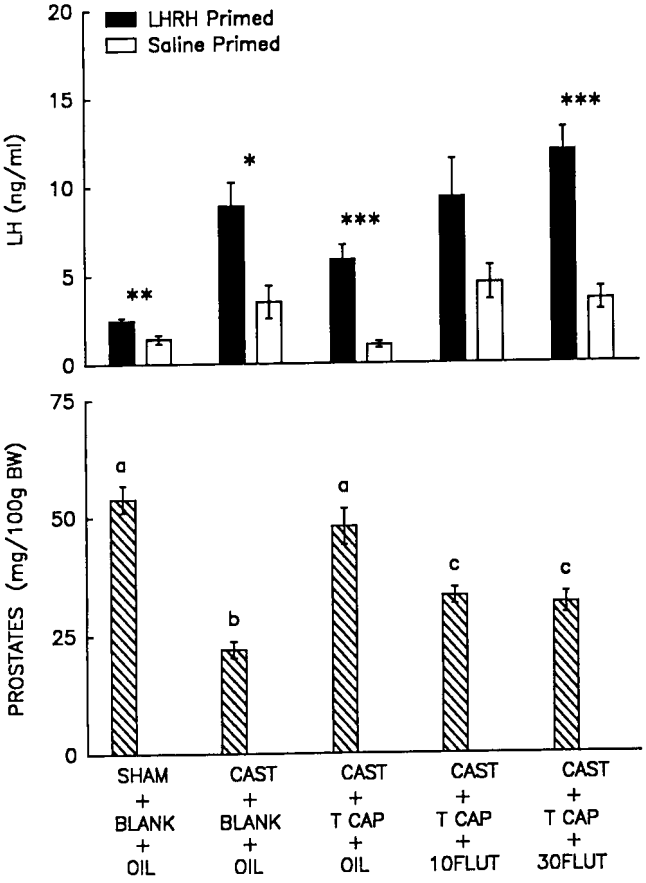

Figure 3. Lower panel, Mean (±SE) relative prostate weight in castrated (CAST) or sham-operated (SHAM) pubertal male rats bearing empty (BLANK) or 100-mm^2 testosterone capsules (T CAP) and treated with vehicle (OIL) or 10 (10FLUT) or 30 (30FLUT) mg of Flutamide/kg body wt. Groups with different letters (a, b, c) are significantly different (a versus b, c: $P < 0.01$; b versus c: $P < 0.05$). Upper panel, Mean (±SE) serum LH concentrations just prior to the LHRH challenge injection. Animals were pretreated with three half-hourly injections of 10 ng of LHRH/100 g body wt (solid bars) or saline (open bars). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

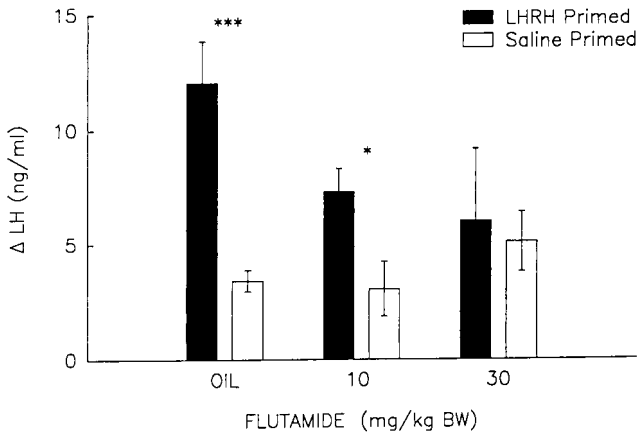

Figure 2. The increment (mean ± SE) in serum LH concentrations 10 min after the 50 ng of LHRH/100 g body wt challenge injection in intact male rats treated with vehicle, 10 or 30 mg of Flutamide/kg body wt. Animals were pretreated with three half-hourly injections of 10 ng of LHRH/100 g body wt (solid bars) or saline (open bars). *$P < 0.05$; ***$P < 0.001$.

Discussion

The self-priming effect of LHRH on LH secretion was originally described in female rats (10–12) where it is believed to play a role in the preovulatory LH surge. Studies in male rats (1–3, 13) have indicated that this effect is absent prior to puberty onset and is, in fact, induced by the peripubertal testosterone rise (1, 2, 13). These observations suggested that the development of the effect was an important component of the pubertal process. Nansel *et al.* (14) have suggested that the

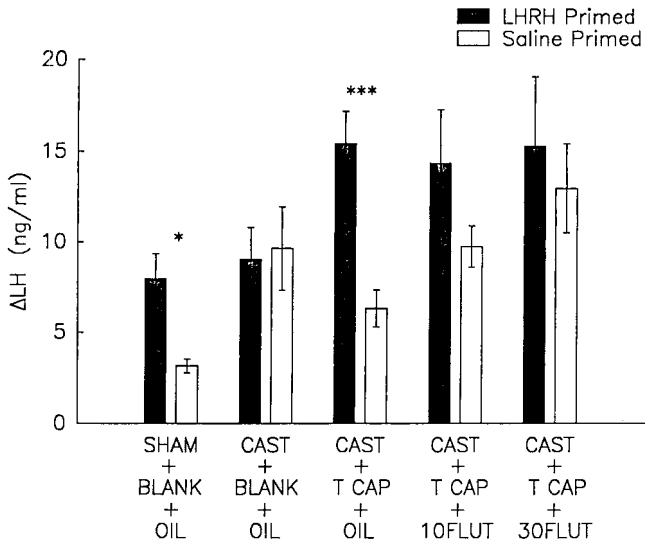

Figure 4. The increment (mean ± SE) in serum LH concentrations 10 min after the 50 ng of LHRH/100 g body wt challenge injection in castrated (CAST) or sham-operated (SHAM) pubertal male rats bearing empty (BLANK) or 100-mm^2 testosterone capsules (T CAP) and treated with vehicle (OIL) or 10 (10FLUT) or 30 (30FLUT) mg of Flutamide/kg body wt. Animals were pretreated with three half-hourly injections of 10 ng of LHRH/100 g body wt (solid bars) or saline (open bars). *$P < 0.05$; ***$P < 0.001$.

inhibitory actions of androgens on the pituitary is accomplished by an alteration in the self-priming effect. The increasing evidence (reviewed in 15) that in the male rat a large component of the negative feedback control of LH secretion by androgens is at the level of the pituitary suggests that the self-priming effect also plays an important role in the control of LH secretion in adult males. Previous studies (3, 4) established that in the pubertal and young adult male rat, the self-priming effect of LHRH on LH secretion could be abolished by castration. It was also demonstrated (4) that testosterone treatment could maintain the effect. A logical question arose from these findings: What was the mechanism of action of testosterone in maintaining this effect? Since reduction to dihydrotestosterone (16–18) or aromatization to estradiol (16, 19) are the commonly suggested mechanisms for testosterone action, the ability of these steroids to maintain the self-priming effect in castrated male rats was determined (4). Rather surprisingly in view of the generally held notion that dihydrotestosterone cannot be aromatized (20), both of these compounds were capable of maintaining a self-priming effect (4). As a result of these observations, it was hypothetized (4) that dihydrotestosterone and estradiol maintain the self-priming effect by different mechanisms. It was also suggested that, alternatively, the self-priming effect was maintained strictly by an estrogen-mediated effect. Support for this latter possibility came from the observation that dihydrotestosterone was metabolized to both ADIOL and BDIOL (21, 22) and the BDIOL binds to the estrogen receptor and apparently activates it in the male rat pituitary (5). Dihydrotestosterone, then, could maintain the self-priming effect by the action of its metabolites on the estrogen receptor, while estradiol acted directly. Based on more recent studies (6), however, it is unlikely that this is true. Neither ADIOL or BDIOL were capable of maintaining the self-priming effect in castrated rats.

In both intact animals (Fig. 2) and castrated males treated with testosterone (Fig. 4), Flutamide was effective in blocking the self-priming effect. Several recent studies have suggested that Flutamide may have antiovulatory and/or antiprogestational activity (10, 23–25). However, it is generally considered to be an antiandrogen devoid of androgenic or antiestrogenic activity (26, 27) that acts by interfering with receptor binding (28). These observations, thus, strongly support the hypothesis that activation of the androgen receptor is required for the maintenance of the self-priming effect in the male rat. The inability of the lower dose of Flutamide to block the self-priming effect in intact rats is probably due to the presence of the testes. The increase in serum testosterone noted in this group (Fig. 1, lower panel) is probably sufficient to overcome the antiandrogenic activity of the Flutamide. The higher dose of Flutamide was sufficient to block the androgen receptor even in the face of the higher testosterone levels that it induced. Such an effect was obviously not possible in the castrated rats. In these groups the serum testosterone concentrations were "fixed" by the presence of a Silastic capsule. Thus, both doses of Flutamide were capable of blocking the self-priming effect in this experiment.

It has also been suggested (3, 4) that the ability of androgens and estrogens to maintain the self-priming effect is related to endogenous LHRH secretion. As discussed in detail previously (4), this hypothesis requires that castration increase and steroid treatment decrease endogenous LHRH release. However, as recently reviewed by Kalra and Kalra (15), evidence based on direct measurements of LHRH release *in vivo* or *in vitro* suggest that LHRH secretion is decreased after castration and increased by androgens and estrogens. Therefore, this explanation seems unlikely.

There appears to be a discrepancy in the response of the prostate and the LH secretory apparatus to Flutamide in these experiments. In intact rats, prostate weight was reduced only slightly (but significantly) by the higher dose of Flutamide. This is most likely explained (as described above) by the compensatory testosterone increase. In the castrated, testosterone-implanted rats such a scenario is not possible. One possible explanation of these data is related to the duration of Flutamide treatment. In the castrated controls, testosterone secretion was abruptly and permanently terminated. In animals treated with testosterone capsules and Flutamide, it probably takes several days to achieve a corresponding reduction in androgen receptor occu-

pancy. Thus, over a 4-day period, the reduction in prostate weight in these groups will lag behind that in the castrated controls. The self-priming effect is eliminated by castration within 24 hr (29). Thus, even a 3-day delay in the establishment of effective androgen receptor blockade would still result in the elimination of the effect in these experiments. Alternatively, there may be a difference in the effectiveness of Flutamide in blocking prostatic receptors compared with pituitary receptors. Such a difference is not, however, evident when Flutamide treatment extends to longer periods than that used in these experiments (26, 27).

It is difficult to reconcile the data reported here which strongly support the idea that maintenance of the self-priming effect requires the androgen receptor, with previous studies (4) that just as clearly indicated that estradiol is capable of maintaining the self-priming effect in castrated male rats. One possible explanation, however, is that the estrogen receptor is capable of maintaining the self-priming effect when large amounts of estrogen are present. Since in intact male rats the endogenous production of estradiol and the conversion of androgens to estrogens are usually relatively low, this mechanism would not normally be operative. It would maintain the self-priming effect only when the estrogen concentrations are abnormally high, such as would have been the case in the previous studies (4). Alternatively, it is possible that androgens and estrogens are both affecting the self-priming effect by a similar mechanism, separately induced by both receptors. This mechanism might be related to basal LH secretion. In general, in those groups where the self-priming effect is not maintained, the starting LH concentrations (as judged from the prechallenge blood sample from saline-primed animals) is elevated. Clearly, however, under normal conditions, the androgens, and most probably primarily dihydrotestosterone, play the dominant role in the maintenance of the self-priming effect of LHRH on LH secretion in the male rat.

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