

# Differential Regulation of Gonadotropin Secretion by Testosterone in the Human Male: Absence of a Negative Feedback Effect of Testosterone on Follicle-Stimulating Hormone Secretion\*

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## ABSTRACT

Studies of sex steroid regulation of gonadotropin secretion in the human male have focused primarily on the respective site(s) of negative feedback of testosterone (T) and estradiol ( $E_2$ ). The use of pharmacological doses of sex steroids in these studies has precluded conclusions about the relative roles of T and  $E_2$  in gonadotropin feedback. Thus, the aims of the present study were to 1) determine the relative contributions of T vs.  $E_2$  to the sex steroid component of gonadotropin regulation, and 2) distinguish the feedback effects of T that are direct (i.e. mediated by the androgen receptor) vs. indirect (mediated by aromatization to  $E_2$ ).

Two experimental interventions were used: 1) inhibition of aromatization by a selective aromatase inhibitor to examine the impact of selective  $E_2$  withdrawal; and 2) acute medical castration to examine the effect of ablating both T and  $E_2$ . Sixteen normal (NL) men (mean age,  $30.5 \pm 2.2$  yr) were studied. Nine NL subjects were treated with the aromatase inhibitor, anastrozole (10 mg, orally, daily, for 5 days). Twelve NL men underwent medical castration with ketoconazole (1-g loading dose followed by 400 mg, orally, four times a day for 5 days). Ketoconazole-treated subjects received concomitant treatment with dexamethasone (0.5 mg twice daily) to prevent the development of adrenal insufficiency. Single blood samples were drawn daily between 0800–1000 h. To ensure that dexamethasone was not altering the gonadotropin response to sex steroid ablation by a direct pituitary effect, five GnRH-deficient men (mean age,  $37.6 \pm 3.9$  yr) underwent GnRH dose-response studies at baseline and after treatment with dexamethasone (0.5 mg twice daily).

Aromatase blockade caused significant lowering of  $E_2$  ( $33 \pm 3$  to

$14 \pm 1$  pg/mL;  $P < 0.0005$ ) with a corresponding increase in T levels ( $563 \pm 42$  to  $817 \pm 81$  ng/dL;  $P < 0.05$ ). Treatment with ketoconazole resulted in equivalent suppression of  $E_2$  ( $41 \pm 4$  to  $14 \pm 1$  pg/mL;  $P < 0.0005$ ), but also induced castrate levels of T ( $491 \pm 28$  to  $40 \pm 3$  ng/dL;  $P < 0.0005$ ). Both treatment regimens were associated with a significant increase in gonadotropin levels. For LH, the percent increase in serum levels after castration was almost 3-fold greater than that seen after selective  $E_2$  withdrawal ( $275 \pm 23\%$  with ketoconazole vs.  $95.6 \pm 21\%$  with anastrozole;  $P < 0.005$ ). Despite the divergent changes in T levels with these two maneuvers (a marked decrease after ketoconazole and a significant increase with anastrozole), the percent rise in FSH levels was similar in the two protocols ( $91 \pm 6\%$  vs.  $71 \pm 7\%$ , respectively;  $P = \text{NS}$ ). Inhibin B levels were unchanged after selective  $E_2$  withdrawal ( $156 \pm 23$  vs.  $176 \pm 19$  pg/mL), but decreased slightly with ketoconazole ( $156 \pm 15$  to  $131 \pm 11$  pg/mL;  $P < 0.05$ ). In contrast to the effects of glucocorticoid administration on gonadotropin secretion in women, no significant changes were observed in the GnRH-deficient men treated with dexamethasone in terms of mean LH levels ( $19.8 \pm 3.2$  vs.  $23.3 \pm 5.4$  IU/L), mean LH pulse amplitude after GnRH ( $16.0 \pm 2.5$  vs.  $19.0 \pm 5.1$  IU/L), or mean FSH levels ( $8.0 \pm 1.9$  vs.  $9.2 \pm 2.4$  IU/L, pre vs. post).

These studies provide evidence of differential regulation of gonadotropin secretion by T in the human male. T exerts both direct and indirect feedback on LH secretion, whereas its effects on FSH appear to be mediated largely by aromatization to  $E_2$ . From these data we conclude that in terms of sex steroid feedback,  $E_2$  is the predominant regulator of FSH secretion in the human male. (*J Clin Endocrinol Metab* 86: 53–58, 2001)

REGULATION OF gonadotropin secretion in the human male involves a complex interplay between stimulation by GnRH from the hypothalamus and inhibition by sex steroids [testosterone (T) and estradiol ( $E_2$ )] from the gonads. For FSH, there is additional regulation by nonsteroidal factors comprising a negative endocrine feedback loop mediated by inhibin B secretion from Sertoli cells (1–6) as well as

autocrine/paracrine modulation within the pituitary mediated by activin and follistatin (7). Thus, an integrated approach to the study of FSH regulation requires the use of models that permit selective manipulation of sex steroids and nonsteroidal factors.

To date, clinical investigation has focused on the sex steroid component of gonadal feedback employing a variety of paradigms, including the response to estrogen receptor blockade as well as administration of pharmacological doses of T (with or without aromatase inhibitor), dihydrotestosterone (DHT), or  $E_2$  (8–15). These studies were important in helping to delineate the site(s) of sex steroid feedback. However, the administration of pharmacological doses of sex steroids in these studies has precluded any conclusions about the relative importance of T and  $E_2$  to gonadotropin feedback. Thus, the aims of the present study were to 1) determine the relative contributions of T vs.  $E_2$  to the sex steroid

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component of gonadotropin regulation, and 2) distinguish the feedback effects of T that are direct (*i.e.* mediated by the androgen receptor) *vs.* indirect (mediated by aromatization to  $E_2$ ).

To address these issues in the human, we manipulated sex steroid secretion in normal (NL) men to produce a hormonal milieu characterized by selective  $E_2$  suppression (aromatase inhibition protocol), on the one hand, and suppression of both T and  $E_2$  (biochemical castration protocol), on the other. We used the potent, highly selective aromatase inhibitor, anastrozole (16), as we have demonstrated recently that this agent reliably suppresses  $E_2$  secretion in normal men (17). In the absence of drug distribution data, one cannot determine definitively whether anastrozole crosses the blood-brain barrier. However, the physical characteristics of this compound (molecular size, 300 kDa; plasma protein binding, <40%; essentially neutral physical properties) combined with the demonstration that chronic anastrozole administration impedes mating behavior in male rats (18) suggest that anastrozole may inhibit aromatase both centrally and peripherally. To ablate both T and  $E_2$ , we induced acute biochemical castration using high dose ketoconazole. Ketoconazole blocks multiple steps in the steroidogenic pathway, in particular  $C_{17-20}$  lyase (19–22) and has been shown to induce castrate levels of T both in NL volunteers (23) and in men with prostate cancer (24, 25).

## Materials and Methods

### Subjects

**Normal men.** Sixteen NL men (mean age,  $30.5 \pm 2.2$  yr) participated in the study. All study subjects met the following criteria: 1) normal pubertal development, sexual function, and general health; 2) normal physical examination, including a testicular volume of 20 mL or more; 3) normal serum levels of T,  $E_2$ , LH, FSH, TSH, and PRL; and 4) normal semen analysis according to WHO criteria (26).

### GnRH-deficient men

Five men (mean age,  $37.6 \pm 3.9$  yr) with isolated GnRH deficiency secondary to idiopathic hypogonadotropic hypogonadism were studied. The diagnosis of idiopathic hypogonadotropic hypogonadism was based on the following criteria: 1) failure to undergo spontaneous puberty by the age of 18 yr, 2) serum T below 100 ng/dL (3.5 nmol/L) in association with inappropriately low gonadotropin levels, 3) absence of endogenous gonadotropin pulsations during a 12- to 24-h period of blood sampling, 4) otherwise normal reserve testing of anterior pituitary function, and 5) normal magnetic resonance imaging of the hypothalamic-pituitary region. At the time of participation in the study, all had normal serum concentrations of T, LH, and FSH for at least 3 months as a result of treatment with pulsatile sc GnRH therapy delivered at 2-h intervals (27).

The study was approved by the human research committee at the Massachusetts General Hospital, and all subjects provided written informed consent.

### Study protocol

**Exp 1: selective  $E_2$  withdrawal.** Nine NL men were treated with the aromatase inhibitor, anastrozole (10 mg/day for 5 days). T,  $E_2$ , LH, FSH, and inhibin B were measured daily in samples drawn between 0800–1000 h. Results are expressed as the mean  $\pm$  SEM of the 5 days on treatment.

**Exp 2(a): acute medical castration.** Acute medical castration was induced in 12 NL men using high dose ketoconazole. Five of the 12 subjects had already participated in the anastrozole protocol. In each instance an interval of at least 3 months had elapsed between the studies. The

ketoconazole regimen consisted of a loading dose of 1 g administered at midnight followed by a maintenance dose of 400 mg four times daily for 5 days. Because this dose of ketoconazole has the potential to impair cortisol biosynthesis, glucocorticoid replacement was provided in the form of dexamethasone (0.5 mg twice daily) for the duration of the study. T,  $E_2$ , LH, FSH, and inhibin B levels were measured daily in samples drawn between 0800–1000 h as in Exp 1. In view of the potential hepatotoxicity of ketoconazole (21, 28), liver function tests were monitored daily. Participants were withdrawn from the study if hepatic transaminases increased to greater than 3 times the upper limit of normal.

**Exp 2(b): potential impact of dexamethasone on gonadotrope function.** To ensure that dexamethasone was not altering the gonadotropin response to sex steroid ablation by a direct pituitary effect, GnRH dose-response studies were conducted in five GnRH-deficient men maintained on long-term GnRH therapy before and after 7 days of treatment with dexamethasone (0.5 mg twice daily). GnRH was administered at 2-h intervals at doses of 2.5, 7.5, 25, 75, and 250 ng/kg as previously described (29). Samples were drawn 0, 1, 2, 3, 5, 10, 15, 20, 30, 40, 60, 80, 100, and 120 min after each dose. LH was measured in all samples, whereas FSH was measured in hourly pools. Mean LH and FSH levels as well as the mean amplitude of the LH response to each GnRH dose were calculated at baseline and on day 7 of dexamethasone therapy.

### Hormone assays

Serum LH and FSH concentrations were determined by microparticle enzyme immunoassay using an automated Abbott AxSYM system (Abbott Laboratories, Chicago, IL). The Second International Reference Preparation was used as the reference standard. The assay sensitivity for both LH and FSH is 1.6 mIU/mL. The intraassay coefficients of variation (CVs) for LH and FSH are less than 7% and less than 6%, respectively, with interassay CVs for both hormones of less than 7.4%. Serum T concentrations were measured using the Coat-A-Count RIA kit (Diagnosics Products, Los Angeles, CA), which has intra- and interassay CVs of less than 10%.  $E_2$  was measured by the Abbott AxSYM system (Chicago, IL), which has an analytical sensitivity of 10 pg/mL (37 pmol/L), an intraassay CV of less than 6.4%, and an interassay CV of less than 10.6%. Neither ketoconazole nor dexamethasone cross-reacted in the  $E_2$  assay; at a concentration of 10 mg/mL the percent interference by anastrozole was 0.001. In a subset of three patients from both protocols,  $E_2$  was also measured in a more sensitive RIA using hexane ethylacetate extraction and LH-20 chromatography (Endocrine Sciences, Inc., Calabasas Hills, CA). The Endocrine Sciences, Inc.,  $E_2$  assay has a sensitivity of 5 pg/mL (18 pmol/L) and, based on a male serum pool, has an intraassay CV of 4.9% and an interassay CV of 15%. Inhibin B was measured using a commercially available, double antibody, enzyme-linked immunosorbent assay (Serotec, Oxford, UK) as previously described (30). In our use, the clinical detection limit of this assay is 50 pg/mL, with a CV of 4–6% within plate and 15–18% between plates.

### Statistical methods

For both the anastrozole and ketoconazole protocols, mean daily hormone levels of the NL men over the 5 days of therapy were analyzed using ANOVA for repeated measures followed by *post-hoc* Newman-Keuls testing for individual differences. To compare the gonadotropin responses of the NL men to the two treatment regimens, the data were expressed as the percent change from baseline and the mean levels were compared using an unpaired *t* test. In the GnRH-deficient subjects, mean LH, LH pulse amplitude, and FSH levels before and after dexamethasone were compared using a two-tailed paired *t* test.  $P < 0.05$  was taken to be statistically significant.

## Results

### Exp 1: selective $E_2$ withdrawal

Aromatase blockade resulted in a fall in  $E_2$  ( $33 \pm 3$  to  $14 \pm 1$  pg/mL;  $P < 0.0005$ ) with a corresponding increase in T levels ( $563 \pm 42$  to  $817 \pm 81$  ng/dL;  $P < 0.05$ ; Fig. 1). Suppression of  $E_2$  was accompanied by an increase in both LH ( $11.6 \pm 1.4$  to  $21.2 \pm 1.8$  IU/L;  $P < 0.0005$ ) and FSH ( $7.9 \pm$

FIG. 1. Changes in T and  $E_2$  in normal men in response to anastrozole (left panels) and ketoconazole (right panels). Individual and mean  $\pm$  SEM data are depicted for subjects before and after selective  $E_2$  suppression by aromatase blockade [ $E_2(-)$ , T(+)] and after biochemical castration [ $E_2(-)$ , T(-)]. Asterisks represent a significant change from baseline: \*,  $P < 0.05$ ; \*\*,  $P < 0.0005$ . To convert the values for T to nanomoles per L, multiply by 0.03467. To convert the values for  $E_2$  to picomoles per L, multiply by 3.671.

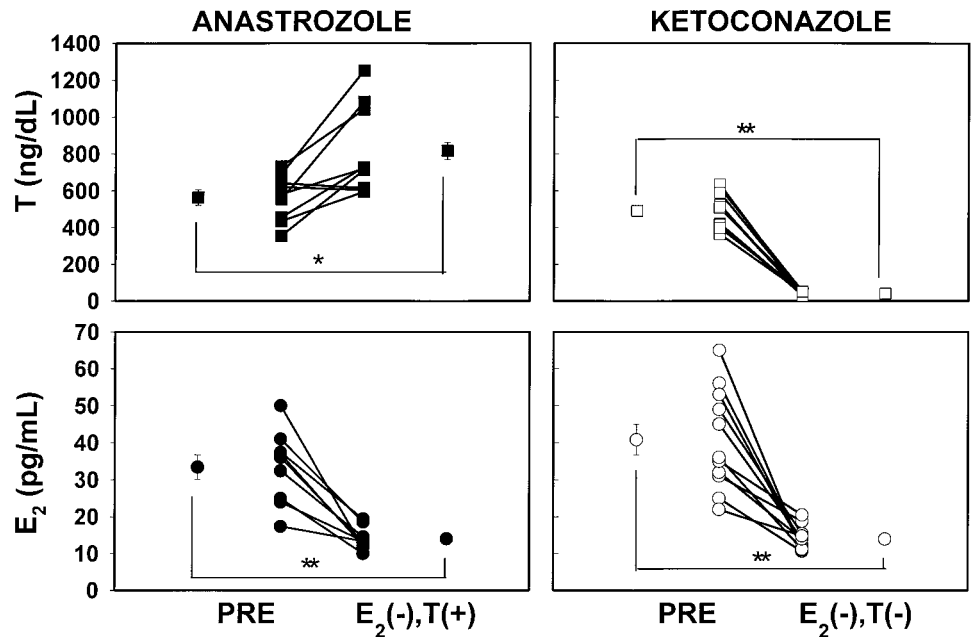
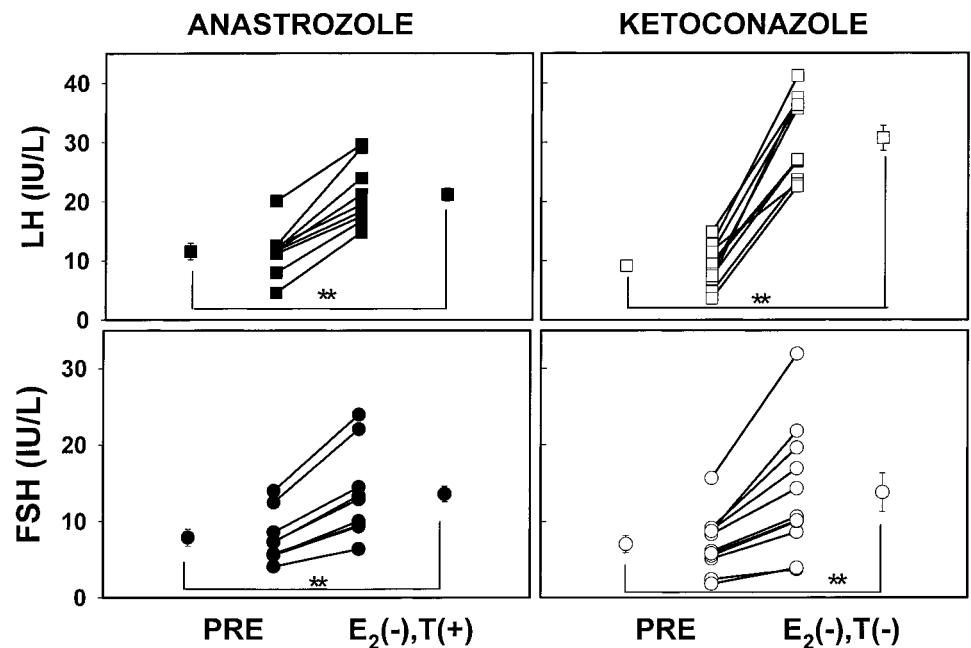


FIG. 2. Changes in gonadotropin concentrations in normal men in response to anastrozole (left panels) and ketoconazole (right panels). Individual and mean  $\pm$  SEM data are depicted for subjects before and after selective  $E_2$  suppression by aromatase blockade [ $E_2(-)$ , T(+)] and after biochemical castration [ $E_2(-)$ , T(-)]. Asterisks represent a significant change from baseline (BL): \*\*,  $P < 0.0005$ .



1.1 to  $13.5 \pm 2.0$  IU/L;  $P < 0.0005$ ; Fig. 2). Mean LH levels reached a steady state by day 2 of anastrozole administration, whereas FSH levels reached a plateau on day 4. No significant change was observed in inhibin B levels ( $156 \pm 23$  vs.  $176 \pm 19$  pg/mL).

#### Exp 2(a): acute medical castration

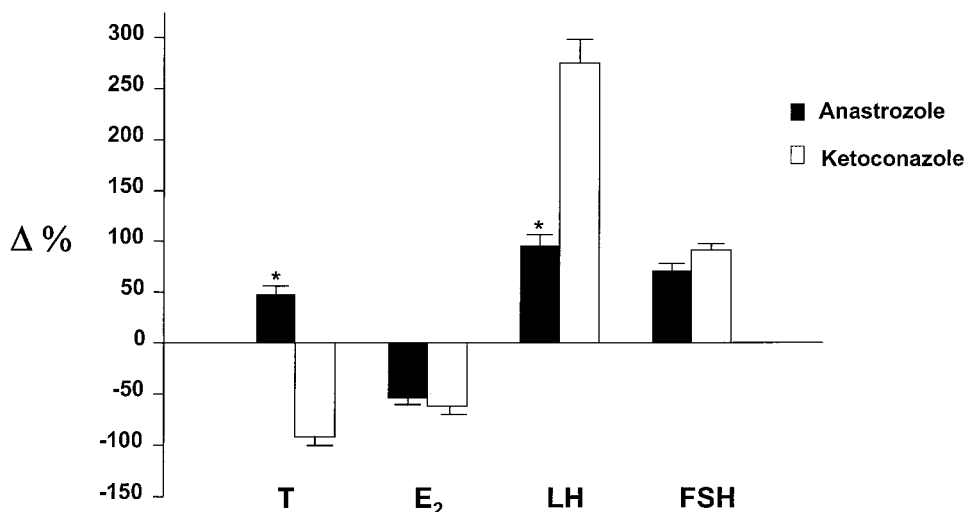
The data reported for the castration protocol is based on the 11 subjects who completed 5 days of ketoconazole therapy. One individual was withdrawn from the study on day 2 of ketoconazole administration because of a greater than 3-fold increase in hepatic transaminases. Administration of ketoconazole induced castrate levels of both T ( $491 \pm 28$  to

$40 \pm 3$  ng/dL;  $P < 0.0005$ ) and  $E_2$  ( $41 \pm 4$  to  $14 \pm 1$  pg/mL;  $P < 0.0005$ ; Fig. 1). Suppression of sex steroids was associated with a greater increase in LH ( $9.2 \pm 1.0$  to  $30.7 \pm 2.1$  IU/L;  $P < 0.0005$ ) than FSH ( $7.0 \pm 1.1$  to  $13.8 \pm 2.5$  IU/L;  $P < 0.0005$ ; Fig. 2). Levels of LH and FSH were stable by day 2 of ketoconazole administration. Inhibin B levels showed a slight, but statistically significant, decrease ( $156 \pm 15$  to  $131 \pm 11$  pg/mL;  $P < 0.05$ ).

#### Comparison of the response to ablation of $E_2$ alone vs. $E_2$ plus T

Anastrozole and ketoconazole caused equivalent  $E_2$  suppression in the NL subjects (Fig. 3). However, T levels moved

FIG. 3. Percent change from baseline in T, E<sub>2</sub>, LH, and FSH in the estrogen-deplete state [E<sub>2</sub>(-), T(+)] after administration of anastrozole (■) and in the castrate state [E<sub>2</sub>(-), T(-)] after ketoconazole (□). Asterisks represent a significant difference between the two protocols: \*,  $P < 0.005$ .



in opposing directions in the two protocols, increasing to the high normal range with anastrozole and decreasing to the castrate range with ketoconazole. Both treatment regimens were associated with significant increases in gonadotropin levels (Fig. 3). For LH, the increase in serum levels after biochemical castration was almost 3-fold greater than that seen after selective E<sub>2</sub> suppression ( $275 \pm 23$  vs.  $96 \pm 11\%$ , respectively;  $P < 0.005$ ). However, the percent rise in FSH levels was similar in the two groups despite the marked differences in T levels ( $91 \pm 6\%$  vs.  $71 \pm 7\%$ , ketoconazole vs. anastrozole;  $P = \text{NS}$ ). Given the possibility that the failure to observe a higher FSH response in the ketoconazole vs. anastrozole protocols was due to a difference in E<sub>2</sub> levels that was not detected by our E<sub>2</sub> assay, E<sub>2</sub> measurements were repeated in a more sensitive RIA in three individuals from each protocol. However, absolute E<sub>2</sub> levels using this more sensitive assay were, in fact, lower at baseline and after treatment in the ketoconazole than in the anastrozole group ( $17 \pm 2$  to  $7 \pm 1$  vs.  $32 \pm 3$  to  $15 \pm 1$  pg/mL, respectively;  $P < 0.05$ ), although the percent inhibition was similar in both protocols.

#### Exp 2(b): impact of dexamethasone on gonadotrope function

In the GnRH-deficient men treated with dexamethasone, no significant change was observed in mean LH levels ( $19.8 \pm 3.2$  vs.  $23.3 \pm 5.4$  IU/L), mean LH pulse amplitude after GnRH ( $16.0 \pm 2.5$  vs.  $19.0 \pm 5.1$  IU/L), or mean FSH levels ( $8.0 \pm 1.9$  vs.  $9.2 \pm 2.4$  IU/L, pre- vs. posttreatment).

### Discussion

Selective suppression of E<sub>2</sub> secretion with an aromatase inhibitor results in a significant increase in both gonadotropins, with the increase in LH approximating that in FSH. Suppression of both T and E<sub>2</sub> to castrate levels using ketoconazole causes a 3-fold greater increase in LH than FSH. Comparing the gonadotropin responses to selective E<sub>2</sub> inhibition vs. complete castration demonstrates that T has both direct negative feedback effects on LH presumably mediated by the androgen receptor as well as indirect effects mediated by aromatization to E<sub>2</sub>. In contrast, T's effects on FSH appear to be mediated exclusively by aromatization to E<sub>2</sub>.

In the experimental paradigms used in this study, similar

E<sub>2</sub> levels were achieved in the two protocols, whereas T levels were markedly divergent. In an ideal model of selective E<sub>2</sub> withdrawal, the concentrations of other sex steroids should be unaltered. However, when studies are conducted in an intact system such as the human, endogenous counterregulatory responses inevitably come into play. Accordingly, T levels increased after the administration of anastrozole as a consequence both of aromatase blockade as well as increased LH stimulation of the Leydig cells. Bearing in mind the marked differences in T levels, the identical increase in FSH levels in the two protocols is all the more significant.

These studies were designed to address the sex steroid component of gonadotropin feedback. However, inhibin B levels decreased by approximately 15% during ketoconazole therapy, but were unchanged after anastrozole administration. This reduction in inhibin B after castration occurred in the face of a 2-fold increase in FSH levels. These data are thus in keeping with recent observations in the adult monkey that Sertoli cell number, rather than FSH, is the major physiological regulator of inhibin B secretion (31). One could speculate that the minor, albeit statistically significant, changes in inhibin B with ketoconazole are not of physiological relevance in terms of gonadotropin secretion. However, if the differences in inhibin B levels in the two protocols were of physiological significance, they should have facilitated a greater increase in FSH in the ketoconazole-treated compared with the anastrozole-treated subjects. The basis for this fall in inhibin B levels after biochemical castration is unclear. Available data on the hormonal regulation of inhibin B in the male are limited to studies conducted in immature rat Sertoli cell cultures and suggest that addition of T inhibits, whereas E<sub>2</sub> stimulates, inhibin B production (32). The demonstration that T inhibits inhibin B secretion *in vitro* in the rat is in conflict with what we observed *in vivo* in the human, where suppression of T was associated with a fall, rather than an increase, in inhibin B levels. This differential regulation of inhibin B by sex steroids in these two experimental models may be explained by the known importance of both species (33, 34) and developmental setting (33) in inhibin B regulation.

The conclusion from these studies that E<sub>2</sub> is the major sex



steroid negative feedback regulator of FSH is supported by experiments of nature that have resulted in models of unopposed T action (congenital estrogen deficiency) and unopposed E<sub>2</sub> action (androgen insensitivity syndrome). Men with E<sub>2</sub> receptor mutations (35) and congenital aromatase deficiency (36, 37) have a 2- to 3-fold increase in FSH despite elevated T levels similar to what we observed with anastrozole. Similarly, adult male mice with targeted disruption of the aromatase CYP19 gene (ArKO mice) exhibit elevated levels of gonadotropins despite high circulating T concentrations (38). Patients with congenital androgen insensitivity syndrome (AIS) provide further evidence for the differential regulation of gonadotropin secretion by T in men, with the demonstration of normal (39–43) or minimally elevated (44–46) FSH despite markedly elevated LH levels. Using models of selective sex steroid withdrawal different from ours, Lacroix *et al.* treated normal men and AIS patients with the antiestrogen clomiphene citrate to produce models of selective estrogen insensitivity and combined androgen and estrogen insensitivity, respectively (43). Consistent with our data, the FSH response to clomiphene was identical in the two groups (43). However, interpretation of data from the AIS model is confounded by the knowledge that estrogen production rates are increased in this disorder (47, 48).

Because androgens can undergo aromatization to estrogens in a variety of tissues, including adipose tissue, brain, and testis (49), it is important to be able to distinguish T effects that are mediated directly by the androgen receptor as opposed to those indirect effects that only occur after aromatization to E<sub>2</sub>. If the hypothesis is correct that T has no direct negative feedback effects on FSH, it follows that administration of T in conjunction with an aromatase inhibitor or administration of nonaromatizable androgens should not inhibit FSH secretion. Previous studies from our group indicate that addition of the aromatase inhibitor, testolactone, abolished the 40% suppression of FSH observed when T was infused to normal men (12). However, the use of testolactone makes this study difficult to interpret given the knowledge that this agent has antiandrogenic properties due to its ability to bind to the androgen receptor (50). Administration of nonaromatizable androgens, such as DHT or fluoxymesterone, has been shown to have no impact on FSH secretion (8, 15, 51–54) except at very high doses (55–57). It is possible that given the high affinity of DHT for sex hormone-binding globulin, such high doses of DHT may displace E<sub>2</sub> and T from sex hormone-binding globulin, increasing free levels of these sex steroids and thus confounding the impact of what was presumed to be a pure DHT effect.

The ketoconazole regimen employed to induce castration included concomitant administration of dexamethasone to prevent the development of adrenal insufficiency. Data on the impact of glucocorticoids on the hypothalamic-pituitary-gonadal axis are conflicting (58–67). Interpretation of these studies is confounded by differences in the glucocorticoid regimen employed (type, dose, and duration), the model system studied (human *vs.* animal models *vs.* pituitary cell cultures), and the state of the CRF axis (suppressed *vs.* activated). In general, women appear to be more sensitive to the gonadotropin inhibitory effects of glucocorticoids than men. Accordingly, glucocorticoid doses that inhibit gonadotropin

secretion in women (63, 65) have been shown to have no negative impact in men (64, 66). Our interpretation from the available literature that the dose and duration of dexamethasone therapy used in this study were unlikely to impact on gonadotropin secretion was confirmed by the GnRH dose-response studies conducted in men with isolated GnRH deficiency before and after treatment with dexamethasone. The failure to observe any change in the gonadotropin response to five doses of GnRH provides conclusive evidence that the dexamethasone regimen used in this study did not impair the gonadotropin response to biochemical castration.

Using *in vivo* human models of selective sex steroid withdrawal, these data indicate that in terms of sex steroid negative feedback, E<sub>2</sub> is the predominant regulator of FSH secretion in the human male. Comparing the gonadotropin responses to complete castration *vs.* selective estrogen inhibition demonstrates that T exerts both direct and indirect negative feedback effects on LH secretion, whereas its effects on FSH appear to be mediated exclusively by aromatization to E<sub>2</sub>.

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