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Effects of In Vivo Administration of Testosterone Propionate on In Vitro Production of Follicle-Stimulating Hormone and Luteinizing Hormone by Pituitaries of Pony Mares

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ABSTRACT

The in vitro incorporation of [^{3}H] leucine into immunoprecipitable follicle-stimulating hormone (FSH) and luteinizing hormone (LH) was assessed for pituitaries from pony mares treated with testosterone propionate (TP) or oil (controls). Mares were treated every other day with TP (n=4) at 350 μg/kg of body weight or with an equivalent volume of oil (n=4). One day following the sixth injection of TP, each mare received an intravenous injection of gonadotropin releasing hormone (GnRH) at 1.0 μg/kg body weight and was bled frequently for 4 h. Treatment of mares with TP reduced FSH ($P<0.05$) and LH ($P<0.01$) concentrations in daily blood samples and increased ($P<0.01$) the amount of FSH secreted in response to GnRH compared with control mares. Incorportion of [^{3}H] leucine into immunoprecipitable FSH was also greater ($P<0.01$) in pituitaries from TP-treated mares compared with control mares on both a per mg tissue and per anterior pituitary basis. The amount of LH secreted after GnRH, the amount left in the pituitary and the incorporation of [^{3}H] leucine into LH were not affected by treatment. These results confirm earlier conclusions drawn from indirect evidence that androgens increase the production of FSH in the mare.

INTRODUCTION

Testosterone propionate (TP) treatment causes an apparent accumulation of follicle-stimulating hormone (FSH) in the pituitary of ovariectomized and intact pony mares (Wallace, 1981; Thompson et al., 1984) which is associated with an increase in the FSH response to exogenous gonadotropin releasing hormone (GnRH). In TP-treated geldings, the enhanced FSH response to GnRH corresponded to an eightfold increase in pituitary FSH concentrations (Thompson et al., 1979).

In intact mares, the FSH response to TP treatment was found to be due to a combination of estrogenic and androgenic properties of testosterone and/or its metabolites (Thompson et al., 1983b). That is, treatment with either TP or estradiol benzoate (EB) resulted in a suppression of daily FSH secretion, whereas treatment with dihydrotestosterone benzoate (DHTB) did not (Thompson et al., 1983b). At the same time, treatment with TP or DHTB increased the FSH response to exogenous GnRH, whereas EB treatment did not; thus, the increased FSH response was considered to be an androgenic effect (Thompson et al., 1983b). It was concluded that the accumulation of FSH in the pituitary after TP was probably a result of suppressed daily FSH secretion coupled with a probable increase in FSH production.

The present experiment was designed to determine directly if testosterone treatment causes an increase in FSH production in the pituitary of ovariectomized pony mares. The de novo synthesis of FSH and luteinizing hormone (LH) by equine pituitaries was measured in vitro after in vivo treatment of the mares with TP.

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MATERIALS AND METHODS

In Vivo Procedures

Eight ovariectomized pony mares between 5 and 20 years of age were used during the breeding season. The mares were randomly assigned to one of two treatment groups: *1*) TP-treated mares or *2*) control mares. Daily blood samples were collected via jugular venipuncture beginning 2 days (Day −2) prior to treatment and continued throughout the treatment period. All blood samples were drawn into heparinized tubes and plasma was harvested by centrifugation and stored frozen until assayed.

On Day 0, each mare was fitted with an indwelling jugular catheter prior to the intravenous injection of GnRH (1.0 μg/kg of body weight). Blood samples (10 ml) were collected at −30, −0.5, 15, 30, 45, 60, 90, 120, 180 and 240 min relative to the GnRH injection. Samples were allowed to clot and serum was harvested by centrifugation and stored frozen until assayed. After the last sample was drawn, each mare was given a subcutaneous injection of either TP (350 μg/kg of body weight) or oil (1.5 ml/mare). Treatments were repeated on Days 2, 4, 6, 8 and 10 for a total of six injections.

On Day 11, each mare was again challenged with GnRH and bled as described for Day 0. After completion of the second GnRH bleeding schedule, the mares were euthanized with an overdose of 5% thiamyl sodium and 10% glyceryl guaiacolate. Each pituitary was removed and placed in ice-cold Hanks' buffered saline solution (Gibco Labs., Grand Island, NY) for transport to the laboratory.

In Vitro Procedures

Each pituitary was weighed and aseptically separated into anterior and posterior pituitary. The anterior pituitary was weighed and minced into pieces of approximately 1 mm^3 in size. Four or five pieces (approx. 100 mg) from each anterior pituitary were weighed and placed into a glass vial with 2 ml of leucine-free Eagle's minimum essential medium (Gibco) to which had been added 5 μCi of [^{3}H] leucine (New England Nuclear Corp., Boston, MA). Duplicate vials of tissue were processed for each pituitary. All vials were placed in a shaking water bath for 8 h at 37°C. At the end of the incubation period, the vials containing tissue and medium were placed at −15°C for storage. The remaining pieces of anterior pituitary (not used for incubation) were weighed and frozen for storage.

After all tissues had been harvested, the incubation samples and remaining anterior pituitary samples were thawed and prepared for homogenization. The contents of each vial were transferred to a ground-glass homogenizer with a disposable pipette. The vial was rinsed with 1 ml of 0.01 M phosphate-buffered 0.15 M saline (pH 7.4; PBS) which was transferred to the homogenizer with the same pipette. Each incubation sample was homogenized for 50 strokes. The homogenate was then transferred to a test tube and centrifuged at 1200 × *g* for 20 min. The nonincubated anterior pituitary samples were homogenized as described above except that 3 ml of PBS were added to the homogenizer for each 100 mg of tissue.

Supernatants from the incubation samples (containing [^{3}H] leucine) were dialyzed in double-tied bags in two changes of 0.05 M Tris-HCl buffer (pH 9.8) and then in two changes of PBS for a total of 48 h at 4°C. The sacks were then carefully removed from the buffer and the contents were transferred to tubes. A 1:10 dilution of each dialyzed supernatant was made and all aliquots were frozen.

Immunoprecipitation and Assay Procedures

Immunoprecipitation techniques used in this study were similar to those described by Chowdhury and Steinberger (1975). To reduce nonspecifically bound radioactivity, each of the dialyzed supernatants was mixed (1:1) with undiluted normal rabbit serum (NRS) and incubated for 18–24 h at 4°C. The NRS was precipitated by the addition of undiluted pony anti-rabbit gamma globulin serum (pARGG). After incubation for 24 h at 4°C, the mixture was centrifuged and the supernatant used for the immunoprecipitation reaction.

Duplicate samples of each adsorbed supernatant (equivalent to 50 μl of original dialyzed supernatant) were mixed with 50 μl of undiluted anti-FSH serum (LSU 3D, generated against purified ovine FSH; Thompson et al., 1983c) or 25 μl of anti-LH serum (LSU 2, generated against equine chorionic gonadotropin; Thompson et al., 1983a) preadsorbed 1:1 with anestrous mare serum. Duplicate samples of each supernatant were also mixed with an equivalent amount of undiluted NRS to determine nonspecific binding. After mixing, samples were incubated at 4°C for 48 h. Undiluted pARGG was then added to each tube and all tubes were incubated for 24 h at 4°C. After incubation, 3 ml of cold PBS was added to all tubes which were then centrifuged at 1200 × *g* for 30 min. The resulting pellets were washed once with 4 ml of PBS by vortexing and centrifugation. The final pellets were dissolved in 1.5 ml of 1 N NaOH and quantitatively transferred to scintillation vials. The amount of radioactivity in each vial was determined by liquid scintillation counting. Quench corrections were based on the channels-ratio method.

The potential influence of endogenous LH or FSH in the samples was determined by adding ^{125}I-FSH or ^{125}I-LH to the same amount of incubation sample and antibody used for the immunoprecipitation reaction. These tubes were incubated and washed as described above except that the final pellets were counted directly in a solid scintillation counter. In all cases, the immunoprecipitation of ^{125}I-labeled gonadotropin in buffer (no sample) was comparable to that in each sample; thus, endogenous gonadotropins in the samples did not alter the amount of ^{3}H-labeled gonadotropin precipitated.

Specificity of the FSH and LH immunoprecipitation reactions was confirmed by preincubating a diluted aliquot of antiserum with either highly purified ovine FSH (20 μg/tube of G4-211B, supplied by Dr. H. Papkoff) or with bovine LH (1.15 mg/tube of NIH-bLH-4, supplied through the National Hormone and Pituitary Program, Bethesda, MD). Preincubation with these unlabeled hormones inhibited the specific binding of a pool of preadsorbed, ^{3}H-labeled pituitary homogenate by 100% for FSH and 92% for LH. This agreed well with the previously reported specificities of these antisera (Thompson et al., 1983a,c).

Daily plasma samples, serum samples after GnRH,

and pituitary homogenates were assayed for LH and FSH by radioimmunoassay as described by Thompson et al. (1983a,c). Previous comparisons with split samples confirmed that serum and plasma gave equivalent results in these radioimmunoassays. The diluted aliquots of each homogenate were used for determination of protein concentration as well as LH and FSH concentration. Protein concentration was determined as described by Lowry et al. (1951).

Hormonal concentrations in daily blood samples were adjusted prior to analysis for pretreatment differences between groups by subtracting each animal's pretreatment average from all its subsequent data and then adding back the grand mean for all animals (Thompson et al., 1977). The adjusted data (daily samples only) were then analyzed by analysis of variance in a split-plot design (Gill and Hafs, 1971) with time periods as subplots. The amount of hormone secreted after GnRH was estimated for each pony and each GnRH challenge as follows: *1*) the net difference between the average pre-GnRH concentrations and the maximum post-GnRH concentrations was determined and then *2*) the total plasma volume was calculated at 5% of body weight (Sack and Sadler, 1982) and was multiplied by the net change in hormone concentration. This calculation was based on the fact that the shape of the gonadotropin-response curves in this experiment was similar to that predicted for a bolus-like introduction of hormone into the bloodstream (one-compartment open model, Rodda, 1981). Areas under the response curves were calculated as described by Mongkonpunya et al. (1974). Hormonal and immunoprecipitation data were analyzed by Student's *t* tests (Steel and Torrie, 1960).

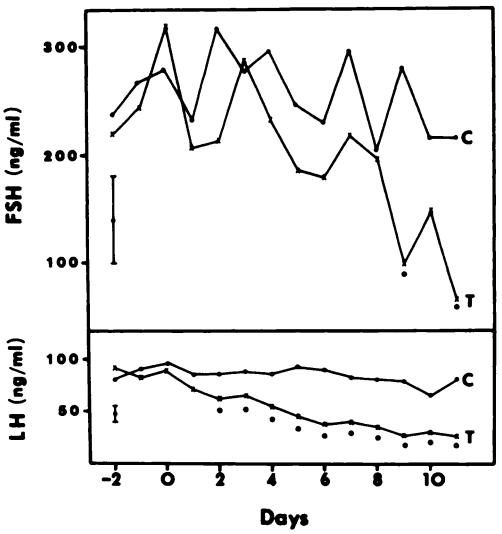

FIG. 1. Concentrations of follicle-stimulating hormone (*top*) and luteinizing hormone (*bottom*) in plasma of control (*C*) and testosterone propionate-treated (*T*) pony mares. Daily samples of blood were drawn. The first treatment injections were given on Day 0 and were repeated every other day through Day 10. *Asterisks* indicate days on which significant differences between groups occurred ($P<0.05$). The *single point* and *vertical lines* in each panel indicate ± the pooled standard error from the analysis of variance.

RESULTS

Concentrations of FSH and LH in daily blood samples of control and TP-treated mares are presented in Fig. 1. There was a slight suppression ($P<0.05$) of FSH concentrations in TP-treated mares after Day 5 of treatment, while concentrations of FSH in control mares remained constant. Concentrations of LH in daily blood samples were significantly suppressed ($P<0.01$) in TP-treated mares compared with control mares.

The LH and FSH response to the first GnRH (pretreatment; Day 0) was similar in all mares (data not shown), indicating that no difference between groups occurred due to chance before the onset of TP treatment. Concentrations of FSH and LH in serum before and after the second GnRH challenge, given after 11 days of TP treatment, are presented in Table 1. FSH concentrations prior to GnRH were lower ($P<0.06$) in TP-treated mares compared with control mares, whereas the maximum concentrations of FSH attained after GnRH were not significantly affected by treatment. The net area under the GnRH-response curve, which is proportional to the amount of hormone secreted and inversely proportional to clearance rate of the hormone, was greater ($P<0.02$) in TP-treated mares than in control mares. The amount of FSH secreted in response to GnRH was greater ($P<0.01$) in TP-treated mares compared with controls. Although absolute concentrations of LH were lower ($P<0.01$) in TP-treated mares both before and after GnRH, the amount of LH released in response to GnRH was similar in the control and treated mares.

The amount of FSH remaining in the pituitaries of TP-treated mares was not significantly different from that in control mares. However, the total amount of FSH in the pituitary prior to the GnRH challenge (sum of amount secreted and amount in pituitary at sacrifice) was greater ($P<0.02$) in TP-treated mares than in control mares. The amount of LH in the pituitary at sacrifice and the total amount of LH in the pituitary before GnRH were not significantly affected by TP treatment, although both tended to be reduced in TP-treated mares.

Results of the immunoprecipitation trials are presented in Table 2. The amount of radiolabeled, immunoprecipitable FSH was greater in

TABLE 1. Effect of in vivo testosterone propionate treatment on serum concentrations and pituitary contents of gonadotropins in pony mares.

Hormone	Control[a]	TP-treated[a]	P[b]
FSH			
Pre-GnRH concentration (ng/ml)[c]	206 ± 36[d]	115 ± 18	0.06
Post-GnRH concentration (ng/ml)[e]	292 ± 36	339 ± 52	0.49
Area under curve ($ng \cdot ml^{-1} \cdot h$)	165 ± 50	600 ± 118	0.02
Amount secreted (μg)[f]	745 ± 143	1681 ± 220	0.01
Amount in pituitary (μg)[g]	905 ± 225	1163 ± 158	0.38
Total amount (μg)[h]	1650 ± 310	2844 ± 175	0.02
LH			
Pre-GnRH concentration (ng/ml)[c]	131 ± 27	10 ± 2	0.01
Post-GnRH concentration (ng/ml)[e]	181 ± 31	50 ± 14	0.01
Area under curve ($ng \cdot ml^{-1} \cdot h$)	80 ± 24	82 ± 32	0.96
Amount secreted (μg)[f]	420 ± 50	325 ± 112	0.47
Amount in pituitary (μg)[g]	493 ± 95	328 ± 170	0.43
Total amount (μg)[h]	913 ± 108	652 ± 271	0.41

[a]Treated with oil (control) or testosterone propionate (TP) for 11 days.

[b]Probability of higher *t* value.

[c]Concentration of hormone before GnRH challenge on Day 11.

[d]Mean ± SEM.

[e]Highest concentration of hormone occurring in first 1.0 h after injection of GnRH.

[f]Calculated from net change in hormonal concentrations × plasma volume for each horse.

[g]After GnRH at sacrifice.

[h]Sum of amount secreted plus amount left in pituitary.

TP-treated mares than in controls when compared on a per μg protein ($P<0.07$), per mg tissue ($P<0.01$) or per anterior pituitary ($P<0.01$) basis. The amount of radiolabeled, immunoprecipitable LH was not significantly affected by treatment when expressed on a per μg protein, per mg tissue or per anterior pituitary basis. As for the pituitary data, the means for LH tended to be reduced in TP-treated mares compared with controls.

TABLE 2. Incorporation of [^{3}H]leucine into immunoprecipitable FSH and LH in pituitary tissue from control and testosterone propionate-treated mares.

Hormone	Control[a]	TP-treated[a]	P[b]
FSH			
dpm/μg protein	20.54 ± 4.72[c]	44.21 ± 6.68[c]	0.07
dpm/mg tissue	668.00 ± 108.00	1948.00 ± 240.00	0.01
dpm/anterior pituitary ($\times 10^{-3}$)	448.00 ± 60.00	1352.00 ± 188.00	0.01
LH			
dpm/μg protein	25.47 ± 7.98	17.76 ± 4.13	0.57
dpm/mg tissue	1076.00 ± 292.00	924.00 ± 176.00	0.77
dpm/anterior pituitary ($\times 10^{-3}$)	660.00 ± 144.00	620.00 ± 100.00	0.86

[a]Treated with oil (control) or testosterone propionate (TP) for 11 days.

[b]Probability of higher *t* value.

[c]Mean ± SEM.

DISCUSSION

Testosterone propionate treatment of these ovariectomized pony mares for 11 days increased FSH secretion in response to GnRH by 2.5-fold. This increased FSH response after TP treatment is similar to that reported for geldings (Thompson et al., 1979), ovariectomized mares and intact mares (Wallace, 1981; Thompson et al., 1983b, 1984). Although Thompson et al. (1979) observed an eightfold increase in pituitary FSH concentrations in TP-treated geldings, those geldings were sacrificed approximately 12 days after GnRH administration, and TP treatment continued until sacrifice. Thus, the lack of greater FSH content in the pituitaries of TP-treated pony mares at sacrifice in the present experiment was probably due to the depletion of extra reserves after GnRH (approximately 5 h before sacrifice). This is supported by the fact that TP-treated mares had greater total FSH content before GnRH, as calculated from pituitary content plus amount secreted.

In conjunction with the increased FSH response to GnRH, we have demonstrated herein that de novo FSH production is also increased by TP treatment of pony mares. Previous studies have supplied indirect evidence that androgens increase FSH production in mares (Thompson et al., 1983b, 1984). However, in those studies, TP treatment also reduced FSH secretion over time, thus the greater response to GnRH could have been due to increased FSH production and/or greater storage of a releasable pool. In contrast, treatment of intact mares with dihydrotestosterone (DHT) increased the posttreatment FSH response to GnRH without suppressing daily FSH secretion (Thompson et al., 1983b), again indicating indirectly that FSH production was increased. The approximately threefold increase in FSH production per anterior pituitary observed in TP-treated mares in the present experiment correlates well with these previous studies.

As in previous studies, TP treatment in the present experiment reduced concentrations of FSH and LH in daily blood samples. Thompson et al. (1983b) reported that the inhibition of FSH secretion in intact mares treated with TP was apparently due to testosterone's estrogenic potential, because treatment of mares with estradiol produced a similar suppression of FSH secretion, whereas treatment with DHT did not. Estradiol treatment of geldings (Thompson et al., 1979) also resulted in a rapid suppression of FSH secretion in conjunction with a stimulation of LH secretion.

Although TP treatment reduced concentrations of LH in daily blood samples, the amount of LH secreted in response to GnRH after TP treatment was not different from that of the control mares. There was a tendency for TP treatment to reduce pituitary concentrations and total amount of LH, but this was not consistent among mares. Treatment of geldings with TP reduced pituitary concentrations of LH by approximately 50% (Thompson et al., 1979); however, this was after 30 days of treatment compared with 11 days in the present experiment. Thus, the lack of effect of TP treatment on de novo LH production reported herein may be due in part to the relatively short duration of treatment.

Treatment of females of other species with testosterone has been reported to increase FSH content and/or secretion by the pituitary. Radford and Wallace (1971) found that TP treatment of anestrous ewes stimulated follicular activity and increased pituitary FSH content. Bogdanove and Gay (1967) reported a similar effect of TP on FSH content in ovariectomized rats. Gay and Tomacari (1974) discovered that endogenous androgens appear to be necessary for the continued estrous secretion of FSH in rats. Although TP treatment of castrated male rats appeared indirectly to increase FSH production (Gay and Bogdanove, 1969), direct in vitro assessment by other researchers (Steinberger and Chowdhury, 1977) did not confirm this effect of TP.

Steroid treatment of gonadectomized animals appears to affect FSH production both qualitatively and quantitatively (reviewed by Bodganove et al., 1975). That is, androgen treatment of castrated rats causes the synthesis of a larger FSH molecule with a longer survival time in the bloodstream, whereas estrogen treatment results in the synthesis of smaller, more rapidly cleared FSH molecule. Because the degree of these qualitative changes may differ between species (Bogdanove et al., 1975), similar research needs to be performed to determine if such qualitative changes occur in the TP-treated horse.

In conclusion, treatment of ovariectomized pony mares with TP decreased daily secretion rates of FSH and resulted in an accumulation of FSH in the pituitary gland that was releasable by exogenous GnRH. Associated with this effect on FSH secretion was a threefold increase in de novo FSH production as assessed via in

vitro incorporation of [^{3}H]leucine into immunoprecipitable hormone. TP treatment also suppressed daily LH secretion rates but did not affect pituitary content of LH, amount released after GnRH or de novo LH production in vitro. This biological effect of testosterone on FSH production is consistent with the hypothesis that androgens from the mare's ovary during estrus may prepare the pituitary for the subsequent high rate of FSH secretion during diestrus (Thompson et al., 1983c).

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