STUDY OF THE DIRECT EFFECT OF LHRH AGONIST ON TESTICULAR 17-HYDROXYLASE AND 5α-REDUCTASE ACTIVITIES IN NON-HYPOPHYSECTOMIZED ADULT RATS TREATED WITH AN ANTI-LUTEINIZING HORMONE SERUM

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ABSTRACT

In order to study both direct and pituitary-mediated mechanisms of action of the LHRH analogue [D-Ser(TBU)^{6}, des-Gly-NH$_{2}^{10}$]LHRH ethylamide upon testicular steroidogenesis in adult rat, we compared the effects of the agonist when administered alone or concomitantly with an anti-LH serum to non-hypophysectomized rats. Testicular steroid contents and *in vitro* progesterone and testosterone metabolism were determined. Anti-LH serum administration was able to prevent 5α-reductase stimulation by the agonistic peptide, but not the inhibition of 17-hydroxylase activity. These data suggest that modulation of 17-hydroxylase involves both direct and pituitary-mediated processes, while 5α-reductase stimulation is mainly if not only due to a pituitary-mediated mechanism.

INTRODUCTION

The inhibitory action of LHRH agonists on reproductive functions in adult male rats is now well known (1-6). It has been demonstrated that administration of LHRH agonists decreases testosterone and androstenedione secretions, accompanied by an accumulation of testicular pregnenolone and progesterone levels due to inhibition of 17-hydroxylase and 17-hydroxyprogesterone-17,20-lyase activities in Leydig cells. Moreover, we have demonstrated that a stimulation of testicular 5α-reductase activity occurs following chronic treatment with an LHRH agonist (6, 7).

Recently, we have been able to reproduce by multiple administration of ovine LH to intact adult rats a testicular desensitization comparable to that observed after LHRH

agonist treatment (8), suggesting that the inhibitory effects of LHRH analogues on testicular steroidogenesis and LH receptor levels are mainly mediated by an increase in the concentration of endogenous LH in the circulation. On the other hand, several observations indicate that LHRH agonists may also interact with a specific gonadal LHRH receptor and exert direct inhibitory effects in male and female rats (9, 10). In fact, administration of pharmacological doses of the LH-releasing peptides to hypophysectomized mature and immature rats induces a loss of LH receptors as well as a marked suppression of testicular 17-hydroxylase activity (15, 16).

In the present study, we investigated the direct effect of LHRH agonist on testicular 17-hydroxylase and 5α-reductase activities in intact adult rats. Since hypophysectomy by itself has profound effects on gonadal enzyme activity, we have used another approach which minimizes hormonal disturbance, namely by using intact animals injected with antibovine LH (anti-LH) serum.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (Crl:CD(SD)Br), weighing 300-325 g upon arrival, were obtained from Charles River Canada Inc., St. Constant, Quebec. Animals were housed 2 per cage in a temperature (20-22°C)- and light (14h light-10h darkness, lights on at 0500h)-controlled room and given food and water ad libitum.

Chemicals. The agonist [D-Ser(TBU)^{6}, des-Gly-NH$_2^{10}$]LHRH ethylamide (LHRH-A) was generously provided by Drs. M. Van der Ohe and J. Sandow, Hoechst, AG, Frankfurt. In addition to the characteristics of the equine antibovine LH serum (JOAN-5-31-67; anti-LH) which have been previously reported (12-14), some additional studies were performed to assess the possible cross-reactivity of our LHRH analog with the anti-LH serum (15). Normal horse serum (NHS) was obtained from Grand Island

Biological Co. (Grand Island, N.Y.). [1,2,6,7-^{3}H]progesterone (92 Ci/mmole) and [1,2,6,7,16,17-^{3}H]testosterone (135 Ci/mmole), were obtained from New England Nuclear and purified on LH-20 columns (16) and by silica gel thin-layer chromatography. Non-radioactive steroids were purchased from Steraloids and from Research Plus Steroid Laboratories Inc. Coated column packing for gas chromatography was obtained from Chromatographic Specialties, Brockville, Canada.

Treatment. The animals were treated as follows: Group I (8 animals per group): 0.5 ml i.v. NHS on days 1, 3 and 5; group II: NHS as in group I, plus 500 ng s.c. of LHRH-A once a day for 5 days; group III: 0.5 ml i.v. anti-LH serum on days 1, 3 and 5; group IV: anti-LH serum as in group III, plus LHRH-A as in group II, the agonist being injected 1h after the anti-LH serum. The animals were sacrificed by decapitation 24h after the last injection.

Isolation of interstitial cells. Interstitial tissue was isolated by a modification of the method reported by Dufau et al. (17). Testes were decapsulated and incubated in groups of 4 in 50 ml flasks containing 10 ml of medium-199 (Gibco) with 0.1% bovine serum albumin (BSA) and 0.18 mg collagenase (Worthington Type III) per ml at pH 7.4. Incubation was performed at 34°C while shaking at 200 cycles/min for about 9-10 min. The volume was then adjusted to 45 ml with incubation medium without collagenase and the tubes were inverted 10 times. After each tube had been allowed to stand vertically for 3 min, the turbid supernatant was filtered and centrifuged at 600 x g for 10 min. The sedimented cells were resuspended in 30 ml of medium-199 containing 0.1% BSA and 0.7 mg trypsin inhibitor (from soybean, type 1-S; Sigma) per ml and centrifuged at 600 x g for 10 min. The sedimented cells were used as the interstitial cells.

Incubation of interstitial cells with ^{3}H-progesterone. The interstitial cells (2×10^5 cells per tube) were incubated in polypropylene tubes in 1 ml of medium-199 containing 0.1% BSA. Each incubation was performed in triplicate. After addition of 3×10^6 dpm of ^{3}H-progesterone, the incubation was carried out (10, 30, 60 or 90 min) in a water bath at 34°C with continuous shaking under a constant stream of 95% O_2 and 5% CO_2. The incubation was stopped by addition of 0.1 ml 1N HCl and the tubes were placed in an ice-water bath at 4°C.

Incubation of testicular homogenates with ^{3}H-progesterone or ^{3}H-testosterone. Testes were homogenized individually after weighing in 10 ml 0.25M sucrose containing 0.001M EDTA and pooled. Buffer co-factor solution (0.1 ml; 0.3 M potassium phosphate, 0.06 M nicotinamide, 0.002 M $MgCl_2$, pH 7.4) containing the tritiated steroid (3×10^6 dpm) was introduced into 2 x 10 cm glass tubes. Each incubation was performed in triplicate. Buffer co-factor solution (0.4 ml) containing 0.8 mg NADPH was then added to each tube before the addition of

testicular homogenate (0.5 ml, equivalent to 20 mg of tissue). The samples were incubated (5, 10, 30 or 60 min) in a shaking water bath under air atmosphere at 34°C. At the end of incubation, the incubate was immediately acidified with 0.1 ml 1N HCl and placed in an ice-water bath at 4°C.

Identification of metabolites. Ethanol (0.1 ml) containing 10 µg of each of the following carrier steroids (progesterone, 5α-pregnane-3,20-dione, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, 17-hydroxyprogesterone, 5α-pregnane-3α,17α-diol-20-one, 5α-pregnane-3β,17α-diol-20-one, androstenedione, 5α-androstane-3,17-dione, 5α-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one, testosterone, dihydrotestosterone, 5α-androstane-3α,17β-diol and androstane-3β,17β-diol) was then added to each tube. Incubation media were extracted three times with ether and the extracts were dried with a stream of nitrogen. No radioactivity can be detected in medium after extraction. The residue obtained from the ether extraction was dissolved in 0.2 ml of the mixture isooctane-benzene-methanol (90:5:5) and then placed on LH-20 columns (4). The eluate was separated in the following fractions: a) progesterone + 5α-pregnane-3,20-dione + 5α-pregnan-3α-ol-20-one (0-15 ml); b) androstenedione + 5α-androstane-3,17-dione + dihydrotestosterone + 5α-androstan-3α-ol-17-one + 5α-androstan-3β-ol-17-one + 5α-pregnan-3β-ol-20-one (16-30 ml); c) 17-hydroxyprogesterone + testosterone + 5α-pregnane-3α,17α-diol-20-one + 5α-androstane-3α,17β-diol (31-60 ml). Polarity of the solvent was then changed to 70: 15:15 (isooctane-benzene-methanol) for the elution of fraction d: 5α-pregnane-3β,17α-diol-20-one + 5α-androstane-3β,17β-diol (61-75 ml). An aliquot of each fraction was then counted and the percentage of total radioactivity present in each zone was calculated. The total radioactivity usually represents more than 90% of radioactivity added at the time of incubation. After evaporation of the solvent, the fractions were acetylated with acetic anhydride in pyridine (2:3; v:v) at room temperature overnight and re-chromatographed on 0.25 mm silica gel thin-layer plates (Merck Silica Gel (GF-254)). Fractions were chromatographed as follows: fraction a, hexane/ether (1:3; v:v), progesterone (Rf = 0.59), 5α-pregnane-3,20-dione (0.78) and 5α-pregnan-3α-ol-20-one (0.90); fraction b, hexane/petroleum ether/ether (1:1:2; v:v:v), androstenedione (0.16), 5α-androstane-3,17-dione (0.40), dihydrotestosterone (0.60), 5α-androstan-3α-ol-17-one (0.72), 5α-androstan-3β-ol-17-one (0.72) and 5α-pregnan-3β-ol-20-one (0.85); fraction c, petroleum ether/ethyl acetate (1:1; v:v), 17-hydroxyprogesterone (0.38), testosterone (0.61), 5α-pregnane-3α,17α-diol-20-one (0.70) and 5α-androstane-3α,17β-diol (0.80); fraction d, petroleum ether/ethyl acetate (1:1; v:v), 5α-pregnane-3β,17α-diol-20-one (0.70) and 5α-androstane-3β,17β-diol (0.80). Radioactivity was detected with a Berthold thin-layer scanner and radioactivity zones were scraped off, eluted with methanol, counted in a liquid scintillation counter or analyzed by radio-gas chromatography

(Varian model 3700 gas chromatograph equipped with a Packard gas proportional counter model 917). The radioactive material isolated with carrier steroid was further analyzed by crystallization to constant specific activity.

Steroid assays. Testicular steroid contents were measured as described (7, 16).

Calculations. RIA data were analyzed using a program based on model II of Rodbard and Lewald (18). Statistical significance was assessed according to the multiple range test of Duncan-Kramer (19).

RESULTS

The rate of tritiated progesterone metabolism by interstitial cells, is illustrated in Fig. 1. It can be seen that administration of an LHRH agonist abolished almost completely the formation of 17-hydroxyprogesterone, androstenedione and testosterone. Moreover, while the basal metabolism is reduced following anti-LH serum administration, the combined treatment with anti-LH and LHRH agonist further decreased the progesterone metabolism.

In fact, the proportion of the initial tritiated progesterone which is already metabolized after 60 min of incubation is reduced from 58 ± 2% to 4 ± 1% ($p < 0.01$) when LHRH-A is injected alone, as compared to a decrease from 25 ± 2% to 8 ± 1% ($p < 0.01$) when anti-LH serum is concomitantly administered.

As shown in Fig. 2A, the administration of LHRH analogue alone or in combination with the anti-LH completely prevent the formation of Δ^4-metabolites (17-hydroxyprogesterone, androstenedione and testosterone) from tritiated progesterone during incubation with testicular homogenates. However, it is

evident from panel B which illustrates the testicular 5α-reductase activity that only the group receiving LHRH-A without anti-LH shows a marked stimulation of 5α-reductase activity. After 10 min of incubation, the 5α-reduced metabolites represent 28 ± 2% of total radioactivity in medium for LHRH-A group, as compared to only 4 ± 1% ($p < 0.01$) for the other three groups, representing a 7-fold increase in the in vitro activity of 5α-reductase. It thus appears that LHRH-A failed in stimulating the 5α-reductase activity when anti-LH serum is concomitantly administered, thus suggesting that activation of 5α-reductase is a pituitary-mediated process.

Figure 1

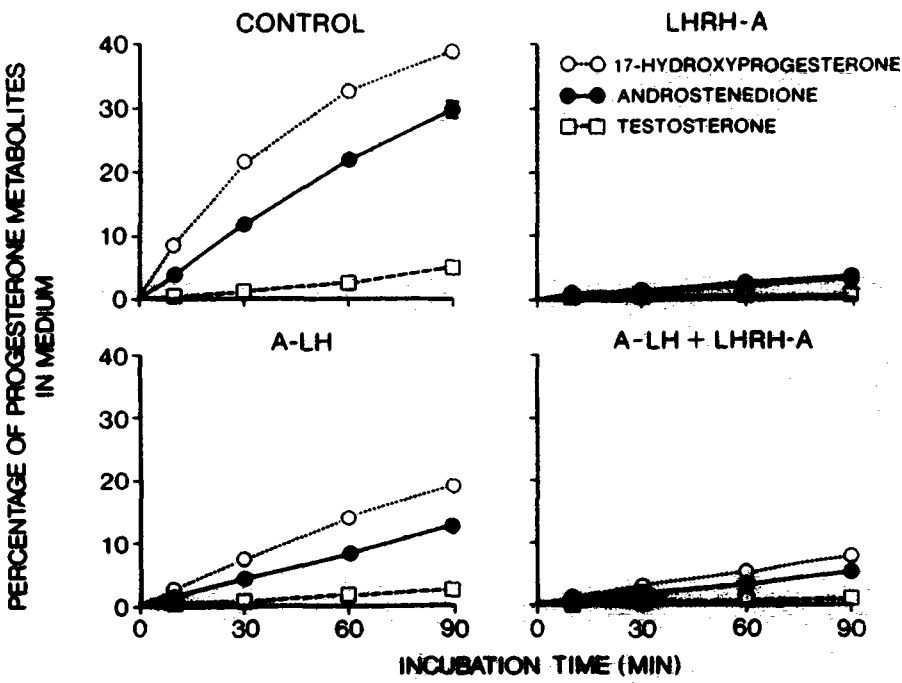

Fig. 1. Tritiated progesterone metabolism by a suspension of testicular interstitial cells. Each point represents the mean ± SEM of results from triplicate incubations.

Figure 2

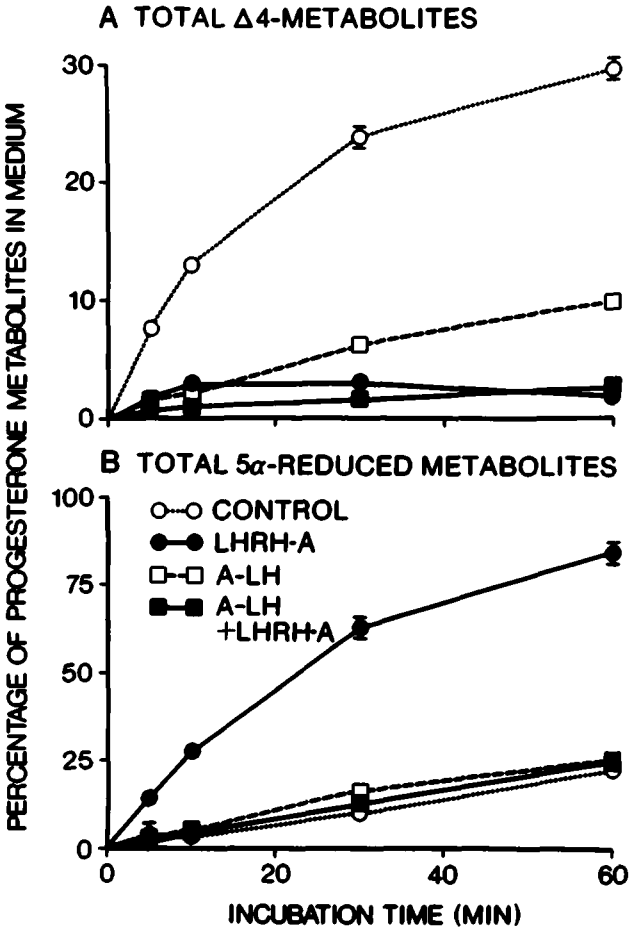

Fig. 2. Formation of Δ^4-metabolites (A) and 5α-reduced metabolites (B) from tritiated progesterone incubation with total testicular homogenates (20 mg of tissue per tube). Δ^4-metabolites: 17-hydroxyprogesterone, androstenedione and testosterone; 5α-reduced metabolites: 5α-pregnane-3,20-dione, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, 5α-pregnane-3α,17α-diol-20-one, 5α-pregnane-3β,17α-diol-20-one, 5α-androstane-3,17-dione, 5α-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one, dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. Each point represents the mean ± SEM of results from triplicate incubations.

In order to further demonstrate the absence of direct action of LHRH-A at the level of 5α-reductase activity, tritiated testosterone was incubated with a testicular homogenate. We can see in Fig. 3 that stimulation of 5α-reductase activity is not present when anti-LH serum is administered before the LHRH agonist.

We next studied the effect of combined treatment of anti-LH and LHRH agonist on testicular steroid contents (Table 1). When LHRH-A is administered alone, there is a

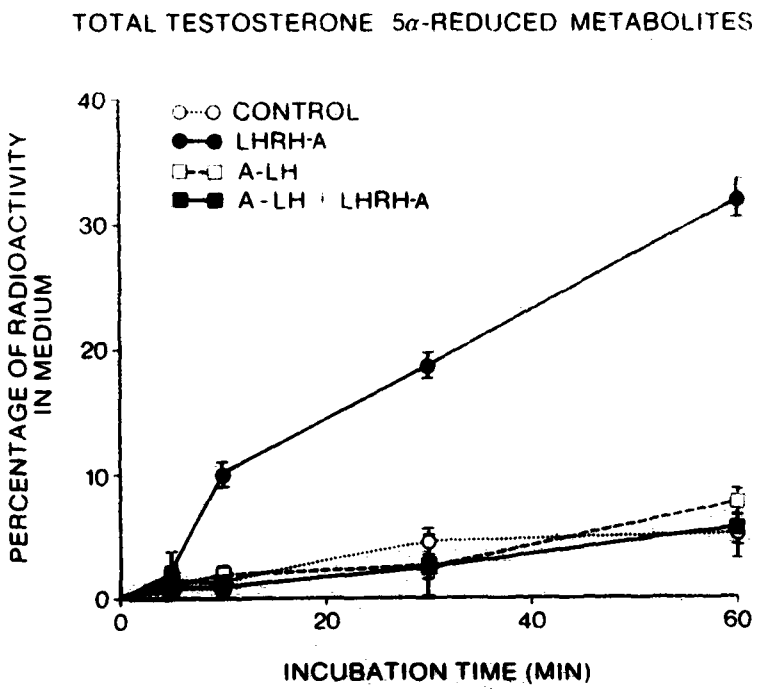

Fig. 3. Formation of 5α-reduced metabolites from tritiated testosterone incubation with total testicular homogenates (20 mg of tissue per tube). 5α-reduced metabolites: 5α-androstane-3,17-dione, 5α-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one, dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. Each point represents the mean ± SEM of results from triplicate incubations.

slight accumu lation of pregnenolone and progesterone levels accompanied by a marked inhibition of Δ^4-steroids (testosterone and androstenedione) concentration (from 34.6 ± 5.1 to 11.3 ± 1.5 ng/g testis, $p < 0.01$). Moreover, we have observed a stimulation of 5α-reduced steroid levels from 20.1 ± 1.9 to 29.2 ± 2.4 ng/g testis; $p < 0.05$).

These data confirm the inhibition of 17-hydroxylase as well as the stimulation of 5α-reductase activities noted in Figs 1, 2 and 3. When LHRH-A is administered concomitantly with anti-LH serum, we observe a marked inhibition of Δ^4-metabolites, confirming the direct effect of LHRH-A at the level of 17-hydroxylase enzyme while there is no increase in the 5α-reduced steroid levels.

Table 1. Testicular steroid contents. Data are expressed as ng/g testis (mean ± SEM from 8 animals). Δ^4-steroids: 17-hydroxyprogesterone, androstenedione and testosterone; 5α-reduced steroids: dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol.

Steroids	Treatment			
	NHS	LHRH-A	A-LH	LHRH-A + A-LH
Pregnenolone + progesterone	4.2 ±0.4	5.8 ±0.6b	1.6 ±0.2	2.0 ±0.3
Δ^4-steroids	34.6 ±5.1	11.3 ±1.5a	6.8 ±0.8	4.0 ±0.7c
5α-reduced steroids	20.1 ±1.9	29.2 ±2.4a	7.4 ±0.8	6.7 ±0.6

a, $p < 0.01$, LHRH-A vs NHS groups
b, $p < 0.05$, LHRH-A vs NHS groups
c, $p < 0.05$, LHRH-A + anti-LH vs anti-LH groups

DISCUSSION

The present data clearly demonstrate that a direct inhibitory action of [D-Ser(TBU)^{6}, des-Gly-NH$_2$^{10}]LHRH ethylamide at the testicular 17-hydroxylase enzyme level is observed after daily injection of as little as 500 ng of the agonistic peptide to non-hypophysectomized adult rats concomitantly treated with an anti-LH serum. This inhibitory effect is reflected by a marked *in vitro* inhibition of progesterone metabolism by interstitial cells and by *in vivo* decrease of testicular Δ^4-androgens contents. Our finding of a direct inhibitory effect of LHRH-A at a 500 ng-dose to rats treated with an anti-LH serum confirms and extends previous studies showing that administration of higher doses of the agonist to hypophysectomized rats can inhibit testicular androgen formation (9, 11).

Moreover, our study shows that the stimulation of 5α-reductase activity observed in intact rats after administration of LHRH analogue alone does not occur when anti-LH serum is concomitantly administered. Thus, the observed stimulatory action on 5α-reductase appears to be a specific LH effect and is not mediated directly at the testicular level by the LHRH agonist.

It is well known that, in adult rats, the testicular 5α-reductase activity is specifically localized in seminiferous tubules. In agreement with these observations, we have found that Leydig cells cannot transform progesterone to 5α-reduced steroids in intact rats as well as those treated

with the LHRH analogue while the 5α-reductase activity detected in testicular homogenate is strongly stimulated after administration of the analogue. The site of specific testicular binding of LHRH and its agonist as well as LH being the Leydig cells (10, 20, 21), we can therefore eliminate a specific action of the hormone. Thus, the mechanism of the stimulatory action of LH upon 5α-reductase activity likely involves an interaction between Leydig cells and the seminiferous tubules and, moreover, this factor of regulation seems to be on the control of LH. Our results further support several observations showing that LHRH analogue can inhibit the androgen secretion by a direct action on Leydig cells and clearly demonstrate that LH is also involved in the mechanism of testicular desensitization.

Trivial names: Androstenedione: 4-androstene-3,17-dione; dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one; 17-hydroxyprogesterone: 17 -hydroxy-4-pregnene-3,20-dione; pregnenolone: 3β-hydroxy-5-pregnen-20-one; progesterone: 4-pregnene-3,20-dione; testosterone: 17β-hydroxy-4-androsten-3-one, 5α-pregnan-3α-ol-20-one: 3α-hydroxy-5α-pregnan-20-one; pregnan-3β-ol-20-one: 3β-hydroxy-5α-pregnan-20-one; 5α-pregnane-3α,17α-diol-20-one: 3α,17-dihydroxy-5α-pregnan-20-one; 5α-pregnane-3β,17α-diol-20-one: 3β,17-dihydroxy-5α-pregnan-20-one; 5α-androstane-3α-ol-17-one: 3α-hydroxy-5α-androstane-17-one; 5α-androstan-3β-ol-17-one: 3β-hydroxy-5α-androstan-17-one.

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