Evaluation of the possible direct effects of gonadotrophin-releasing hormone analogues on the monkey (*Macaca mulatta*) testis

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Summary. In Exp. 1, the effect of treatment with a GnRH agonist on basal concentrations of serum testosterone and peak values of serum testosterone after administration of hCG was determined. One group of adult male monkeys was treated with a low dose (5–10 µg/day) and a second group with a high dose (25 µg/day) of a GnRH agonist for 44 weeks. Basal and peak testosterone concentrations were both significantly reduced by GnRH agonist treatment in all groups compared to untreated control animals, but the % rise in serum testosterone above basal values in response to hCG administration was unchanged by agonist treatment.

In Exp. 2, the GnRH agonist (100 or 400 ng) or a GnRH antagonist (4 µg) was infused into the testicular arteries of adult monkeys. The agonist did not alter testosterone concentrations in the testicular vein or testosterone and LH values in the femoral vein.

In Exp. 3, testicular interstitial cells from monkeys were incubated with three concentrations (10^{-9} , 10^{-7} and 10^{-5} M) of the GnRH agonist or a GnRH antagonist with and without hCG. After 24 h, neither basal nor hCG-stimulated testosterone production was affected by the presence of the GnRH agonist or antagonist.

The results from all 3 experiments clearly suggest that GnRH agonist treatment does not directly alter steroid production by the monkey testis.

Keywords: GnRH; testes; LH; testosterone; rhesus monkey

Introduction

Chronic administration of gonadotrophin-releasing hormone (GnRH) agonists causes azoospermia in monkeys and men (Akhtar *et al.*, 1983a, b; Swerdloff *et al.*, 1985; Mann *et al.*, 1985, 1987). Simultaneous testosterone therapy to maintain libido and potency in men (Swerdloff *et al.*, 1985; Bouchard *et al.*, 1986) and ejaculatory response in monkeys (Mann *et al.*, 1987) does not interfere with agonist-induced azoospermia. Administration of GnRH antagonists also suppresses spermatogenesis in primates although the doses of antagonist required for this effect are much greater than for agonists (Weinbauer *et al.*, 1984; Akhtar *et al.*, 1985). These developments suggest that GnRH analogues may be useful for male contraception.

The mechanism of the antifertility effects of GnRH analogues in primates remains undefined. GnRH agonists reduce the luteinizing hormone (LH) and testosterone response to GnRH (Akhtar *et al.*, 1983b; Mann *et al.*, 1985, 1987), presumably by down-regulating pituitary GnRH receptors, as reported for rats (Heber *et al.*, 1982). GnRH antagonists bind with high affinity to GnRH

receptors in the pituitary, preventing binding by GnRH (Clayton *et al.*, 1982). In the rat, these analogues also directly inhibit gonadal steroidogenesis (Hsueh & Erickson, 1979; Clayton *et al.*, 1980; Hsueh *et al.*, 1981; Sharpe & Cooper, 1982a, b, 1987; Sharpe, 1982; Spona *et al.*, 1985). However, most evidence does not support a gonadal site of action of these analogues in primates. GnRH agonist treatment does not abolish the testicular response to LH or hCG in primates (Mann *et al.*, 1984; Schaison *et al.*, 1984; Heber *et al.*, 1984; Evans *et al.*, 1984), and GnRH receptors were not demonstrated on the primate gonad (Asch *et al.*, 1981; Clayton & Huhtaniemi, 1982).

Evidence for a direct effect of GnRH or GnRH-like peptides on the primate gonad is supported by findings that the seminiferous tubules of the stump-tailed macaque contain low-affinity GnRH-binding sites and GnRH-like biological activity, and human seminal plasma has GnRH-like immunoactivity (Sharpe *et al.*, 1981; Swerdloff *et al.*, 1984). In addition, the presence of a GnRH agonist inhibited progesterone secretion from cultured human granulosa cells (Tureck *et al.*, 1982). Progesterone secretion was normal with simultaneous incubation with a GnRH antagonist (Tureck *et al.*, 1982). These results clearly indicate that the question about a direct effect of GnRH agonists on the primate gonad has not been resolved.

In this study, we tested the hypothesis that there is a direct effect of GnRH analogues on the testis of the rhesus monkey. We examined the effect of: (1) chronic GnRH agonist administration (44 weeks) on the testicular response to a low dose of hCG, (2) acute infusion of a GnRH agonist or antagonist into the testicular artery on testosterone release into the testicular vein in adult monkeys and (3) in-vitro incubation of monkey testicular interstitial cells with a GnRH agonist or antagonist on basal and hCG-stimulated testosterone production.

Materials and Methods

The 23 adult male rhesus monkeys (*Macaca mulatta*) used in this study were housed under a 12-h light:12-h dark lighting schedule (lights on: 06:00 h) at 20–23°C.

Experiment 1: effect of chronic GnRH agonist administration on the response to hCG. Ten monkeys were allocated randomly to two groups. Group 1 animals were treated continuously with a GnRH agonist (D-Trp⁶-N-alpha-Me-Leu⁷-desGly¹⁰-Pro⁹-NHEt-GnRH; Wyeth Laboratories, Philadelphia, PA), 5 µg/day for 24 weeks followed by a dose of 10 µg/day of the same agonist for 20 weeks, using an osmotic minipump as previously described (Mann *et al.*, 1985, 1987). The dose of agonist was doubled from 5 to 10 µg/day after 24 weeks because the 5 µg/day dose was not effective in reducing sperm counts (Mann *et al.*, 1987). Group 2 monkeys received 25 µg agonist/day for 44 weeks. Pumps were replaced at 4-week intervals. All animals were injected i.v. with 50 i.u. hCG during the control (pretreatment) period and at the end of 44 weeks of agonist administration. Blood samples were taken 15 min before and at 0, 30, 60 and 120 min after hCG administration for determination of testosterone by radioimmunoassay.

Experiment 2: influence of acute infusion of a GnRH agonist or antagonist into the testicular artery on serum testosterone concentrations in testicular vein. Eighteen adult male monkeys were used with each animal serving as its own control. Animals were anaesthetized with 30 mg ketamine HCl/kg and prepared for abdominal surgery under sterile conditions. A midline incision was made in the abdominal region and the inguinal canal identified on one side of the animal. The GnRH agonist (100 or 400 ng), a GnRH antagonist (4 µg; Ac-D-pCIPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH₂-CH₃COOH; Organon, Oss, The Netherlands) or saline (0.154 M-NaCl) was infused into the testicular artery (proximal to its involvement with the pampiniform plexus) over a 1-min period, and blood samples were taken from the testicular (0.5 ml) and femoral veins (4 ml) before and at 15, 30, 60 and 120 min after administration of the agonist for testosterone and LH determination. Analgesics were administered during the immediate post-surgical period. After a 2–3-month recovery period, animals were subjected to the identical procedure on the other testis.

Experiment 3: effect of GnRH agonist or antagonist treatment on testosterone production from monkey testicular interstitial cells. Testicular interstitial cells were prepared from 2 sexually mature male monkeys (3.5 and 12 years old). The testes were decapsulated and the remaining tissue was minced into small pieces. The tissue was then incubated in Medium 199 (pH 7.4; GIBCO, Grand Island, NY, USA) containing 1.0 mg collagenase/ml and 100 i.u. penicillin-streptomycin/ml in a shaking water bath at 34°C for 45 min under an atmosphere of 5% CO₂:95% O₂. The cell suspension was then filtered through nylon (Nitex, mesh no. HD3-100, Tetko Inc., Elmsforce, NY, USA) and washed 3 times with Medium 199 without collagenase followed each time with centrifugation (400 g for 5 min at 4°C). Cells were counted with a haemocytometer and viability was assessed with trypan blue. Cells were rediluted to 10⁷ cells per ml in Medium 199 containing 0.1 mM-isobutyl-l-methylxanthine.

In Exp. 3A, cells (10^6) were incubated with 0 , 10^{-9} , 10^{-7} or 10^{-5} M agonist in the absence or presence of hCG (50, 250 or 1000 μ i.u.) for 24 h at 34°C under an atmosphere of 5% CO_2 :95% O_2 in a shaking water bath. In Exp. 3B, the agonist was replaced with 10^{-9} , 10^{-7} and 10^{-5} M antagonist in the presence or absence of hCG. Viability of cells was reassessed at the end of the incubation period. Cells were removed by centrifugation and testosterone concentration was determined in the incubation medium.

Assays. Serum concentrations of LH were measured using the mouse interstitial cell bioassay (Van Damme *et al.*, 1974). A rhesus monkey pituitary gonadotrophin standard (LER 1909-2) provided by the National Institutes of Diabetes and Diseases of the Kidney was used as the reference preparation. The intra- and interassay coefficients of variation were 11.2% and 14.7% respectively. The usable range of the LH bioassay standard curve was from 0.2 to 10 $\mu\text{g/ml}$. Serum testosterone concentrations were measured by radioimmunoassay as described in detail previously (Perachio *et al.*, 1977). The intra- and interassay coefficients of variation were 4.8% and 6.7% respectively. The usable portion of the testosterone standard curve was from 2 to 50 nmol/l.

Statistical analysis. Data from Exps 1 and 2 were evaluated by a one-way analysis of variance (agonist treatment) with repeated measures (blood samples over time) followed by the least significant difference test for multiple comparisons. In Exp. 3, data were evaluated by a two-way analysis of variance (agonist or antagonist treatment in the presence or absence of hCG).

Results

Experiment 1: effect of chronic GnRH agonist administration on the response to hCG

Basal serum concentrations of testosterone were reduced in adult male monkeys in Group 1 and 2 compared to basal values during the control (pretreatment) period (Fig. 1). The peak serum testosterone concentration after an i.v. bolus of hCG was $<40\%$ of the control peak in Group 1 ($P < 0.001$) and $<30\%$ in Group 2 ($P < 0.001$) (Fig. 1). However, when the lower basal values of testosterone in Groups 1 and 2 after agonist treatment were considered, the overall % increase of testosterone in response to hCG after agonist treatment in Groups 1 and 2 ($666\% \pm 117$ and $892\% \pm 436$) did not differ from the control response ($652\% \pm 97$).

Fig. 1. Serum testosterone concentrations before and after hCG administration in adult male monkeys. Ag = agonist. Group 1 ($N = 5$): GnRH agonist, 5 $\mu\text{g/day}$ for 24 weeks and then 10 $\mu\text{g/day}$ for 20 weeks. Group 2 ($N = 5$): GnRH agonist, 25 $\mu\text{g/day}$ for 44 weeks. Animals in Groups 1 and 2 had lower basal values ($P < 0.05$) and reduced peak values of serum testosterone after hCG ($P < 0.001$) than during the pretreatment period.

Experiment 2: influence of acute infusion of GnRH agonist or antagonist into the testicular artery on testosterone concentrations in the testicular and femoral veins

Infusion of 100 or 400 ng GnRH agonist into the testicular artery failed to change testosterone concentrations in the testicular vein or systemic circulation over the next 2 h (Table 1). The infusion of 4 µg GnRH antagonist also failed to influence testicular secretion of testosterone into the testicular vein (Table 1).

Table 1. Effect of infusion (over 1 min) of 100 or 400 ng GnRH agonist or 4 µg antagonist into the testicular artery on testosterone concentrations in the testicular vein and on LH and testosterone concentrations ($\pm$ s.e.m.) in the femoral vein in adult monkeys

	Time (min) after agonist or saline infusion					
	-15	0	15	30	60	120
Testicular vein testosterone (nmol/l)						
Control	198 ± 71	189 ± 52	156 ± 42	170 ± 38	125 ± 34	90 ± 20
Agonist						
100 ng	183 ± 87	237 ± 116	157 ± 74	141 ± 64	253 ± 180	130 ± 44
400 ng	135 ± 41	127 ± 40	234 ± 71	271 ± 70	304 ± 99	122 ± 29
Antagonist						
4 µg	193 ± 124	202 ± 103	155 ± 53	194 ± 44	186 ± 40	135 ± 69
Femoral vein testosterone (nmol/l)						
Control	15 ± 5	21 ± 7	17 ± 6	19 ± 6	16 ± 5	12 ± 4
Agonist						
100 ng	24 ± 9	18 ± 5	15 ± 3	14 ± 3	15 ± 5	11 ± 2
400 ng	14 ± 4	15 ± 4	18 ± 2	17 ± 3	17 ± 3	16 ± 3
Antagonist						
4 µg	11 ± 2	12 ± 2	13 ± 2	14 ± 2	14 ± 2	11 ± 1
Femoral vein LH (µg/ml)						
Control	3.2 ± 0.5	3.3 ± 0.5	2.7 ± 0.4	2.7 ± 0.5	1.9 ± 0.3	1.2 ± 0.2
Agonist						
100 ng	3.5 ± 1.1	3.6 ± 1.2	3.9 ± 1.2	4.1 ± 1.4	3.0 ± 0.9	1.8 ± 0.5
400 ng	4.4 ± 0.8	4.4 ± 0.6	6.8 ± 1.6	6.2 ± 1.9	6.3 ± 1.7	2.9 ± 0.7
Antagonist						
4 µg	3.0 ± 0.8	3.3 ± 0.9	3.5 ± 1.3	2.5 ± 0.8	2.1 ± 0.7	1.6 ± 0.5

Values are mean $\pm$ s.e.m.; N = 6 for each GnRH analogue treated group and each animal served as its own control.

Experiment 3: effect of a GnRH agonist or antagonist on testosterone production from monkey testicular interstitial cells in vitro

As shown in Table 2, incubation of testicular interstitial cells with 10^{-9} , 10^{-7} or 10^{-5} M-GnRH agonist or antagonist failed to alter the amount of testosterone in the incubation medium after 24 h;

testosterone production ranged from 4.4 and 4.7 pmol in Exp. 3A and 1.0 to 1.2 pmol in Exp. 3B. Untreated interstitial cells showed a dose-related increase ($P < 0.001$) in testosterone production when incubated with increasing doses of hCG (50–1000 $\mu\text{i.u.}$) in Exps 3A and 3B. While basal testosterone production was lower in Exp. 3B than in Exp. 3A, the overall response of cells (based on the % increase over basal values) to hCG did not differ between the two experiments (743% and 745%). The response of these cells to hCG was not affected by the presence of the GnRH agonist or antagonist in the incubation medium.

Table 2. Effect of a GnRH agonist and antagonist on testosterone production (pmol/ 10^6 cells/24 h) by isolated testicular interstitial cells from male monkeys

Exp.	Treatment	hCG dose ($\mu\text{i.u.}$)			
		0	50	250	1000
3A	Control	4.7 ± 0.5	7.4 ± 1.0	18.9 ± 1.0	30.0 ± 1.8
	Agonist				
	10^{-9} M	4.6 ± 0.4	7.0 ± 0.5	19.4 ± 0.7	34.2 ± 2.3
	10^{-7} M	4.5 ± 0.4	7.5 ± 0.5	24.5 ± 2.0	32.9 ± 1.4
	10^{-5} M	4.4 ± 0.4	7.5 ± 0.5	21.0 ± 2.8	33.6 ± 2.0
3B	Control	1.1 ± 0.1	2.0 ± 0.1	6.0 ± 0.4	8.2 ± 0.5
	Antagonist				
	10^{-9} M	1.0 ± 0.1	1.7 ± 0.2	3.9 ± 0.2	9.6 ± 0.4
	10^{-7} M	1.2 ± 0.1	1.6 ± 0.1	4.5 ± 0.3	7.4 ± 0.4
	10^{-5} M	1.0 ± 0.1	1.4 ± 0.1	4.7 ± 0.2	8.7 ± 0.7

Values are mean $\pm$ s.e.m. of at least 5 incubates.

Discussion

It has been clearly established that GnRH analogues have direct effects on testicular function in the rat. In hypophysectomized rats, GnRH agonists inhibited steroidogenesis, reduced the responsiveness to LH, and blocked FSH induction of LH receptors on Leydig cells (Bambino *et al.*, 1980; Hsueh *et al.*, 1981). In-vitro incubation of testicular interstitial or purified Leydig cells with GnRH agonist had a biphasic effect on testosterone production. Acutely, GnRH agonists increased testosterone production. However, the effect on testicular steroidogenesis was inhibitory in long-term incubations (24–27 h) (Hsueh *et al.*, 1981; Sharpe & Cooper, 1982a). Binding sites for GnRH were reported on rat Leydig cells (Hsueh & Erickson, 1979; Clayton *et al.*, 1980), and GnRH-like factors have been demonstrated in the rat testis (Sharpe *et al.*, 1981; Swerdloff *et al.*, 1984). These data support the hypothesis that there is a GnRH-like gonadal peptide involved in modulating testicular steroidogenesis in the rat testis.

The results reported here do not demonstrate a direct effect of a GnRH agonist or antagonist on testicular steroidogenesis in the primate testis. Chronic GnRH agonist treatment for 44 weeks inhibited spermatogenesis and reduced basal concentrations of testosterone (Mann *et al.*, 1987),

but failed to alter the testicular testosterone response (based on the % increase of serum testosterone after hCG to the basal testosterone value) of these animals to a low dose of hCG. However, absolute peak concentrations of testosterone after hCG administration were lower in agonist-treated animals than they were during the control period.

Infusion of 100 or 400 ng GnRH agonist or 4 µg antagonist directly into the testicular artery did not significantly alter testosterone concentrations in the testicular vein over the next 2 h. However, serum LH concentrations in animals treated with 400 ng agonist were slightly elevated 15 and 30 min after agonist administration, suggesting that this dose of agonist approaches levels that would stimulate pituitary LH secretion, and therefore, testicular testosterone secretion indirectly.

Finally, in-vitro incubation of testicular interstitial cells from sexually mature male monkeys with the GnRH agonist or antagonist (10^{-9} to 10^{-5} M) for 24 h did not affect either testosterone production or the testosterone response to hCG stimulation. These results do not rule out the possibility that shorter or longer term incubations of the cells with the analogues would have influenced steroidogenesis. However, GnRH agonist treatment of male monkeys for 44 weeks did not alter the testicular testosterone response to a low dose of hCG *in vivo*. Therefore, the in-vivo and in-vitro evidence from the present study does not support the hypothesis that GnRH analogues directly alter testicular steroidogenesis.

The results reported here provide the first direct evidence *in vivo* and *in vitro* that GnRH analogues do not directly alter testicular steroidogenesis in primates. These data are in agreement with a recent report that showed chronic GnRH agonist treatment for 1 year of men with prostatic cancer resulted in an inhibition of the production of Δ^4 and Δ^5 steroids in the testis. This inhibition was overcome by treatment with exogenous hCG (Rajfer *et al.*, 1987). Thus, most evidence does not support the hypothesis that the testis is a site for the inhibitory effects of GnRH agonists on gonadal function. Nevertheless, there is a need to be cautious. Data continue to accumulate suggesting the presence of a GnRH-like protein in the primate gonad (human ovary) (Aten *et al.*, 1987). Moreover, while monkey testicular interstitial cells incubated for 24 h with a GnRH agonist or antagonist failed to show any change in testosterone production in the present study, it is possible that more chronic treatment with the analogues would have altered either basal testosterone secretion or the response of these cells to hCG.

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