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RECEPTOR MEDIATED GONADOTROPIN ACTION IN GONADAL TISSUES: RELATIONSHIP BETWEEN BLOOD CHOLESTEROL LEVELS AND GONADOTROPIN STIMULATED STEROIDOGENESIS IN ISOLATED RAT LEYDIG AND LUTEAL CELLS*

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SUMMARY

The present studies were performed to evaluate the role of steroid precursors and plasma lipoproteins in gonadal tissue steroidogenesis. Leydig cell suspension isolated from rat testes responded to hCG, Bt_2cAMP, 8 Br-cAMP and cholera toxin with an increase in testosterone response. Administration of 4-aminopyrazolo[3,4-d]pyrimidine (4-APP) reduced the plasma cholesterol and testosterone levels in a time and dose dependent manner. This treatment also reduced the steroidogenic capacity of isolated Leydig cells both under basal conditions and in response to trophic hormone. Different doses of 4-APP up to 25 mg/kg BW and up to 4 days of treatment, however, did not modulate cholesterol and cholesterol ester contents of isolated Leydig cells. 4-APP treatment also had no effect on testis weight, phospholipid content, protein synthesis and energy metabolism in isolated Leydig cells. Similarly, administration of 4-APP (12.5 mg/kg) to PMSG-hCG primed rats beginning on day 3, post hCG, drastically reduced the circulating cholesterol and progesterone levels. Injection of the drug also produced an inhibition in *in vitro* luteal cell steroidogenesis and a reduction in cellular cholesterol esters and free cholesterol contents. Addition of LDL or HDL to incubation medium reversed the inhibitory effect of 4-APP on luteal cell steroidogenesis while this inhibition persisted in Leydig cells. Injection of rats with Triton-WR-1339 (mg/kg BW) resulted in a 10-fold increase in plasma cholesterol and a contrasting decrease in testosterone levels. This treatment, however, produced no effect on *in vitro* Leydig cell steroidogenesis or cellular content of cholesterol esters and free cholesterol. It appears that the Leydig and luteal cells process and utilize lipoprotein-delivered cholesterol for steroidogenesis through different mechanism(s). These studies thus demonstrate differential actions and an acute regulatory role of lipoproteins in gonadotropin modulated steroidogenesis in two different gonadal tissue.

INTRODUCTION

The pituitary gonadotropins are the major regulators of steroidogenesis in gonadal tissues [1–5]. In recent years considerable progress has been made in delineating the mode of action of gonadotropins (LH/hCG) in target tissues [1–5]. Initial events in LH/hCG action, including hormone receptor interaction, adenylate cyclase activation and protein kinase stimulation have been extensively studied. However, the role that the steroid precursor, cholesterol, plays in the regulation of steroidogenesis has remained relatively unexplored. The fact that in gonadal tissues, cholesterol is not only utilized for membrane biogenesis but also serves as steroid precursor, suggests that its synthesis and availability could be an important factor in the regulation of steroidogenesis.

Recently, many investigators have demonstrated in the adrenal gland a close correlation between exogenous vs endogenous cholesterol levels and the extent of tissue steroidogenesis [6–14]. In contrast, to date, very limited information is available regarding the role of exogenous vs endogenous cholesterol in the regulation of gonadal tissue steroidogenesis [6, 8, 15–17]. Further, although the steroidogenic tissues, adrenal, testis, ovary, and placenta all secrete steroid, the regulatory role exerted by cholesterol could be entirely different in each of these systems.

In the present studies, attempts were made to analyze systematically the effect of varying the plasma cholesterol levels on testicular cholesterol ester and free cholesterol content and the extent of gonadotropin induced steroidogenesis in isolated Leydig cells. This was accomplished by treating rats with 4-aminopyrazolo[3,4-d]pyrimidine (4-APP) to lower the plasma lipoprotein and cholesterol levels [18, 19] and by injection of Triton WR-1339 to raise the circulating levels of cholesterol [20–22]. Further, attempts were also made to compare the cholesterol require-

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ments and lipoprotein utilization by ovarian (luteal) and testicular (Leydig) cells in gonadotropin induced steroidogenesis. These studies demonstrate that the two gonadal tissues exhibit considerable variation in their cholesterol requirements for gonadotropin induced steroidogenesis.

MATERIALS AND METHODS

Materials

Human chorionic gonadotropin (hCG, CR-119, 11,500 IU/mg) was generously supplied by Dr R. Canfield, Columbia University, New York, through Population Research Branch, National Institutes of Health, Bethesda, MD. 4-Amino-pyrazolo[3,4-d]pyrimidine (4-APP) and 1 methyl-3-isobutylxanthine (MIX) were obtained from Aldrich Chemical Co., Milwaukee, WI. Collagenase (CLS) Type I and deoxyribonuclease I were purchased from Worthington Biochemical Corp., Freehold, NJ. Cholera enterotoxin and pregnant mare's serum gonadotropin (PMSG) were supplied by Schwarz/Mann and Organon OSS, Holland, respectively. 8 bromo-cyclic 3,5 adenosine monophosphate (8 Br-cAMP), Triton WR-1339 (Toxapol) and partially purified human chorionic gonadotropin (hCG) were the products of Sigma Chemical Co., St. Louis, MO. Eagle's Minimum Essential Medium with Earle's salt, Medium 199 and Medium 109 were purchased from Grand Island Biological Co., Grand Island, NY. ^{125}I-choriogonadotropin was prepared according to Catt *et al.*[23].

Isolation of Leydig cells from rat testis

Leydig cells from rat testis were prepared by a combination of procedures of Dufau *et al.*[24] and Moyle and Ramachandran[25]. Testes from 50–60-day old Sprague–Dawley rats were excised free of fat and decapsulated. Groups of four decapsulated testes were added to 50 ml conical polypropylene centrifuge tube containing 10 ml Medium 199, 1 mg/ml bovine serum albumin (BSA) and 0.25 mg/ml collagenase (Type I). The tubes were gassed with O_2/CO_2 (95/5% v/v), tightly capped, placed sideways in Dubonoff metabolic shaker and shaken at 100 cycles/min for 20 min at 37°C. At the end of incubation the tubes were filled to 40 ml mark with Medium 199–0.1% BSA, and gently inverted several times to facilitate the separation of interstitial cells from the tubular mass. The tubules were allowed to settle for 10 min, and the turbid supernatant containing Leydig cells was carefully removed with a plastic disposable syringe attached to Tygon tubing. The sedimented tubular mass was washed once with 50 ml of the same medium and after allowing the tubes to remain for 10 min, the supernatant was carefully removed. Both supernatants were combined and Leydig cells were sedimented at 1000 *g* for 15 min. The sedimented cells were washed two times in Medium 199–0.1% BSA and finally resuspended in a desired volume of the same medium.

Incubation conditions for testosterone production

Rat Leydig cell suspensions (0.1 ml), were incubated in triplicate in a final volume of 1 ml of Medium 199 containing 0.1 mM methyl-3-isobutylxanthine, 1 mg/ml BSA and 20 ng/ml hCG and/or various lipoprotein fractions. After incubation, usually for 3 h at 37°C in an atmosphere of $O_2:CO_2$ (95:5% v/v), the reaction was stopped by placing the sample tubes in a boiling water bath for 3 min. Water (1 ml) and [1,2,6,7-^{3}H(N)]-testosterone (SA 98.8 Ci/mmol, New England Nuclear) (10 μl, 10,000 c.p.m. to monitor testosterone recovery) were added and the samples left in the cold overnight. The next morning the samples were extracted with anhydrous diethyl ether[26] and assayed for testosterone by radioimmunoassay as described by Niswender *et al.*[27].

Binding of [^{125}I]-hCG to testis Leydig cells

Incubation in a final volume of 1 ml Medium 199 contained the testis Leydig cells suspension, 1 mg/ml BSA, [^{125}I]-hCG (200,000 c.p.m., 5–10 ng) and increasing amounts of unlabeled hCG. After incubation at 37°C for 2 h in the presence of $O_2:CO_2$ (95:5 v/v), 2 ml ice cold medium was added and tubes were centrifuged at 100 *g* for 15 min in the cold. The pellets were washed once with 2 ml medium and counted for radioactivity in a gamma counter (Searle). The specific binding was calculated from the difference of total binding to that observed in the presence of 1000-fold excess unlabeled hCG.

Hormonal treatment of female rats

Twenty-two to twenty-four day old female Sprague–Dawley rats (Spartan Research, Inc., Hazlett, MI) were used in the present studies. Highly luteinized ovaries from these rats were obtained following a regimen described by Parlow[28]. Rats were injected subcutaneously with 50 IU of PMSG and followed 56 h later with 25 IU of hCG (Sigma). Day 0 was taken as the day of hCG injection.

Preparation of collagenase-dispersed luteal cells

The collagenase dispersed luteal cells were prepared by the procedure described earlier from this laboratory [29, 30].

Incubation conditions for progesterone measurement

Luteal cells (1.5 to 2 × 10^6) were incubated in 0.4 ml of Medium 109 containing 1 mg/ml BSA, and where required, 10 ng/ml hCG and/or appropriate concentrations of lipoprotein fractions were also added. The incubations were performed at 37°C in a Dubnoff metabolic shaking water bath gassed with $O_2:CO_2$ (95:5 v/v). Following incubation, the reaction was stopped by placing the tubes in a boiling water bath for 3 min. Water (0.5 ml) and [1,2-^{3}H(N)]-progesterone (SA 55 Ci/mmol) (10 μl, 10,000 c.p.m. to monitor progesterone recovery) were added and the samples left in the cold overnight. The next day

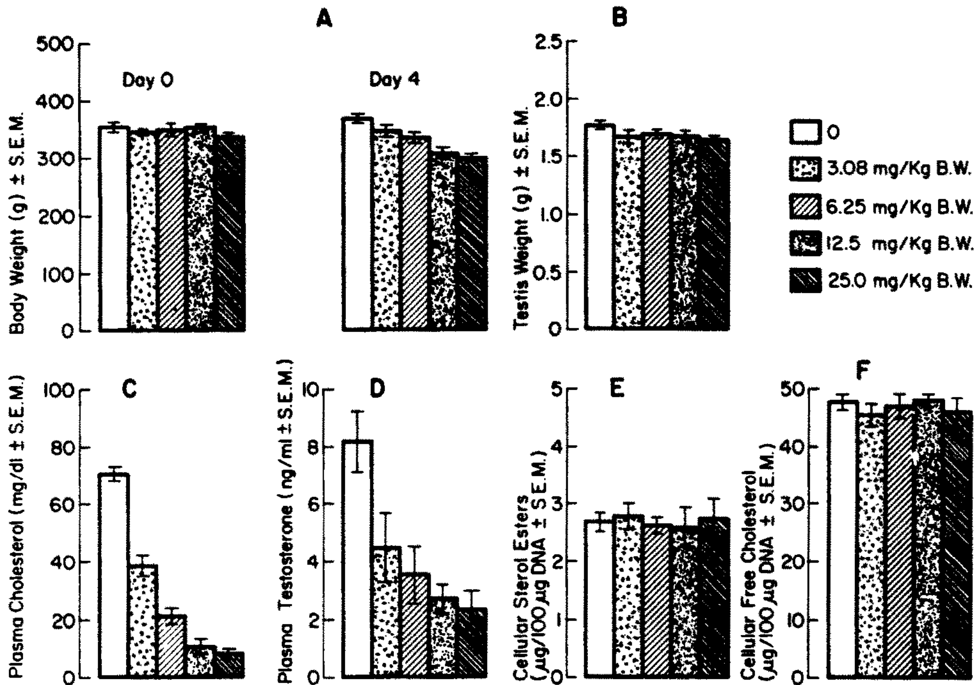

Fig. 1. Effect of injection of different doses of 4-APP on body weight, testis weight, plasma cholesterol, testosterone and free and esterified cholesterol contents in isolated Leydig cell suspension. Groups of four male rats (50–60-day old) were injected with phosphate buffered saline (PBS pH 4.0) or indicated doses of 4-APP every day for 3 days. On the fourth day rats were weighed for body weight and then killed. The pooled testes from each group were processed for the isolation of Leydig cell suspension by collagenase treatment as described in "Materials and Methods." The free cholesterol and cholesterol ester content of Leydig cells were separated by silicic acid/celite column chromatography.

samples were extracted with light petroleum ether and assayed for progesterone by radioimmunoassay as described previously [31, 32].

Analytical procedures

DNA content of the cells was measured by the colorimetric procedure of Burton[33]. Total plasma or serum cholesterol was determined by the procedure of Zak[34]. Cellular free cholesterol and cholesterol esters were separated according to the procedure of Brown *et al.*[35]. Cholesterol esters after saponification in alcoholic KOH and free cholesterol were quantitated by the micromethod of Glick *et al.*[36]. Human plasma LDL (d, 1.019–1.063 g/ml) and HDL (d, 1.09–1.215 g/ml) were isolated by differential ultracentrifugation using KBr for density adjustment [37]. Isolated lipoprotein fractions were dialyzed for 36–48 h against three to four changes of 0.15 M NaCl containing 0.3 mM EDTA. Before use, these fractions were dialyzed against 0.15 M NaCl to remove EDTA. Purity of lipoprotein fractions was checked by agarose gel electrophoresis in 0.05 M barbital buffer pH 6.8 using precast (Bio-Gram A, BioRad) agarose slides. The mass ratio of total cholesterol to protein was 1.4:1 and 1:3.58 for LDL and HDL, respectively. Protein content of lipoproteins was determined by a modification of the procedure of Lowry *et al.*[38] as described by Markwell *et al.*[39].

RESULTS

Effect of different doses of 4-APP injection on plasma cholesterol, testosterone levels and testicular cell cholesterol and sterol ester contents

As shown in Fig. 1, injection of 4-APP at a dose of 12.5 mg/kg BW or 25 mg/kg BW slightly but significantly reduced the body weight. 4-APP treatment, however, produced no significant effect on testis weight. Injection of different doses of 4-APP (3.08, 6.25, 12.5 and 25 mg/kg BW) also reduced the plasma cholesterol and testosterone levels in a dose dependent manner (Fig. 1). In contrast, cholesterol and sterol ester contents of interstitial (Leydig) cells were not affected by 4-APP treatment.

Effect of increasing doses of 4-APP injection on in vitro *steroidogenesis in isolated Leydig cell suspension*

Injection of low dose of 4-APP (3.08 mg/kg BW) to rats caused a significant inhibition in basal as well as hCG, 8 Br-cAMP and cholera toxin stimulated steroid production in isolated Leydig cells (Table 1). Increasing the 4-APP dose to 6.25 mg/kg BW further reduced the steroidogenic capacity of isolated Leydig cells both under basal condition and in response to various stimulators. Maximum inhibition of basal and hormone-stimulated *in vitro* steroidogenesis was observed when rats were pretreated with 4-APP at a

Table 1. Effect of different doses of 4-APP injection on hCG, cholera toxin and 8 Br-cAMP stimulated steroidogenesis in isolated Leydig cells

4-APP (mg/kg BW)	Testosterone (pg μg DNA^{-1} ± SEM) Basal	hCG (20 ng/ml)	8 Br-cAMP (1.5 mM)	Cholera toxin (1 μg/ml)
0	181 ± 10	1070 ± 30	1100 ± 31	430 ± 38
3.08	152 ± 6	787 ± 60	730 ± 6	320 ± 9
6.25	108 ± 10	584 ± 43	710 ± 31	340 ± 15
12.50	77 ± 7	550 ± 35	620 ± 37	289 ± 29
25	95 ± 10	570 ± 64	640 ± 33	280 ± 18

Groups of four rats were injected with indicated doses of 4-APP every 4 h for 3 days and rats were killed on day 4. The testes were treated with collagenase for the isolation of crude Leydig cell suspension. The isolated cells were incubated for 3 h with hCG, 8 Br-cAMP or cholera toxin as described in "Materials and Methods." After the incubation, samples (medium + cells) were extracted and assayed for testosterone by radioimmunoassay. The results are mean of three different experiments.

dose of 12.5 mg/kg BW. In subsequent experiments rats were injected with this dose of 4-APP (12.5 mg/kg BW) to reduce the circulating levels of cholesterol and to monitor the effect on *in vitro* steroidogenesis under variable physiological conditions.

Time dependent effect of 4-APP treatment

Results presented in Fig. 2 and Table 2 demonstrate time-dependent effect of 4-APP (12.5 mg/kg BW) injection on plasma cholesterol, testosterone and Leydig cell steroidogenesis and sterol contents. Drug treatment resulted in a 50% decrease in plasma cholesterol levels after 1 day of injection and by 2 days the cholesterol levels were reduced to 25% of control values. Cholesterol was maximally reduced (85%) between days 3 and 4. Similarly, 4-APP treatment decreased serum testosterone levels approximately 40 and 50% on day 1 and day 2 after the injection. Maximum decrease in testosterone levels was observed on days 3 and 4. In contrast, cellular cholesterol and cholesterol ester contents remained unchanged following 4-APP injection (Fig. 2). Leydig cell suspension prepared from 4-APP injected rats exhibited ap-

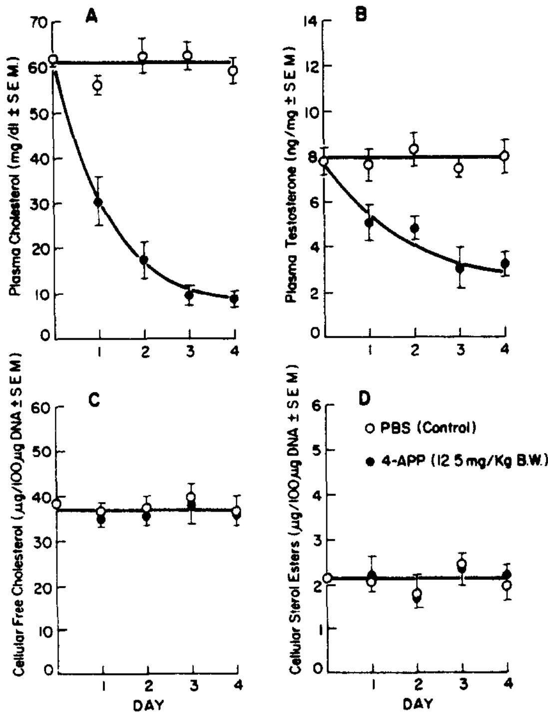

Fig. 2. Effect of varying 4-APP injection time on plasma cholesterol, plasma testosterone, Leydig cell free cholesterol and cholesterol ester contents. Groups (6) of 50–60-day old rats were injected with PBS or 4-APP (12.5 mg/kg BW) every day for 4 days. After 24 h following last injection, the rats were killed and plasma cholesterol and testosterone measurements were made using plasma of collected blood. Leydig cell isolation and separation of cellular cholesterol and cholesterol ester content were carried out as described in "Materials and Methods."

Table 2. Time-dependent effect of 4-APP injection on *in vitro* steroidogenesis in isolated Leydig cells

	Testosterone (pg·μg DNA^{-1} ± SEM) Basal	hCG (20 ng/ml)
Day 1		
PBS	175 ± 7	831 ± 45
4-APP	192 ± 39	784 ± 41
Day 2		
PBS	197 ± 11	872 ± 53
4-APP	99 ± 4	456 ± 22
Day 3		
PBS	204 ± 39	1083 ± 64
4-APP	120 ± 25	559 ± 13
Day 4		
PBS	185 ± 21	1400 ± 19
4-APP	87 ± 32	730 ± 59

Groups of rats were injected with saline or 4-APP (12.5 mg/kg BW) every 24 h for up to 4 days. Rats were killed on day 1, 2, 3 and 4. The testes were removed, decapsulated, and interstitial (Leydig cell) suspensions were prepared by collagenase digestion. Aliquots of isolated cells were incubated with or without hCG (20 ng/ml) and *in vitro* testosterone production was followed for 3 h. The results are mean of three different experiments.

Table 3. Time-dependent effect of 4-APP injection on [^{125}I]-hCG binding to isolated Leydig cell suspension

4-APP (12.5 mg/kg BW) Treatment	[^{125}I]-hCG Binding* (% of control)
Day 1	73.3
Day 2	64.9
Day 3	60.8
Day 4	53.9

Experimental details were the same described in "Materials and Methods."

* Results are mean of three separate experiments.

proximately 50% inhibition in basal and hCG stimulated testosterone production compared to cells from saline injected rats. The inhibitory effect of 4-APP was first observed after two days of drug injection, and the effect persisted and remained unchanged after 3 and 4 days of injection (Table 2). Surprisingly, 4-APP treatment also produced a significant inhibition in gonadotropin (^{125}I-hCG) binding to Leydig cells (Table 3).

Effect of lipoprotein additions on in vitro *steroidogenesis by Leydig and luteal cells obtained from 4-APP injected rats*

Results presented in Fig. 3 demonstrate the effect of 4-APP injection on plasma testosterone and progesterone levels in male and pseudopregnant female rats. As shown in Fig. 1 and also in Fig. 3, injection of 4-APP (12.5 mg/kg BW) for 3 days significantly decreased the plasma cholesterol and testosterone levels in male rats. Similarly in pseudopregnant female rats, 4-APP injection drastically reduced the plasma progesterone and cholesterol levels. The *in vivo* treatment of pseudopregnant rats with 4-APP resulted in significant inhibition of basal and hCG stimulated steroidogenesis in isolated luteal cells (Fig. 3). Addition of h-LDL (750 μg protein/ml) or h-HDL (700 μg protein/ml) to luteal cells isolated from PBS injected rats caused about 2-fold increase in basal progesterone production and both lipoproteins greatly potentiated the gonadotropin-stimulated steroid production. Interestingly, addition of h-LDL to luteal cells from 4-APP injected rats completely reversed the inhibitory action of the drug on hCG-stimulated progesterone accumulation. h-LDL also partially reversed the inhibition of basal steroid production seen in luteal cells of 4-APP injected rats. h-HDL, although producing identical effects, was slightly less effective than h-LDL. In contrast, neither h-LDL nor h-HDL was able to overcome the inhibitory effect of 4-APP seen in Leydig cell steroidogenesis (Fig. 3). Further, compared to its lack of effect on Leydig cell cholesterol and cholesterol ester contents (Fig. 1 and 2), injection of the drug 4-APP to pseudopregnant rats

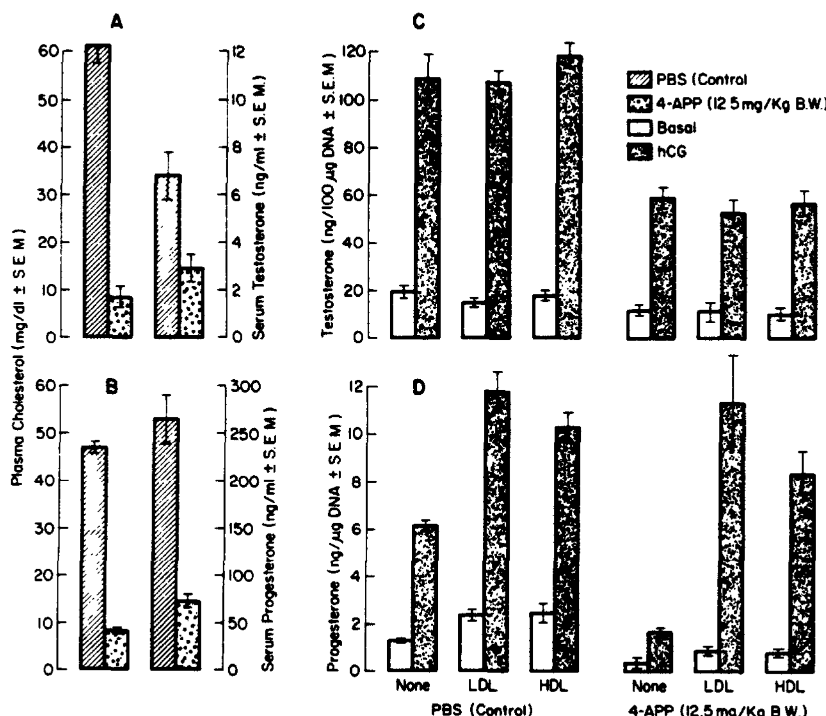

Fig. 3. Effect of 4-APP injection to male rats or pseudopregnant rats on steroidogenesis in isolated cells in response to gonadotropins and lipoproteins. Pseudopregnancy in 23-day old rats was induced by injection of 50 IU PMSG followed 56 h later with a single injection of 25 IU hCG (day 0). On day 3 PBS or 4-APP (12.5 mg/kg BW) were injected every day for 3 days. Similarly, male rats were injected with PBS or 4-APP every day for 3 days. Twenty-four hours after last injection, Leydig cells and luteal cells from testes and luteinized ovaries, respectively, were isolated and incubated with lipoproteins and/or hCG for steroid production. Testosterone and progesterone on extracted samples were assayed by specific radioimmunoassays. A and C refer to males and B and D to females.

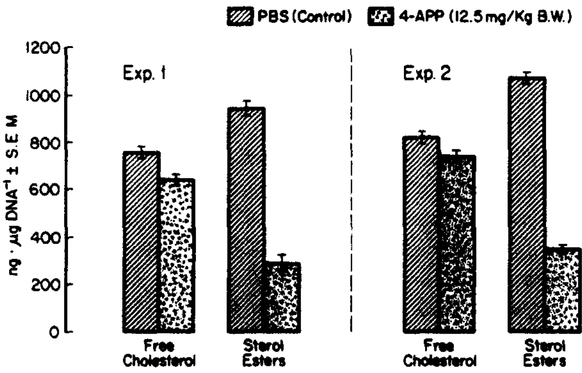

Fig. 4. Effect of 4-APP injection to pseudopregnant rats on cholesterol and cholesterol ester contents in luteal cells. Groups (4) of rats were injected with PBS or 4-APP (12.5 mg/kg BW) as described in Fig. 3. Cholesterol and cholesterol ester from luteal cells were separated by silicic acid/celite column chromatography.

caused a 60–70% decrease in cholesterol ester content and a slight but significant reduction in free cholesterol levels in luteal cells (Fig. 4).

Effect of Triton WR-1339 injection

Further experiments were performed to monitor the effect of raising plasma cholesterol levels on Leydig cell steroidogenesis. This was accomplished by treating rats with Triton WR-1339, a pharmacological agent known to increase circulating cholesterol levels [20–22]. Results presented in Fig. 5 demonstrate that treatment of rats with Triton WR-1339 caused a 6-fold increase in plasma cholesterol levels after one day of detergent injection. The maximum increase in plasma cholesterol (10–11-fold) levels was observed after 2 days of treatment and then remained elevated up to 3 days. In contrast, Triton WR-1339 injection caused a maximum reduction in plasma

Table 4. Effect of Triton-Wr-1339 injection on [^{125}I]-hCG binding to isolated Leydig cell suspension

Days after Triton Wr-1339 injection	[^{125}I]-hCG Binding (% of control)
Day 1	89
Day 2	72
Day 3	94

Groups of four rats were injected with normal saline or Triton WR-1339 (1 g/kg BW) every 12 h for 1, 2 and 3 days and killed 12 h after last injection. [^{125}I]-hCG binding to isolated Leydig cell suspension was carried out as described in "Materials and Methods." Results are mean of four different experiments.

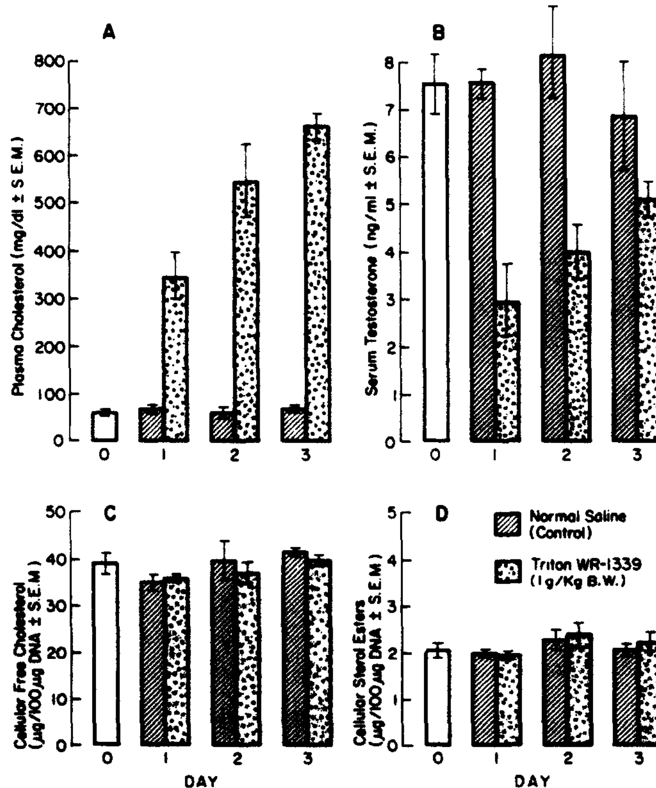

Fig. 5. Effect of varying Triton WR-1339 injection time on plasma cholesterol and testosterone levels and Leydig cell cholesterol and sterol ester contents. Groups of four rats (50–60-day old rats) were injected with normal saline or Triton-Wr-1339 (lg/kg BW) every 12 h for 1, 2 or 3 days. After 12 h following last injection, plasma cholesterol, testosterone and Leydig cell free cholesterol and sterol esters were measured as described in "Materials and Methods."

Table 5. Effect of Triton WR-1339 injection on *in vitro* steroidogenesis in isolated Leydig cell suspension

Treatment (days)	Testosterone (pg · μg DNA^{-1} ± SEM) Basal	hCG (20 ng/ml)	h-LDL (750 μg protein/ml)	h-HDL (700 μg protein/ml)	h-LDL + hCG	h-HDL + hCG
Day1						
Saline	172 ± 9	1400 ± 84	220 ± 23	239 ± 16	1310 ± 22	1290 ± 33
Triton	188 ± 12	1540 ± 160	220 ± 17	181 ± 16	1270 ± 180	1310 ± 210
Day 2						
Saline	176 ± 17	1389 ± 104	185 ± 10	173 ± 17	1435 ± 65	1175 ± 210
Triton	163 ± 5	1404 ± 170	201 ± 40	187 ± 43	1395 ± 33	1371 ± 81
Day 3						
Saline	176 ± 3	1300 ± 66	193 ± 13	165 ± 7	1350 ± 49	1480 ± 600
Triton	187 ± 9	1346 ± 220	1737 ± 8	205 ± 13	1070 ± 600	1240 ± 87

Groups of six rats were injected with saline or Triton WR-1339 (1 g/kg BW) every 12 h for 1, 2 or 3 days. Twenty-four hours after the last injection, the rats were killed and Leydig cells suspension were prepared by collagenase treatment as described in the "Materials and Methods". Aliquots of cells were incubated with indicated concentrations of various substnaces. After incubation for 3 h, samples (Medium + cells) were extracted and assayed for testosterone by radioimmunoassay.

testosterone levels after day 1. Although plasma testosterone levels slightly increased on day 2 and day 3, the drug-mediated inhibition persisted in treated groups during the entire period of experiment. Detergent treatment, however, produced no significant effect on Leydig cell cholesterol and cholesterol ester contents (Fig. 5).

In vivo treatment of rats with detergent also caused a slight decrease in [^{125}I]-hCG binding activity to isolated Leydig cell suspension (Table 4). Similarly, Triton treatment was without any discernable effect on steroidogenesis in isolated Leydig cell suspension (Table 5). Further addition of h-LDL or h-HDL failed to modulate steroidogenesis in Leydig cell suspension isolated from testes of rats pretreated with normal saline or Triton Wr-1339.

DISCUSSION

In the present studies, attempts were made to explore the role of circulating plasma cholesterol on gonadal steroidogenesis with special emphasis on Leydig cell steroidogenesis. The drug 4-APP, an adenine analog, has previously been reported to block secretion of all major classes of plasma lipoproteins and subsequently lead to a decrease in plasma cholesterol levels [6, 18, 19, 40]. This drug was therefore selected to lower circulating plasma cholesterol levels and to monitor Leydig cell steroidogenesis *in vitro* and *in vivo* under identical experimental conditions.

Subcutaneous administration of 4-APP markedly reduced the circulating levels of cholesterol in male rats in a dose and time-dependent manner. The drug-induced decrease in plasma cholesterol was closely associated with a parallel decrease in plasma testosterone levels. Further, Leydig cell suspensions isolated from testes of 4-APP injected rats exhibited a decreased steroidogenic activity both under basal conditions and also in response to trophic hormone. Although maximum testosterone production was considerably decreased in Leydig cells of 4-APP injected rats, these cells still retained the capability to respond to gonadotropins. These observations thus support the notion that the various enzymes and steps involved in testosterone synthesis are probably not affected by this treatment. Surprisingly, 4-APP treatment also slightly decreased the gonadotropin binding activity of Leydig cells. However, inhibition of gonadotropin-receptor–adenylate cyclase system by 4-APP cannot be ruled as its sole point of action, since Leydig cells also showed decreased steroidogenesis in response to Bt_2cAMP and 8 Br-cAMP, agents which bypass initial steps of gonadotropin action [1–5]. Further, since in Leydig cells only about 10% receptors need to be occupied for maximum expression of steroidogensis [41], the 60–70% receptor concentration retained by cells from 4-APP injected rats should be sufficient to carry on maximum rate of testosterone synthesis. In contrast to the inhibition of steroidogenesis seen, 4-APP treatment failed to modulate the cholesterol esters and free cholesterol contents of isolated Leydig cell suspension. These results are in agreement with those reported previously by Andersen and Dietschy[8] for intact rat testis. In addition, our observations that 4-APP did not affect testes weight, phospholipid, protein synthesis and energy metabolism of Leydig cells (Azhar and Menon, unpublished observation) further rule out the possibility that the inhibitory effect of drug on steroidogenesis was due to general cytotoxic action.

Similarly Gerson *et al.*[42] found no change in testicular cholesterol contents following treatment of rats with β-sitosterol although this plant sterol under identical experimental conditions produced a signifi-

cant drop in plasma cholesterol levels. Further studies by Morris and Chaikoff[43], based upon the [^{14}C]-acetate incorporation into cholesterol concluded that most of the testicular cholesterol is synthesized *de novo*. Although in the present studies 4-APP treatment failed to modulate testicular cholesterol and sterol ester contents, it drastically reduced the steroidogenic capacity of isolated Leydig cells. Thus, the present studies as well as *in vivo* studies reported by Andersen and Dietschy[8] support a regulatory role of plasma lipoproteins in testicular steroidogenesis.

Further studies were performed to see if the inhibitory action of 4-APP could be reversed by the addition of various lipoproteins. The *in vivo* studies reported recently suggest that testes preferentially utilize HDL for steroid synthesis [8], while in adrenals cholesterol for steroidogenesis can be obtained from HDL [6, 8] and/or LDL [10]. The demonstration of specific receptors for HDL on testicular interstitial cell plasma membrane further suggests that Leydig cells are probably equipped with the necessary machinery to bind and metabolize the lipoproteins [44]. However, all our efforts to reverse the inhibitory action of 4-APP by *in vitro* lipoprotein addition were unsuccessful. Further, while 4-APP treatment leads to stimulation of *de novo* synthesis of cholesterol in testis [8], the amount produced probably is not sufficient to support steroidogenesis maximally. Contrary to these observations, in luteal cells the inhibitory action of 4-APP on steroidogenesis was reversed by the addition of LDL or HDL. In addition, 4-APP treatment drastically reduced the cholesterol ester content in luteal cells while this treatment had no effect on Leydig cell cholesterol and cholesterol ester content. Thus, results of the present study as well as those reported by Schuler *et al.*[17] and Schreiber *et al.*[45] for granulosa cells, suggest that cells of ovarian origin can effectively utilize LDL and/or HDL delivered cholesterol for steroidogenesis, whereas in short-term incubated Leydig cells, lipoproteins probably do not support steroidogenesis. However, the possibility that during isolation Leydig cells lose their cell surface components necessary for lipoprotein binding and/or processing cannot be ruled out at present.

Injection of rats with Triton WR-1339 produced approximately 10-fold increase in plasma cholesterol, an effect observed earlier by Goldfarb[21] and Anderson and Dietschy[46]. The detergent mediated increase in plasma cholesterol levels is due to its capacity to block lipid clearance [47–50]. Although feeding high cholesterol diet could also lead to increase in serum cholesterol levels [43, 51] it is not clear if dietary ingested cholesterol totally gets incorporated into lipoproteins. In addition, Triton WR-1339 mediated increase in plasma cholesterol can be achieved within 2–3 days, compared to 6–12 days required for diet-induced hypercholesterolemia [43, 51]. Finally, diet-induced hypercholesterolemia may also affect lipoprotein secretion [52]. In contrast, to increase in plasma cholesterol levels, detergent injection caused a significant inhibition in plasma testosterone levels. However, Triton WR-1339 injection did not affect the steroidogenic capacity of isolated Leydig cell suspensions either under basal conditions or in response to trophic hormone. Since we could not detect any change in cholesterol and cholesterol ester contents of Leydig cells following Triton WR-1339 injection, the inhibition of plasma testosterone could be due to a secondary effect. It is quite possible that Triton WR-1339 treatment somehow interfered with the release of LH from the pituitary and a subsequent inhibition in the *in vivo* testosterone production and release into the plasma.

In summary, the present studies demonstrate that reduction in plasma cholesterol levels by 4-APP treatment leads to a significant inhibition of *in vitro* steroidogenic capacity of gonadal cells. This inhibition of steroidogenesis in luteal cells was effectively reversed by *in vitro* addition of LDL or HDL. In contrast, coincubation of Leydig cells with LDL or HDL did not reverse the inhibitory action of 4-APP on testosterone synthesis. It appears, therefore, that Leydig and luteal cells probably process and utilize lipoprotein delivered cholesterol for steroidogenesis through different mechanisms.

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