Receptor-mediated Gonadotropin Action in the Ovary

RAT LUTEAL CELLS PREFERENTIALLY UTILIZE AND ARE ACUTELY DEPENDENT UPON THE PLASMA LIPOPROTEIN-SUPPLIED STEROLS IN GONADOTROPIN-STIMULATED STEROID PRODUCTION

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These studies were aimed at evaluating the role of steroid precursor and circulating plasma lipoproteins in gonadotropin induced steroidogenesis. Rat luteal cells responded to luteinizing hormone, human chorionic gonadotropin, isotroperenol, and cholera toxin with an increase in progesterone and cyclic AMP responses. Administration of 4-aminopyrazolo(3,4-d)pyrimidine (4-APP) (12.5 mg/kg body weight) for 3 consecutive days reduced the circulating cholesterol level from 47.32 ± 1.12 mg/dl to 8.16 ± 0.43 mg/dl and plasma progesterone from 280 ± 26 ng/ml to 96 ± 6 ng/ml. Cellular steroid ester content was reduced to 25% following 4-APP injection. Injection of drug also produced a small but significant decrease in luteal cell free cholesterol. Treatment of rats with 4-APP also reduced the in vitro basal and hormone-stimulated progesterone production. This treatment showed no effect on luteal cell 125I-human chorionic gonadotropin binding or gonadotropin and cholera toxin stimulated cyclic AMP synthesis. Exposure of luteal cells isolated from control and 4-APP injected groups to homologous or heterologous lipoproteins (low and high density lipoproteins) produced significant stimulation in steroidogenesis, both under basal conditions and in response to gonadotropins. Similarly, luteal cells isolated from 4-APP-injected rats incorporated [3H]cholesterol from [3H]cholesterol into progesterone at a much faster rate compared to control cells, and this effect was further enhanced in response to human chorionic gonadotropin. Injection of rats with Triton WR 1339 (1 g/kg body weight) resulted in a 10-fold increase in circulating cholesterol level. This treatment, however, produced a significant decrease in plasma progesterone and cyclic AMP and progesterone responses in isolated cells. The inhibitory effect of Triton injection on steroidogenesis could not be reversed by lipoprotein addition. These studies suggest that rat luteal cells are acutely dependent upon exogenous lipoprotein cholesterol for the maximum expression of steroidogenic response.

The steroidogenic tissues including ovary are unique in the sense that they require cholesterol not only for membrane biogenesis but also as a precursor for steroid hormone synthesis. The ovarian tissues can obtain cholesterol needed for steroidogenesis either by intracellular de novo synthesis from acetate (1-4), by hydrolysis of stored cholesterol esters (1-3), or from lipoprotein-delivered cholesterol (3, 5). Although all these pathways are potentially important and could supply precursor needed for steroidogenesis, the principal source by which ovarian cells meet their cholesterol requirement under normal physiological conditions is not clearly understood. The slow progress in this direction is a reflection of the fact that it is not always possible to observe precursor requirement under normal physiological situations.

Recently, studies have been reported which suggest that lowering of blood cholesterol by 4-aminopyrazolo(3,4-d)-pyrimidine leads to inhibition of in vivo secretion of steroid by all three (ovary, testis, and adrenal) steroidogenic tissues (6-9). These studies are compatible with the idea that steroidogenic tissues could take up and utilize blood cholesterol. However, little information is available regarding the role of cholesterol and its utilization in isolated cell system. Studies reported by Brecher and Hyun (10) suggest that adrenal cells isolated from 4-APP-treated rats secrete less corticosterone in response to adrenocorticotropic hormone. The dependence of adrenocortical cells (11, 12) granulosa cells (9), and choriocarcinoma cells (13) on exogenous cholesterol source has been established following prolonged incubation of these cells in lipoprotein-deficient medium. While this manuscript was in review, Gwynne and Hess (14) have reported that adrenal cells isolated from 4-APP-injected rats respond to HDL with an increase in corticosterone production.

The purpose of this study was to determine the regulation of steroidogenesis in the ovarian cells by exogenously supplied lipoproteins and gonadotropins without prolonged culture in cholesterol-deficient medium. We also determined the effect of lowering and increasing blood cholesterol with 4-APP (15-18) and Triton WR-1339 (19-22), respectively, on subsequent steroidogenesis in isolated cells, and reversal of their effects by lipoproteins in vitro. Our results show that the ovarian cells are unique among various steroid-producing cells (11-13) in their ability to take up and metabolize lipoprotein cholesterol upon isolation from tissue with no apparent need for culturing for prolonged periods of time.

EXPERIMENTAL PROCEDURES

Materials

Purified human choriogonadotropin (CR-119, 11,500 I.U./mg) was generously supplied by Dr. R. Canfield, Columbia University, New York.

The abbreviations used are: 4-APP, 4-aminopyrazolo(3,4-d)-pyrimidine; HDL, high density lipoprotein; PMSG, pregnant mare's serum gonadotropin; hCG, human chorionic gonadotropin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LH, luteinizing hormone; LDL, low density lipoprotein; VLDL, very low density lipoprotein; Br-AMP, dibutyryl cAMP; 8-Br-cAMP, 8-bromo-cAMP.
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York, through Population Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. Bovine luteinizing hormone (NIH-LH89) was a gift from Hormone Distribution Program, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 1,25-Dihydroxycholecalciferol (vitamin D3), 25-hydroxycholecalciferol (vitamin D3), progesterone, 17beta-estradiol, testosterone, cortisol, Dexamethasone, 17a-methyl testosterone, and gonadotropin were purchased from Grand Island Biological Co., Grand Island, NY, and Organon, Oss, Holland, respectively. [\(^{3}H\)-Choriogonadotropin (CG) was obtained following a regimen described by Parlow (23). All other reagents were used as of analytical reagent grade.

Methods

Animals and Hormonal Treatment—Twenty-two- to 24-day-old female Sprague-Dawley rats (Spartan Research Inc., Hazlet, NJ) were employed in the present studies. Highly luteinized ovaries from these rats were isolated by a slight modification of the procedure described by Burton (24). Rats were injected subcutaneously with 50 I. U. of PMSG and followed 56 h later with 25 I. U. of hCG. Day 0 was taken as the day of hCG injection. Where required, rats were injected with 4-APP (intraperitonially, 12.5 mg/kg body weight) in PBS, pH 3.0, between 09:00 and 11:00 h of day 12. Control animals were killed on day 12 h after the last Triton WR-1339 injection. Control animals received the vehicle.

Preparation of Collagenase-Dispersed Luteal Cells—Collagenase-dispersed luteal cells were prepared by a slight modification (25, 26) of the procedure described earlier from this laboratory (27-29). DNA content of the cells was measured by the colorimetric procedure of Burton (30).

Incubation Conditions for Progesterone Measurements—Unless otherwise stated, aliquots of luteal cells (1.5 to 2.0 x 10\(^6\) cells in 0.1 ml) were transferred into plastic tubes (12 x 75 mm) containing 0.3 ml of Medium 109, 0.1% BSA, and, where required, appropriate concentrations of hCG, LH, cholesterol, testosterone, 8-Br-cAMP, or various lipo-protein fractions were also added. The incubations were carried out at 37 °C in a Dubnoff metabolic shaking incubator gassed with O\(_2\)/CO\(_2\) (95:5, v/v) for 4 h in an atmosphere of O\(_2)/CO\(_2\) (95:5, v/v), 1 ml of phosphate-buffered saline was added to each tube and transferred to boiling water bath for 3 min. The samples were extracted with light petroleum ether and assayed for progesterone by radioimmunoassay (RIA) as described earlier (31).

Determination of \(^{3}H\)hCG Binding to Luteal Cells—The binding of \(^{3}H\)hCG was determined by a minor modification of a procedure of Clark and Menon (28) as described under Table II. Briefly, cells (2 x 10\(^6\) cells) were incubated with \(^{3}H\)hCG (approximately 150,000 cpm, 25 to 30,000 cpm/ng) in 0.4 ml of Medium 109/0.1% BSA and in the presence or absence of 10 ng/ml of unlabeled hCG. After incubation at 37 °C for 3 h, the tubes were centrifuged at 500 X g for 30 min. The supernatant was withdrawn and the radioactivity of washed pellet was determined in a automatic gamma counter. The specific binding was calculated from the difference of total binding, which observed in the presence of a 1000-fold excess of unlabeled hCG.

Isolation of Lipopolysaccharides—Human VLDL (d < 1.006 g/ml), LDL (d, 1.019-1.063 g/ml), HDL (d, 1.063-1.215 g/ml), and rat serum VLDL (d < 1.006 g/ml), LDL (d, 1.017-1.215 g/ml), and HDL (d, 1.07-1.215 g/ml) were fractionated by differential ultracentrifugation using KBr for density adjustment (33). Human lipoprotein fractions were isolated from blood plasma collected in 0.1% EDTA from healthy donors. Rat lipoprotein fractions were isolated from pooled sera. Purity of the lipoprotein fractions was checked by cellulose acetate (34) and agarose gel electrophoresis in 0.05 M barbital buffer, pH 6.8, using precast (Bio-Gram A, Bio-Rad) agarose slides (35). The mass ratio of total cholesterol to protein was 1:1.4 and 0.28:1 for human LDL and human HDL, respectively. Similarly, rat LDL and rat HDL had a mass ratio of 1:1.3 and 1.6:1, respectively. Human LDL radiolabeled with [\(^{3}H\]cholesterol/linoleate was prepared according to the procedure of Krieger et al. (36).

Miscellaneous Procedures—Total plasma or serum cholesterol was determined by the procedure of Zak (37). For lipid analysis, lipids were extracted from the lipoprotein fraction in chloroform/methanol (2:1, v/v) (38). Lipoprotein phospholipid phosphorus was determined by the procedure described previously (39, 40). The relative contribution of individual phospholipids to total phospholipids and free cholesterol and cholesterol ester to total cholesterol was determined after the separation of individual lipid by thin layer chromatography (41).

The separation of neutral lipids, including cholesterol and cholesterol esters, was accomplished by single dimension, two-step development thin layer chromatography (41), using isopropyl ether/acetone (96:4, v/v) as the first developing solvent and petroleum ether/diethyl ether/acidic acid (70:30:2, v/v) as the second solvent. Cholesterol and cholesterol esters were eluted successively with diethyl ether (20 ml); chloroform/methanol (10 ml); aqueous buffer (10 ml); and, followed by chloroform/methanol (10 ml) (2:1, v/v). Cholesterol esters after saponification in alcoholic KO\(_2\) were quantitated by the colorimetric procedure of Zak (37) and, if necessary, by the micromethod of Glick et al. (42).

Protein content of lipoproteins was determined by a modification of the procedure of Lowry et al. (43) as described by Markwell et al. (44).

Incorporation of \(^{3}H\)Cholesterol from \(^{3}H\]Cholesterol Linolate—Human LDL (0.100 g/ml, 1000 cpn/ml), 20 a-hydroxypregn-4-ene-3,1-one (50 μg), and progesterone (20 μg) were added as carriers, and steroids were extracted two times with 5 volumes of light petroleum ether and two times with 5 volumes of ethyl acetate. The petroleum ether and ethyl acetate fractions were dried separately under nitrogen. The residues from two fractions were dissolved in chloroform/methanol (2:1), combined, and redissolved under a stream of nitrogen. The resulting redissolved lipid was transferred to a solvent system of isopropyl ether/diethyl ether (90:10, v/v) (45) three times in a system of isopropyl ether/diethyl ether (90:10, v/v) (45) and, if necessary, by the micromethod of Glick et al. (42).

Steroids in methanolic phase was separated by thin layer chromatography using Silica G as glass plates. The plates were developed either one time in a solvent system of chloroform/diethyl ether (9:1, v/v) (46) three times in a solvent system of isopropyl ether/diethyl ether (9:1, v/v) (46) or, three times in a solvent system of isopropyl ether/petroleum ether/acidic acid (70:20:1, v/v) (47). Authentic standards, cholesteryl oleate, cholesterol, progesterone, 20 a-hydroxypregn-4-ene-3-one, and pregnenolone were always run simultaneously.

Individual steroids were eluted in chloroform/methanol (2:1) and counted for radioactivity determinations.

**RESULTS**

Luteal Cell Steroidogenesis in Response to Gonadotropins, Cholera Enterotoxin, and Cyclic Nucleotide Derivatives—The results presented in Fig. 1 (A-D) show progesterone production by luteal cells in response to various stimulators. Sensitivity of the luteal cells to physiological concentrations of hCG is shown in Fig. 1A. Concentrations of hCG as low as 0.01 ng/ml significantly stimulated progesterone production and this effect was enhanced with increasing concentrations of hCG reaching a maximum at 0.1 ng/ml of hormone. The concentration of hormone required for half-maximal stimulation (ED\(_{50}\)) was 0.02 ng/ml. The dose-dependent stimulatory effect of bovine LH (lutropin) on steroid synthesis by luteal cells is shown in Fig. 1B. The ED\(_{50}\) of LH was around 0.5 ng/ml.
CHORIOGONADOTROPIN (ng/ml)

FIG. 1. Effect of increasing concentrations of hCG, LH, cholera toxin, and 8-Br-cAMP in progesterone production in luteal cells. The luteal cells were isolated from luteinized ovaries (6 days post-hCG) by collagenase digestion procedure. Aliquots of isolated cells (2 x 10^6) were incubated with indicated concentrations of hCG, LH, cholera toxin, and 8-Br-cAMP. CHORIOGONADOTROPIN (ng/ml)

Similarly, cholera toxin, a universal stimulator of adenylate cyclase in various mammalian systems (44-46) also effectively enhanced steroidogenesis in a dose-related manner, with ED₅₀ equivalent of 4 ng/ml (Fig. 1C). Results presented in Fig. 1D show the stimulatory action of 8-Br-cAMP on luteal cell steroid production. These cells were also very responsive to 8-Br-cAMP and maximum stimulatory effect of the nucleotide was at a concentration of 0.5-1 mM with ED₅₀ 0.1 mM. These results present the stimulatory conditions of luteal cell steroidogenesis.

The results presented in Fig. 2 show time-dependent increases in progesterone production under basal conditions and in response to hCG (10 ng/ml). In the presence of hormone, progesterone synthesis increased almost linearly with increase in incubation time reaching a maximum at 120 min. Basal production of progesterone also increased up to 60-90 min.

Effect of Various Hormones on cAMP Response in Luteal Cells—The results presented in Fig. 3 compare the ability of hCG, LH (NIH-LH-B9), and cholera toxin to stimulate cAMP accumulation in luteal cells. LH and hCG produced about 8- to 10-fold stimulation of cAMP accumulation. Cholera toxin also effectively enhanced cAMP synthesis, although maximum effect was of a lower magnitude compared to that observed with gonadotropin. The concentration of hCG, LH, and cholera toxin required for half-maximal stimulation of cAMP production (ED₅₀) were found to be 5.5, 20, and 10 ng/ml, respectively. Addition of 1-methyl-3-isobutylxanthine (0.5 mM), an inhibitor of cyclic nucleotide phosphodiesterase, greatly potentiated the stimulatory effect of tropic hormone and cholera toxin. Other hormones, including follicle-stimulating hormones, thyroid-stimulating hormone, growth hormone, and prolactin did not affect cyclic AMP synthesis (data not presented). Further experiments reported below were performed to examine the effect of modulation of blood cholesterol levels by pharmacological agents on cAMP and progesterone responses.

Effect of 4-APP Treatment for Rat on Subsequent Hormonal Steroidogenesis in Isolated Luteal Cells—Results presented in Table I show the effect of 4-APP injection on plasma cholesterol, progesterone, luteal cell cholesterol, and cholesterol esters and in vitro steroidogenic response of luteal cells. 4-APP injection (12.5 mg/kg body weight) reduced plasma cholesterol level from 47.32 ± 1.12 mg/dl (control) to 8.16 ± 0.43 mg/dl (4-APP). This treatment also reduced the plasma progesterone level from 280 ± 26 ng/ml to 96 ± 6 ng/ml. Similarly, cellular cholesterol ester content was reduced to about 25% in luteal cells isolated from 4-APP-injected rats. Free cellular cholesterol content, however, was less affected by this treatment.

Results presented in Table IA also compare the in vitro progesterone response of luteal cells isolated from PBS (control) and 4-APP-injected rats. 4-APP injection resulted in a
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experiments. were processed for CAMP determination. The production by luteal cells in response to hCG. The incubation steroidogenic response of luteal cells to LH (100 ng/ml), hCG ---, of three separate experiments. methyl-3-isobutylxanthine. volume cholera toxin on cyclic luteal cells (approximately concentrations of various hormones in the presence 6-fold decrease in basal production of progesterone. Similarly, steroidalogenic response of luteal cells to LH (100 ng/ml), hCG (10 ng/ml), cholera enterotoxin (1 pg/ml), Bt-cAMP (1.5 mM), and 8-Br-cAMP (1.5 mM) was also decreased following 4-APP injection. Further, stimulatory effect of DL-isoproterenol (1 pg/ml) was reduced by 3-fold in luteal cells isolated from 4-APP-treated rats. Among various doses of 4-APP (3.12, 6.25, 12.5, and 25 mg/kg body weight) tried, maximum effect on plasma cholesterol and in vitro luteal cell steroidogenesis was observed at 12.5 mg/kg body weight when this dose was injected every 24 h for 3 days beginning on day 3 of post-hCG treatment. Subsequent experiments were therefore performed with this dose of 4-APP.

Fig. 2. Effect of increasing incubation time on progesterone production by luteal cells in response to hCG. The incubation conditions were similar to those described under “Experimental Procedures.” The results represent the means (± S.E.) of three separate experiments. △ --- △, basal; ▲ --- ▲, hCG (10 ng/ml).

FIG. 3. Effect of increasing concentrations of hCG, LH, and cholera toxin on cyclic AMP synthesis in rat luteal cells. Rat luteal cells (approximately 2 × 10^6 cells) were incubated in a final volume of 0.4 ml of Medium 109 containing 0.1% BSA and indicated concentrations of various hormones in the presence of 0.5 mM 1 methyl-3-isobutylxanthine. After incubation at 37°C for 2 h in the atmosphere of O_2/CO_2 (90:10, v/v) the samples (cells + medium) were processed for cAMP determination. Results represent the means of three separate experiments. △ --- △, hCG; ---- ---- LH (NIH-LHB9); ---, cholera toxin.

Table 1

| Conditions                  | PBS control | 4-APP (125 mg/kg body weight) |
|-----------------------------|-------------|--------------------------------|
| A. Luteal cell steroidogenesis in vitro | ng progesterone µg DNA^-1± S.E.M.) | 4-APP (125 mg/kg body weight) (n = 6) |
| Basal                       | 1.35 ± 0.05 | 0.23 ± 0.02                    |
| hCG (10 ng/ml)              | 6.13 ± 0.13 | 1.23 ± 0.04                    |
| LH (NIH-LHB9, 100 ng/ml)    | 5.00 ± 0.47 | 1.25 ± 0.05                    |
| Cholera enterotoxin (1 μg/ml) | 5.02 ± 0.81 | 1.31 ± 0.08                    |
| DL-Isoproterenol (1 μg/ml)  | 2.14 ± 0.16 | 0.72 ± 0.04                    |
| Bt-cAMP (1.5 mM)            | 5.80 ± 0.30 | 0.82 ± 0.02                    |
| 8-Br-cAMP (1.5 mM)          | 4.95 ± 0.51 | 0.78 ± 0.13                    |
| B. Plasma cholesterol (mg/dl ± S.E.) | 47.32 ± 1.12 | 8.16 ± 0.43                   |
| C. Plasma progesterone (ng/ml ± S.E.) | 280 ± 26 | 96 ± 6                      |
| D. Luteal cell cholesterol (ng µg DNA^-1 ± S.E.) | 760 ± 33 | 648 ± 25                     |
| E. Luteal cell cholesterol ester (ng µg DNA^-1 ± S.E.) | 950 ± 47 | 290 ± 51                     |

Effect of Various Lipoprotein Fractions on Luteal Cell Steroidogenesis—Since 4-APP treatment reduced circulating blood cholesterol level and inhibited ovarian steroidogenesis, it was of interest to delineate the underlying mechanism. Initial attempts were made to reverse the inhibitory action of 4-APP by the addition of various lipoprotein fractions. Incubation of luteal cells from PBS (control)-injected rats with increasing concentrations of human LDL resulted in significant stimulation of steroid production. Under basal conditions, human LDL at a concentration of 40-600 μg of protein/ml maximally enhanced progesterone synthesis (Fig. 4A) with no further effect by increasing human LDL concentration. Coincubation of luteal cells with hCG and increasing concentrations of human LDL also enhanced steroidogenesis. The maximum stimulatory effect of human LDL in the presence of hCG was observed at a concentration around 600 μg of protein/ml and concentrations over 1600 μg of protein/ml reduced the maximum response. Under identical conditions human LDL also potentiated the stimulatory effect of 8-Br-cAMP. Similarly, human HDL (570 μg of protein/ml) also exhibited stimulatory action.

Results presented in Fig. 4B demonstrate the reversal of the inhibitory action of 4-APP treatment by human LDL. Treatment with 4-APP reduced the steroidalogenic capacity of the isolated luteal cells. Addition of increasing concentrations of human LDL enhanced steroidogenesis in a dose-dependent manner and completely reversed the 4-APP effect at a protein


### Table II

**Effect of 4-APP injection of \( ^{125}I\)-hCG binding and hormone-stimulated cyclic AMP accumulation in isolated luteal cells**

Female rats pretreated with PMSG and hCG were injected with PBS or 4-APP (12.5 mg/kg body weight) under the conditions described under "Experimental Procedures." The luteal cells isolated from both groups of ovaries were used for the determination of \( ^{125}I\)-hCG binding and cyclic AMP accumulation in response to various stimulators. For \( ^{125}I\)-hCG binding, luteal cells (approximately \( 2 \times 10^9 \)) were incubated with \( ^{125}I\)-hCG (180,000 cpm, 30 cpm/pg) in the presence or absence of 2.5 \( \mu \)g/ml of unlabeled hCG in 0.4 ml of Medium 109/0.1% BSA. The samples were incubated for 3 h at 37 °C in the presence of \( O_2/CO_2 \) (95/5, v/v). At the end of incubation, binding was stopped by the rapid addition of 2 ml of incubation medium and centrifugation at \( 250 \times g \) for 10 min. The sedimented cells after an additional wash in medium were counted for radioactivity. The results are expressed as specific binding, computed from the difference of total binding with that observed in the presence of unlabeled hCG. Conditions for cyclic AMP production were the same as described in Table I except 1-methyl-3-isobutylxanthine (0.5 mm) was also present in the incubation medium.

| Treatment                              | \( ^{125}I\)-hCG bound cpm/10 \( \mu \)g DNA ± S.E. | Basal hCG ng/ml pmol \( \mu \)g DNA ± S.E. | Cholera enterotoxin (1 \( \mu \)g/ml) pmol \( \mu \)g DNA ± S.E. |
|----------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Luteal cells from PBS-injected rats (control) | 13059 ± 395                    | 1.95 ± 0.10                     | 6.0 ± 0.57                      |
| Luteal cells from 4-APP-injected rats (12.5 mg/kg) | 10783 ± 276                    | 1.44 ± 0.21                     | 6.48 ± 0.14                     |

Fig. 4. Effect of increasing concentrations of human LDL (h-LDL) on progesterone production in luteal cells of PBS (Panel A)- and 4-APP (Panel B)-injected rats in response to hCG and 8-Br-cAMP. The cells isolated from PBS- or 4-APP-injected rats (for details, see Table II) were incubated with indicated concentrations of human LDL in the presence and absence of hCG or 8-Br-cAMP. At the end of 4 h incubation the samples were quantitated for progesterone by radioimmunoassay. For comparison results obtained with optimum concentration of human HDL (h-HDL) are also presented. The results represent the means (± S.E.) of three separate experiments on triplicate determinations.
that various lipoproteins could effectively deliver cholesterol into the luteal cells for steroid syntheses.

Effect of Triton WR-1339 Injection on Progesterone and Cyclic AMP Responses in Rat Luteal Cells—The studies reported thus far suggest that reduction in circulating blood cholesterol levels leads to subsequent inhibition of luteal cell steroidogenesis. We then evaluated the possibility of increasing blood cholesterol levels on the luteal cell steroidogenic capacity. To accomplish this, rats were injected with Triton WR-1339, which is known to increase circulating blood cholesterol level (19-22). Injection of rats with Triton WR-1339 (1 g/kg body weight) every 12 h for 2 days raised the plasma

![Graph showing effect of increasing concentrations of human HDL (h-HDL) on gonadotropin- and 8-Br-cAMP-induced steroidogenesis in luteal cells of PBS (Panel A) - or 4-APP (Panel B) injected rats.](image)

![Graph showing stimulatory effect of increasing concentrations of rat LDL (r-LDL) and rat HDL (r-HDL) on hCG-stimulated progesterone accumulation in luteal cells of PBS- or 4-APP-injected rats.](image)

**Table III**

Incorporation of [3H]cholesterol from [3H]cholesterol linolate-LDL into progesterone of luteal cells isolated from PBS- or 4-APP-injected rats

Incubation conditions were similar to those described under "Experimental Procedures."

| Experiment | Radioactivity (fmol µg DNA⁻¹ ± S.E.) |
|------------|-------------------------------------|
| Basal      | 272 ± 14                          |
| hCG (10 ng/ml) | 1159 ± 13                        |
| h-HDL      | 673 ± 4                           |
| Human LDL (700 µg protein/ml) | 5349 ± 54                        |
| Human HDL (730 µg protein/ml) | 59 ± 13                           |
| Rat LDL (350 µg protein/ml) | 95 ± 11                           |
| Rat HDL + hCG | 73 ± 12                          |
| Human HDL + hCG | 485 ± 69                         |
| Rat HDL + hCG | 597 ± 85                          |
| 4-APP (12.5 mg/kg body weight) | 627 ± 51                         |

* Injected every 24 h for 3 days.

**Table IV**

Effect of Triton WR-1339 injection on subsequent steroidogenesis in isolated luteal cells in response to hCG and various lipoproteins

Twenty-four-day-old female rats were injected with 50 IU of PMSG followed 65 h later with a single injection of 25 IU of hCG (day 0). On day 4 (post-hCG) rats were injected with normal saline or Triton WR-1339 (1 g/kg body weight) every 12 h for 2 days. Twelve h after last injection, the rats were killed and luteal cells were isolated by collagenase digestion. Aliquots of isolated cells (approximately 2 × 10⁷ cells) were incubated with or without hCG in a final volume of 0.4 ml of Medium 109 containing 0.1% BSA and indicated final concentrations of various lipoproteins. After 4 h incubation in the presence of O₂/CO₂ (95:5, v/v) the samples were processed for progesterone radioimmunoassay. Aliquots of luteal cells were also extracted and quantitated for cellular cholesterol ester and free cholesterol contents as described under "Experimental Procedures." The concentration of lipoprotein is expressed as micrograms of cholesterol/ml.

| Conditions | Saline (control) (ng (control)) | Triton WR-1339 (1 g/kg body weight) (n = 5) |
|------------|-------------------------------|-------------------------------------------|
| A. Luteal cell steroidogenesis in vitro (ng progesterone µg DNA⁻¹ ± S.E.) |                           |
| Basal      | 1.91 ± 0.14                   | 0.291 ± 0.01                              |
| hCG + human VLDL | 5.54 ± 0.18                  | 0.836 ± 0.04                              |
| Human LDL (50 µg/ml) | 4.93 ± 0.17                  | 1.080 ± 0.05                              |
| Human HDL (210 µg/ml) | 6.00 ± 0.16                  | 0.897 ± 0.09                              |
| Rat LDL (750 µg/ml) | 4.11 ± 0.16                  | 0.45 ± 0.03                               |
| Rat HDL (600 µg/ml) | 5.00 ± 0.32                  | 0.76 ± 0.16                               |
| hCG (10 ng/ml) | 8.88 ± 0.32                  | 1.05 ± 0.05                               |
| hCG + human VLDL | 8.12 ± 0.44                  | 1.30 ± 0.04                               |
| hCG + human LDL | 16.10 ± 1.70                 | 5.60 ± 0.46                               |
| hCG + human HDL | 14.64 ± 1.20                 | 3.76 ± 0.13                               |
| hCG + rat VLDL | 8.76 ± 0.54                  | 1.20 ± 0.01                               |
| hCG + rat LDL | 12.84 ± 0.86                 | 3.02 ± 0.20                               |
| hCG + rat HDL | 14.82 ± 0.22                 | 3.50 ± 0.24                               |
| B. Plasma cholesterol (mg/dl ± S.E.) | 47 ± 1 | 467 ± 41 |
| C. Plasma progesterone (ng/ml ± S.E.) | 357 ± 33 | 126 ± 20 |
| D. Luteal cell cholesterol ester (ng/µg DNA ± S.E.) | 1015 ± 12 | 908 ± 15 |
| E. Luteal cell free cholesterol (ng/µg DNA ± S.E.) | 788 ± 12 | 676 ± 13 |
cholesterol levels from 47 ± 1 to 467 ± 41 mg/dl (± S.E.) (Table IV). In contrast, this treatment reduced circulating plasma progesterone from 357 ± 33 to 126 ± 20 ng/ml (± S.E.). Similarly, detergent injection also drastically reduced the basal as well as hCG-stimulated steroid production in isolated luteal cells. Contrary to the 4-APP effect, the inhibitory action of Triton WR-1339, however, was not reversed by subsequent exposure of luteal cells to various lipoproteins (Table IV). Finally, Triton WR-1339 injection produced only a slight decrease in luteal cell cholesterol ester and free cholesterol contents.

Results of Table V show the effect of Triton WR-1339 on 125I-hCG binding and cyclic AMP accumulation in luteal cells in response to various stimulators. As evident, there was no change in 125I-hCG binding to luteal cells of Triton WR-1339 injected rats compared to saline-injected rats. However, this treatment significantly reduced the extent of cyclic AMP synthesis by luteal cells in response to hCG or cholera enterotoxin.

**DISCUSSION**

The present studies were aimed at evaluating the role of precursor and circulating plasma lipoproteins in gonadotropin-induced steroidogenesis in ovary. Luteal cells isolated from PMSG/hCG-primed pseudopregnant rat ovaries were used as a model system. These cells were chosen because of their extreme sensitivity to physiological doses of gonadotropin. Studies reported here demonstrate that these cells respond to LH, hCG, and isoproterenol with an increase in progesterone production (48-50). Further studies were undertaken to study systematically the effect of varying culturing blood cholesterol on progesterone synthesis. The 4-APP, which is an adenine analog, has previously been shown to inhibit secretion of all major classes of plasma lipoproteins and causes a decrease in circulating levels of plasma cholesterol (17-19). In the present studies, 4-APP-mediated decrease in circulating level of cholesterol was closely correlated with the decrease in plasma progesterone levels. Interestingly, administration of 4-APP to rats greatly reduced the in vitro steroidogenic capacity of isolated luteal cells both under basal conditions and in response to various stimulators. While maximum steroid production was decreased considerably, cells isolated from 4-APP-injected rats still retained the ability to respond to gonadotropins. The lack of effect of 4-APP on 125I-hCG binding and gonadotropin-stimulated cyclic AMP production, along with the decreased responsiveness of cells to 8-Br-cAMP and Bt2cAMP, further support the notion that the 4-APP effect was primarily at a step after the hormone/receptor/adenylate cyclase system and, more specifically, at the precursor level. Indeed, in the present studies, 4-APP administration resulted in a major decrease in luteal cell steroid esters and a small but significant decrease in free cholesterol content.

Our next approach was to see if 4-APP-mediated reduction in steroidogenic response could be reversed in vitro by lipoproteins. To our surprise, incubation of luteal cells from PBS (control)-injected rats, with human LDL or human HDL significantly enhanced the steroidogenesis over that observed in the absence of lipoprotein addition. These cells are, thus, clearly different from those of adrenocortical cells (11, 12), granulosa cells (9) and choriocarcinoma cells (13) which require prolonged exposure to lipoprotein-deficient serum before becoming dependent on exogenous lipoprotein cholesterol for steroid synthesis. Luteal cells are therefore unique in their ability to take up and metabolize lipoprotein cholesterol immediately upon the isolation from the tissue with no apparent need for prolonged culture. Various lipoproteins effectively reversed the inhibition of steroidogenesis seen in cells isolated from 4-APP-pretreated rats. It was also demonstrated that cells of 4-APP-injected rats incorporated a higher amount of [3H]cholesterol linolate-LDL delivered cholesterol into progesterone compared to cells of PBS-injected rats. The extensive in vivo studies reported by Anderson and Dietschy (6) suggest that three major steroid producing tissues, ovary, adrenal, and testis, preferentially take up and incorporate cholesterol from HDL rather than LDL into steroid. Further, infusion of HDL from human or rat plasma to 4-APP-treated rats was reported to block the [1-14C]acetate incorporation into digitonin precipitable sterols in ovary and adrenal gland (18). However, in contrast to the adrenal gland, infusion of LDL failed to exert any effect on [1-14C]acetate incorporation into digitonin precipitable sterols in the ovary. In contrast, mouse adrenal glands were shown to obtain cholesterol in vivo from two lipoprotein systems, one specific for LDL and the other specific for HDL (7). The present studies, as well as those reported earlier for cultured granulosa cells (9), suggest that either LDL or HDL can supply cholesterol for steroidogenesis in cells of ovarian origin, although possibly not to the same extent.

The elegant work of Brown and Goldstein suggests that, in many LDL-responsive cells, the lipoprotein is bound and taken up by a saturable membrane-associated process (51-54). Following internalization and degradation of LDL particle, the released cholesterol could be utilized for membrane biosynthesis or steroid synthesis or stored as cholesterol ester. Relatively higher intracellular concentrations of cholesterol lead to suppression of activity of hydroxymethylglutaryl CoA reductase and in some instances, such as in human fibroblasts, reduction in the number of binding sites on cell surface, so as to further reduce the uptake of lipoprotein cholesterol (53-54). We have recently demonstrated the presence of both LDL and HDL receptors in the luteal cells. Thus, it is conceivable that a similar mechanism is operating in the luteal cells.

Injection of rats with Triton WR-1339 produced a 10-fold

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**TABLE V**

Effect of Triton WR-1339 injection on 125I-choriogonadotropin binding and hormone-stimulated cAMP accumulation in isolated luteal cells

| Treatment                      | 125I-hCG bound | Cyclic AMP                        |
|-------------------------------|----------------|----------------------------------|
|                               | cpm/10 µg DNA ± S.E. | pmol µg DNA⁻¹ ± S.E. |
| Luteal cells from saline-injected rats (control) | 15278 ± 329 | 1.94 ± 0.12       |
| Luteal cells from Triton WR-1339-injected rats | 13773 ± 649 | 2.25 ± 0.26       |

Note: The values are mean ± standard error (S.E.)
increase in plasma cholesterol, an effect observed earlier and notably by Anderson and Dietzchy (18) and by Goldfarb (21). However, detergent administration leads to a significant reduction in circulating progesterone level, an effect in contrast to an increase in plasma cholesterol. Since Triton WR-1339 causes a marked increase in hepatic cholesterol synthesis (22, 55, 56) and an increase in circulating level of cholesterol (19–22), we were interested to test whether plasma cholesterol had any effect on luteal cell steroidogenesis.

Administration of Triton WR-1339 significantly reduced the steroidogenic capacity of luteal cells in response to gonadotropin. Our efforts to reverse the inhibitory effect by the addition of various lipoproteins were unsuccessful. Further, since detergent injection also resulted in a decrease in cyclic AMP response, it is possible that detergent produced a general decrease in overall metabolic activity of luteal cells. However, the possibility that detergent treatment may change the metabolism of lipoproteins in luteal cells cannot be ruled out.

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REFERENCES

1. Armstrong, D. T. (1968) Recent Prog. Horm. Res. 24, 255–319
2. Marsh, J. M. (1976) Biol. Reprod. 14, 30–53
3. Dorrington, J. H. (1977) in The Ovary (Zuckerman, L., and Weir, B. J., eds) Vol. 3, pp. 359–412, Academic Press, New York.
4. Dorfman, R. I. (1973) in Handbook of Physiology (Greep, R. O., and Astwood, E. B., section eds) Section 7, Vol. 2, Part I, pp. 537–546, American Physiological Society, Washington, D. C.
5. Bolte, E., Coudert, S., and Lefebvre, Y. (1974) J. Clin. Endocrinol. Metab. 39, 384–403
6. Andersen, J. M., and Dietzchy, J. M. (1978) J. Biol. Chem. 253, 9024–9032
7. Kovanen, P. T., Schneider, W. J., Hillman, G. M., Goldstein, J. L., and Brown, M. S. (1979) J. Biol. Chem. 254, 5498–5505
8. Christie, M. H., Strauss, J. F., III, and Flickinger, G. L. (1979) Endocrinology 105, 92–98
9. Schuler, L. A., Scavo, L., Kirseb, T. M., Flickinger, G. L., and Strauss, J. F., III (1979) J. Biol. Chem. 254, 8662–8668
10. Brecher, P. L., and Hyun, Y. (1978) Endocrinology 102, 1404–1413
11. Faust, J. R., Goldstein, J. L., and Brown, M. S. (1977) J. Biol. Chem. 252, 4861–4871
12. Kovanen, P. T., Faust, J. R., Brown, M. S., and Goldstein, J. L. (1979) Endocrinology 104, 599–609
13. Simpson, E. R., Porter, J. C., Milewich, L., Bilheimer, D. W., and MacDonald, P. C. (1978) J. Clin. Endocrinol. Metab. 47, 1099–1105
14. Gwynne, J. T., and Hess, B. (1980) J. Biol. Chem. 255, 10875–10883
15. Henderson, J. F. (1963) J. Lipid Res. 4, 68–74
16. Shiff, T. S., Roheim, P. S., and Eder, H. A. (1971) J. Lipid Res. 12, 596–603
17. Balsubramaniam, S., Goldstein, J. L., Faust, J. R., and Brown, M. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2564–2568
18. Andersen, J. M., and Dietzchy, J. M. (1977) J. Biol. Chem. 252, 3852–3859
19. Kuroda, M. K., Tanazawa, Y., Tsujita, Y., and Endo, A. (1977) Biochim. Biophys. Acta 489, 119–125
20. Andersen, J. M., and Dietzchy, J. M. (1977) J. Biol. Chem. 252, 3646–3651
21. Goldfarb, S. (1978) J. Lipid Res. 19, 489–494
22. Ishikawa, T., and Fidge, N. (1979) J. Lipid Res. 20, 254–264
23. Cast, K. J., Dufau M. L., and Tsuruhara, T. (1972) J. Clin. Endocrinol. Metab. 34, 123–132
24. Parlow, A. F. (1958) Fed. Proc. 17, 402
25. Menon, K. M., Azhar, S., and Menon, K. M. J. (1980) Am. J. Obstet. Gynecol. 136, 594–530
26. Azhar, S., and Menon, K. M. J. (1981) Biochem. J. 194, 19–27
27. Kawano, A., Gunaga, K. P., and Menon, K. M. J. (1975) Biochim. Biophys. Acta 385, 88–100
28. Clark, M. R., and Menon, K. M. J. (1976) Biochim. Biophys. Acta 444, 23–32
29. Sen, K. K., Azhar, S., and Menon, K. M. J. (1979) J. Biol. Chem. 254, 5664–5671
30. Burton, K. (1956) Biochem. J. 62, 315–323
31. Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U. S. A. 65, 305–312
32. Azhar, S., and Menon, K. M. J. (1979) Biochem. J. 180, 201–211
33. Havel, R. J., Eder, H. A., and Bradgon, J. H. (1955) J. Clin. Invest. 34, 1345–1353
34. Fletcher, M. J., and Styliou, M. H. (1970) Clin. Chem. 16, 362–365
35. Noble, R. P. (1978) J. Lipid Res. 9, 693–700
36. Krieger, M., Brown, M. S., Faust, J. R., and Goldstein, J. L. (1978) J. Biol. Chem. 253, 4093–4101
37. Zak, B. (1965) Stand. Methods Clin. Chem. 5, 79–89
38. Radin, N. S. (1969) Methods Enzymol. 14, 245–254
39. Ames, B. N. (1966) Methods Enzymol. 8, 115–118
40. Azhar, S., Haaja, A. K., and Menon, K. M. J. (1976) J. Biol. Chem. 251, 7405–7412
41. Skipski, V. P., and Barclay, M. (1969) Methods Enzymol. 14, 530–599
42. Glick, D., Fell, B. F., and Sjolin, K. E. (1964) Anal. Chem. 36, 1119–1121
43. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
44. Markwell, M. A. K., Hass, S. M., Bieher, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
45. Channing, C. P., and Ville, C. A. (1966) Biochim. Biophys. Acta 127, 1–17
46. Kovanen, P. T., Goldstein, J. L., and Brown, M. S. (1978) J. Biol. Chem. 253, 5126–5132
47. Dimino, M. J. (1977) Endocrinology 101, 1844–1849
48. Finchelli, R. A. (1973) CRC Crit. Rev. Microbiol. 2, 553–623
49. Van Heyningen, S. (1977) Biol. Rev. 52, 509–549
50. Vaughan, M., and Moss, J. (1978) J. Supramol. Structure. 8, 473–488
51. Goldstein, J. L., and Brown, M. S. (1977) Annu. Rev. Biochem. 46, 897–930
52. Brown, M. S., and Goldstein, J. L. (1979) Harvey Lect. 73, 163–201
53. Brown, M. S., and Goldstein, J. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3330–3337
54. Brown, M. S., and Goldstein, J. L. (1976) Science 191, 150–154
55. Frantz, I. D., and Hinkelman, B. J. (1975) J. Exp. Med. 141, 225–232
56. Bucher, N. L. R., Mcgarrah, K., Gould, E., and Loud, A. V. (1959) J. Biol. Chem. 234, 262–267