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Steroidogenic Effects of Lipoproteins and 25-Hydroxycholesterol on Luteal and Ovarian Cells: A Comparison of Two Pseudopregnant Rat Models (42248)

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Abstract. Steroidogenesis was compared between luteal cells from immature pseudopregnant (PSP) rats induced by either 5 IU pregnant mare serum gonadotropin (PMSG) alone or 50 IU PMSG combined with 25 IU human chorionic gonadotropin (hCG). It was also determined whether differences in steroidogenesis existed when the entire ovary (ovarian cells) or just luteal cells from Day 4 PSP rats were exposed *in vitro* to lipoproteins or 25-hydroxycholesterol (25-OH chol). In the absence of luteinizing hormone (LH), basal steroid accumulation, especially progesterone (P_4) was around fourfold greater in luteal cells from rats treated with PMSG alone than from rats receiving PMSG–hCG. However, serum P_4 and LH were about fivefold greater in the latter group. It is therefore likely that net cellular cholesterol uptake per luteal cell is lower in the PMSG–hCG treated rats, but this is offset by a much greater mass and number of corpora lutea. Lipoproteins (HDL and LDL) and 25-OH chol stimulated *in vitro* luteal steroidogenesis from rats treated with PMSG alone or PMSG–hCG, and their responses were virtually identical. Therefore, luteal steroidogenesis in the rat always depends on exogenous cholesterol even though treatment in the preovulatory period with PMS or PMSG–hCG and serum LH and follicle-stimulating hormone (FSH) levels on Day 4 PSP are very different. When ovarian cells from PMSG–hCG treated rats were incubated with LH plus HDL or 25-OHP, the production of 20α-DHP was considerably greater than luteal cell production which may be due to a contribution from nonluteal cells. Indeed, about 30% of the cells in the PMSG–hCG group represent nonluteal components as estimated by weight and deoxyribonucleic acid content. © 1986 Society for Experimental Biology and Medicine.

Most mammalian steroidogenic tissues use extracellular cholesterol carried by lipoproteins as a source of steroid hormone precursor (1). However, we previously reported that lipoproteins and 25-hydroxycholesterol (25-OH chol) were only minimally effective in increasing luteal steroidogenesis in immature pregnant and pseudopregnant rats in which pseudopregnancy (PSP) was induced by injecting 5 IU pregnant mare serum gonadotropin (PMSG) (2). These results were quite different from previous reports in which immature rats were treated with 50 IU PMSG followed by 25 IU human chorionic gonadotropin (hCG) and the *entire* ovary was used on Days 4–6 PSP (3–5).

The aim of this study was to explore this difference by using luteal cells from PSP rats injected with 50 IU PMSG followed by 25 IU hCG, which produces massive ovarian luteinization; the other group received 5 IU PMSG alone which results in a normal ovulation of 9 ova (6). Another objective was to discover whether a different steroidogenic response to lipoproteins exists between ovarian and luteal cells from PMSG–hCG primed immature rats since about 75% of the cells have been reported to be luteal-like (3).

Materials and Methods. *Animal treatment.* Twenty-one-day-old Holtzman rats from SASCO Laboratories (Omaha, Nebr.) were maintained under standard lighting conditions (14 L: 10 D; lights on 0500 hr). They were injected sc with 50 IU PMSG (Sigma, St. Louis, Mo.) on Day 23 (Day 1 = day of birth) followed 66 hr later by 25 IU hCG (Ayerst, New York, N.Y.). The day of hCG treatment was designated Day 0 PSP. Another group of rats was injected sc with 5 IU PMSG on Day 30. These animals ovulated early on the morning of Day 33 therefore corresponding to Day 1 PSP. Animals were decapitated at 0900 hr on Day 4 PSP and trunk blood was saved for determination of progesterone (P_4) and 20α-dihydroprogesterone (20α-DHP).

Estimation of luteal mass in the highly luteinized PMSG–hCG treated ovary. Dispersed ovarian cells in the PMSG–hCG primed rat

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model have been estimated by transmission electron microscopy to consist of approximately 75% luteal cells (3). For our purpose, a more quantitative estimate was provided by weighing the CL and nonluteal ovary and determining cellular deoxyribonucleic acid (DNA) content of each compartment. The ovaries from PMSG–hCG primed rats were removed on Day 4 PSP, placed in ice-cold saline, and all corpora lutea (CL) were dissected and the remaining nonluteal tissue was collected. After weighing CL and nonluteal tissue, the CL and the nonluteal tissue were homogenized in saline and the DNA concentration of homogenates was measured by the method of Burton (7) using calf thymus DNA (Sigma) as a standard. The cell number of CL and nonluteal tissues were calculated from the total DNA content since each cell contains approximately 6.4 pg of DNA (8).

Preparation and incubation of dispersed ovarian and luteal cells. Ovaries from PMSG–hCG treated rats were divided on Day PSP into two groups. For the left ovary the entire ovary was used, and in the other, CL were dissected. In rats treated with PMSG alone, on Day 4 PSP, CL were dissected from the ovary.

Collagenase (Type V, Sigma) dispersed luteal and ovarian cells were prepared as described earlier (9). Aliquots of dispersed cells (approximately 5×10^4 viable cells) were incubated at 37°C for 2 hr in 1 ml of Medium 199 (GIBCO, New York, N.Y.) containing increasing amounts of human low density lipoprotein (LDL), human high density lipoprotein (HDL), or 25-OH chol (Steraloid, Wilton, N.H.) in the presence or absence of 100 ng of ovine luteinizing hormone (LH; ovine LH-S24, NIAMMD). The incubation was stopped by placing the tubes in an ethanol–dry ice bath. All samples were stored at −20°C until assayed. The samples were thawed and cells were broken using a microultrasonic cell disruptor (Kontes Glass Co., Vineland, N.J.) at 540 W/in^2 for 5 sec. Cell viability was checked before and after incubation by the trypan blue exclusion test and was always greater than 85%.

Preparation of lipoproteins. Human LDL (density, 1.019–1.063 g/ml) and human HDL (density, 1.063–1.21 g/ml) were prepared by a density-gradient ultracentrifugation method (10) and dialyzed against 0.15 M NaCl, pH 7.4, for 48 hr with 2 medium changes. The dialyzed lipoproteins were stored at 4°C and cholesterol and protein content were measured. The concentration of lipoprotein was expressed in terms of cholesterol content.

Radioimmunoassays (RIAs) of steroids and gonadotropins. The methods for steroid RIAs were described previously (11) using P_4 antisera (12) and 20α-DHP antisera (Dr. Pang and Dr. Hilliard, UCLA). The lower limit of sensitivity in the assay was 2 pg per assay tube for both steroids.

Serum levels of follicle-stimulating hormone (FSH) and LH were measured with RIA kits provided by NIADDK. Rat FSH I-1 and rat LH I-7 were radioiodinated by the lactoperoxidase method (13). Anti-rat FSH S-11 and anti-rat LH S-7 were used in the FSH and LH RIAs, with serum FSH and LH concentrations expressed in terms of NIAMMD rat FSH-RP-2 and NIAMMD rat LH-RP-2. The lower limits of the assays were 1 ng/ml for FSH and 0.1 ng/ml for LH. The details of the gonadotropin assays have been described previously (14). Antibodies for rat FSH and LH did not cross-react with PMSG or hCG (data not shown).

Statistics. Comparison of effects of lipoproteins between luteal and ovarian cells was done by Duncan's multiple range comparison test and the dose response of lipoproteins was analyzed by Dunnet's multiple comparison test. Statistical significance was determined at the level of $P < 0.05$.

Results. In the PMSG–hCG primed pseudopregnant rat, 69.9 ± 2.1% (n = 8) of the ovary by weight represented CL. Also, as measured by DNA content, luteal-derived cells constituted 68.0 ± 1.9% (n = 8) of the total ovary. It must be kept in mind that the corpus luteum contains endothelial cells and other cell types as well as luteal cells. Yields of luteal and nonluteal cells per ovary were $65.5 \pm 2.3 \times 10^6$ and $30.8 \pm 2.2 \times 10^6$ cells, respectively.

Serum P_4, 20α-DHP, and LH were much greater in PMSG–hCG treated rats than in rats receiving PMSG alone. Serum FSH, however, was higher in the PMSG alone group (Table I). In the absence of LH, the basal accumulation of P_4 in luteal cells from rats treated only with PMSG was around four- or ninefold higher than in luteal or ovarian cells, respectively, from the PMSG–hCG treated group (Fig. 1). In the latter group, luteal cells not

TABLE I. SERUM LEVELS OF PROGESTERONE (P_4), 20α-DIHYDROPROGESTERONE (20α-DHP) AND GONADOTROPINS IN IMMATURE DAY 4 PSEUDOPREGNANT RAT INDUCED BY DIFFERENT TREATMENTS

Treatment	P_4[a] (ng/ml)	20α-DHP[a] (ng/ml)	FSH[b] (ng/ml)	LH[b] (ng/ml)
50 IU PMSG + 25 IU hCG	173 ± 9.2	194 ± 17.8	4.5 ± 0.5	2.0 ± 0.2
5 IU PMSG alone	37 ± 4.1	16 ± 1.6	9.7 ± 0.9	0.4 ± 0.05

Note. All values in 5 IU PMSG alone group are significantly different ($P < 0.01$ or $P < 0.05$) from values of 50 IU PMSG + 25 IU hCG.
[a] $n = 12$.
[b] $n = 14$ for PMSG and hCG group; $n = 7$ for PMSG alone group.

exposed to LH were more active than ovarian cells in secreting P_4; Basal 20α-DHP production, however, was almost the same between ovarian and luteal cells (Fig. 1). Luteinizing hormone, without lipoproteins, stimulated production of P_4 and 20α-DHP in both treatment groups; P_4 accumulation in ovarian or luteal cells from rats primed with PMSG–hCG was 4–5 times greater than basal values after adding 100 ng LH; these increments are almost the same as in previous studies (4, 15, 16). In contrast, luteal P_4 synthesis in rats primed with PMSG alone was increased only twofold by LH. Without lipoproteins, LH stimulated 20α-DHP to almost the same extent for ovarian and luteal cells from the PMSG–hCG treated

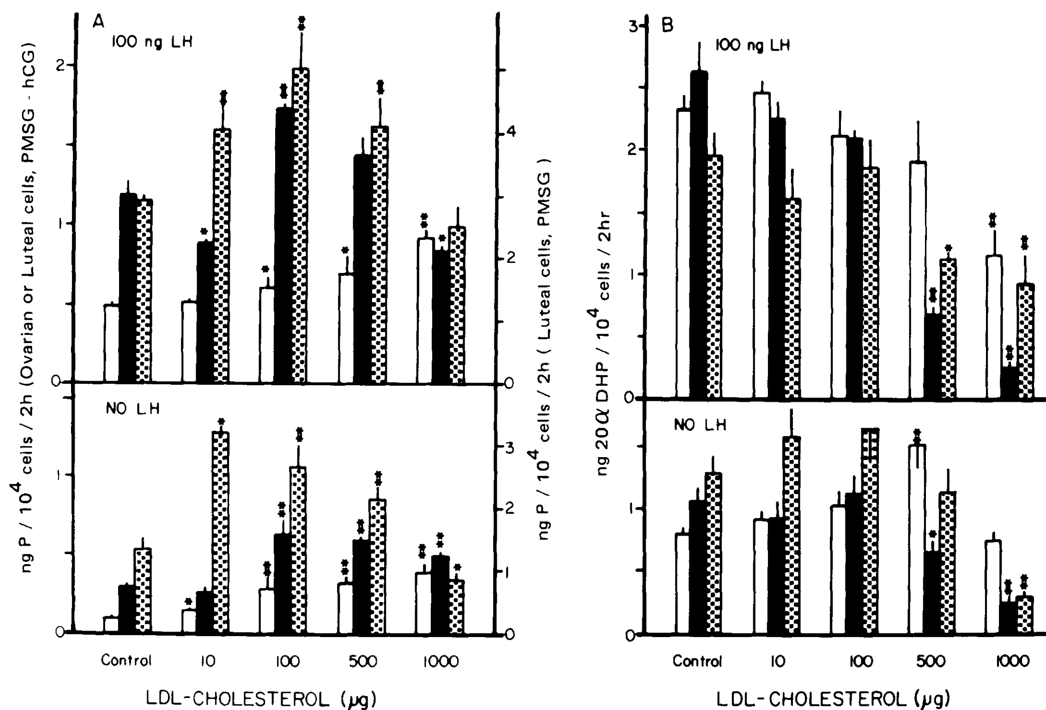

FIG. 1. Effect of LDL in the presence or absence of LH on accumulation of (A) progesterone (P_4) and (B) 20α-dihydroprogesterone (20α-DHP) in ovarian cells isolated from the entire ovary (blank bar) and luteal cells (filled bar) from PMSG–hCG treated rats and luteal cells (dotted bar) from rats treated with PMSG alone. Note the difference in scale for the latter group. In all figures, results are expressed as means + SEM of three to four duplicated observations. In this and subsequent figures, $^*P < 0.05$ and $^{**}P < 0.01$ versus the control value for each group.

group but was less effective in increasing 20α-DHP secretion by luteal cells from the PMSG alone group (Fig. 1).

In response to LDL, with or without LH, ovarian cells from rats injected with PMSG–hCG produced P_4 in a dose-dependent manner (Fig. 1). Luteal cells from PMSG–hCG treated rats also produced P_4 when LDL was added; the maximal effect occurred with 100 μg of LDL and the response then decreased. For PMSG alone treated rats, 10 μg of LDL increased P_4 accumulation in luteal cells. In the presence or absence of LH, up to 100 μg of LDL had no effect on accumulation of 20α-DHP but 500 μg or more decreased the hormone in PMS and PMS–hCG groups (Fig. 1).

HDL increased basal accumulation of P_4 and 20α-DHP in all cell groups except for 20α-DHP by luteal cells from the PMSG group (Fig. 2). On addition of LH, HDL was more effective in increasing P_4 in luteal cells from PMSG–hCG treated rats or PMSG alone treated rats whereas for ovarian cells it was more effective in increasing 20α-DHP. Five hundred micrograms of HDL, with or without LH, inhibited 20α-DHP in all groups except for basal accumulation by ovarian cells (Fig. 2).

Incubation with 25-OH chol enhanced basal and LH-stimulated production of P_4 and 20α-DHP in a dose-dependent manner in ovarian and luteal cells from PMSG–hCG-treated rats except for 20α-DHP accumulation by luteal cells (Fig. 3). In luteal cells from rats injected only with PMSG, the production of P_4 was increased by 25-OH chol but 20α-DHP was unaffected.

Discussion. Basal luteal production of progestins (P_4 and 20α-DHP) from the PMSG alone group was considerably higher than in the PMSG–hCG treated rats; however, serum levels of steroids were much higher in the latter group (Table 1). This implies that the number of CL or total mass of CL was much greater in the PMSG–hCG group. The number of CL in the PMSG only group was around 10, comparable with a previous study in which the same treatment caused an average ovulation of 8.3 eggs (6). In the PMSG–hCG group, however, there were more than 50 CL per

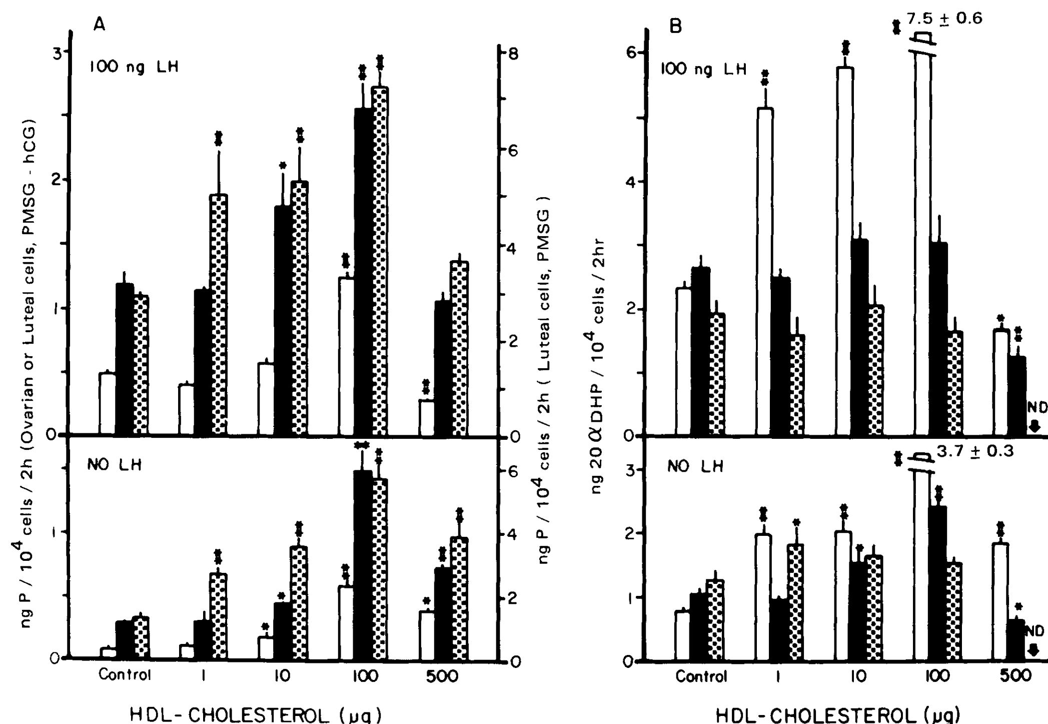

FIG. 2. Effect of HDL on the production of P_4 and 20α-DHP in various cells. All symbols are the same as in Fig. 1. ND represents nondetectable.

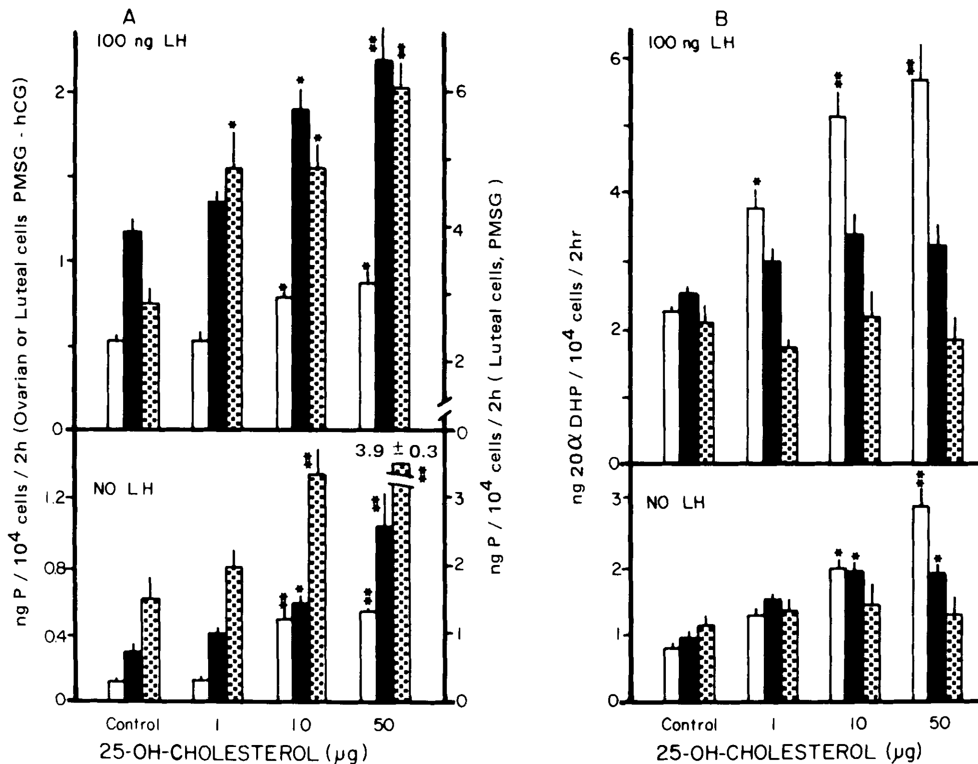

FIG. 3. Effect of 25-OH chol on the production of P_4 and 20α-DHP in various cells. Symbols are the same as in Fig. 1.

ovary and total ovarian mass was greater. Injection of the immature hypophysectomized rat with 50 IU PMSG followed by 10 IU hCG induces superovulation of around 40 eggs (17). Then why is basal luteal steroidogenesis lower in the PMSG–hCG treated rat? If the body pool of cholesterol or total amount of circulating cholesterol is not changed by the different gonadotropin pretreatments, it is possible that net cellular cholesterol uptake per luteal cell may be lower after PMSG–hCG treatment than PMSG alone because the mass or the number of CL is much greater in the former group. Alternatively, the amount of endogenous cholesterol in luteal cells may be lower in the PMSG–hCG treated rat because the luteal cells use most of their endogenous sterol pool to produce steroids to account for the high serum levels of progestins (Table I). This may correlate with elevated LH levels which act on the rate limiting step of transfer of cholesterol to the mitochondria (18).

Luteal steroidogenesis in rats treated with PMSG alone was similar to intact immature PSP rats (9) and normal pregnant rats (19). The results with PMSG–hCG can not be compared directly with previous studies because other authors have expressed luteal steroidogenesis as nanogram steroid per microgram DNA, per milligram tissue or per milligram protein instead of per cell. Since each cell contains approximately 6.4 pg of DNA (8), however, the present data can be converted to nanogram P_4 production per microgram DNA as 1.4 ng P_4/μg DNA/2 hr in ovarian cells and 4.6 ng P_4/μg DNA/2 hr in luteal cells from rats receiving PMSG and hCG. These values are comparable to those of previous reports where basal P_4 production in ovarian cells from the PMSG–hCG treated rat was 0.5–1.9 ng/μg DNA/3 hr (4, 15) or 4.7 ng/μg DNA/hr (16).

An interesting finding is that serum LH is higher and FSH lower in the PMSG–hCG treated rats than in the group receiving PMSG alone although serum P_4 is much higher in the former animals (Table I). This was unexpected since elevated serum P_4 normally acts to sup-

press LH release (20, 21). The high serum LH in the PMSG–hCG group can not be attributed to the lingering effects of the injected PMSG and hCG because neither cross-reacts with anti-rat FSH and anti-rat LH sera. In intact immature rats there is also no difference in serum LH or FSH levels between 29 and 36 days old (22) which eliminates the age difference at necropsy as a factor. The elevated LH in the PMSG–hCG group may be due to the positive feedback of estrogen and P_4 on LH release in immature rats (23, 24).

In the present study, luteal cells from rats treated with PMSG–hCG or PMSG alone showed no significant differences in steroidogenic response to lipoproteins and 25-OH chol but slight differences existed in 20α-DHP production with HDL or 25α-OH chol even though their production of progestins differed markedly. Injection of hCG in the immature rat increases both LDL and HDL receptors (25, 26) without altering binding affinity (26). Also, uptake of labeled HDL by ovaries of immature rats treated with PMSG alone is lower than in rats receiving PMSG and hCG (25). Thus, *in vitro* HDL uptake is directly related to ovarian steroidogenic activity as reflected by serum levels of P_4 and 20α-DHP (25); unfortunately, luteal or ovarian steroidogenesis was not measured in these studies (25, 26). We suggest that the amount of cholesterol entering luteal cells through membrane lipoprotein receptors may be enough to synthesize steroids in rats treated with PMSG alone even though the numbers of lipoprotein receptor sites may be considerably lower than in rats treated with PMSG and hCG (25, 26). How much of the lipoprotein–cholesterol complex entering luteal cells is incorporated into the steroidogenic pathway is unknown. The present results suggest that *in vitro* steroidogenesis always depends upon exogeneous cholesterol if luteal cells are still steroidogenically active and healthy even though the pretreatment schedule of gonadotropin(s) and/or present gonadotropin levels are different. Therefore, luteal cells from Day 10 PSP rats that are beginning to undergo luteolysis respond minimally to exogeneous cholesterol (2).

The present study shows that the utilization of exogenous cholesterol carried by lipoprotein is different between ovarian and luteal cells from the same PSP animal. Patterns of P_4 synthesis after adding lipoproteins or 25-OH chol were similar between ovarian and luteal cells from PMSG–hCG treated rats but accumulation of 20α-DHP after addition of lipoproteins or 25-OH chol was different between the two cell types. This suggests that the synthesis of 20α-DHP from nonluteal tissues, i.e., interstitial cells and follicles, was markedly increased by addition of HDL or 25-OH chol, especially in the presence of LH. This is consistent with the finding that 30% of ovarian cells represent nonluteal tissue and that the nonluteal ovary secretes 20α-DHP in response to LH in hypophysectomized rats (6). The remaining portion (about 70%) of ovarian cells, however, are not all luteal cells, since it also includes endothelial cells, macrophages, etc. We cannot explain why LDL failed to increase LH-stimulated 20α-DHP production in ovarian cells. However, numerous studies have shown that LDL is less effective in enhancing secretion of progestin in ovarian cells from rats treated with PMSG and hCG (3, 27). Therefore, it is possible that nonluteal tissue, such as interstitial cells, have no or few LDL binding sites.

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