Indirect regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by oestradiol in the rabbit corpus luteum*

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Summary. Rabbits were given 50 i.u. hCG, i.v., to initiate ovulation and pseudopregnancy (Day 0) and were treated, s.c., with or without a 1-cm Silastic oestradiol implant. Serum progesterone concentrations were measured at 4-day intervals and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was estimated by the conversion of HMG to mevalonate in microsomes from corpora lutea removed on Days 4, 8, 12, 16 and 20 of pseudopregnancy (4 rabbits/day). Total HMG-CoA reductase activity was significantly \((P < 0.05)\) higher in control rabbits on Days 8 and 12 \((5.29 \pm 0.63\) and \(5.5 \pm 0.28\) nmol/min/mg protein, respectively) compared to oestradiol-treated rabbits \((2.57 \pm 0.25\) and \(4.03 \pm 0.23\) nmol/min/mg protein, respectively). On Days 16 and 20, total HMG-CoA reductase activity was not different in control and oestradiol-treated animals. There was no difference in the levels of the active fraction of HMG-CoA reductase, which represented \(< 20\%\) of the total enzyme activity, in control and oestradiol-treated rabbits \((< 780\) pmol/min/mg protein, Day 12). These results indicate that oestradiol does not alter the active form, but can reduce the total activity of HMG-CoA reductase in the rabbit corpus luteum without a decline in serum progesterone. Therefore, neither total nor active forms of HMG-CoA reductase are directly related to progesterone secretion. This suggests that other sources of cholesterol may contribute to progesterone production in the rabbit.

Keywords: HMG-CoA reductase; corpus luteum; progesterone synthesis; oestradiol action; lipoproteins

Introduction

As early as 1933, Schoenheimer & Breusch (1933) had deduced that cholesterol regulated its own synthesis. The enzyme responsible for this process was found to be 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the rate-limiting enzyme for de-novo cholesterol biosynthesis (Shapiro & Rodwell, 1972). This enzyme occurs exclusively in the microsomal membrane fraction of cell homogenates, with over 80% of the enzyme being associated with the smooth endoplasmic reticulum, Golgi and plasma membrane (Goldfarb, 1972). HMG-CoA reductase exists in an active (dephosphorylated) and an inactive (phosphorylated) form (Beg & Brewer, 1978).

Several hormones have been implicated in the regulation of HMG-CoA reductase. Those that increase HMG-CoA reductase activity include insulin (White, 1972), thyroid hormone (Guder et al., 1968), adrenaline and noradrenaline (Edwards, 1973). Hormones which have been shown to decrease HMG-CoA reductase include glucagon (Huber et al., 1973) and the glucocorticoids (Dugan et al., 1974). The sex steroids, oestrogens, progestagens and androgens, can also suppress

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reductase activity of human fibroblasts when added to culture medium (Brown & Goldstein, 1974) or hepatic reductase activity when fed or injected into rats or mice (Mukherjee & Bhose, 1968; Kandutsch & Packie, 1970). In contrast, oestradiol stimulates HMG–CoA reductase activity in uterine epithelial cells (Wilce et al., 1984) and rat corpora lutea (Azhar et al., 1985). This suggests that oestradiol may regulate HMG–CoA reductase activity differently in target tissues that have high oestrogen receptor levels than in non-target tissues.

Oestradiol is the principal luteotrophic hormone in the rabbit, and it can maintain the usual patterns of serum progesterone concentrations during pseudopregnancy in the hypophysectomized animal (Bill & Keyes, 1983). Although it is presumed that oestradiol acts via an oestrogen receptor to regulate luteal function (Mills & Osteen, 1977; Miller & Keyes, 1978; Miller & Toft, 1983), the cellular mechanisms through which oestradiol regulates steroidogenesis in this tissue are as yet unknown. In the rabbit corpus luteum, the level of HMG–CoA reductase activity peaks on Day 5 of pregnancy or pseudopregnancy, then rapidly declines on Days 5–7 to intermediate values which are maintained throughout the remainder of pregnancy or pseudopregnancy (Kovanen et al., 1978). Coincident with the decrease in HMG–CoA reductase activity is an increase in oestradiol binding in corpora lutea (Mills & Osteen, 1977; Miller & Toft, 1983) and a decrease in plasma cholesterol (Zilversmit et al., 1972). These changes occur within a span of 2–3 days during early luteal development. This suggests that oestradiol may regulate de-novo cholesterol synthesis or its utilization by the corpus luteum. The purpose of the present study was therefore to examine the effect of (1) oestradiol on HMG–CoA reductase activity during pseudopregnancy and (2) decreased serum cholesterol concentrations on luteal HMG–CoA reductase activity and progesterone production in the rabbit. Some of the results have been presented in abstract form (McLean & Miller, 1986b).

Materials and Methods

**Chemicals and materials.** Mevalonic acid, HMG–CoA, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, hCG, and a cholesterol kit were obtained from Sigma Chemical Co. (St Louis, MO). 4-Aminopryrazole-(3,4-d)pyrimidine was a product of Aldrich Chemical Co. (Milwaukee, WI). Silica gel G thin-layer chromatography plates were obtained from Analtech (Newark, DE). DL-3-Hydroxy-3-methyl-[3-14C]glutaryl-CoA, [5-3H]mevalonic acid, [1,2,6,7-3H]progesterone and [2,4,6,7-3H(N)]oestradiol were obtained from New England Nuclear Co. (Boston, MA). Xylazine (Rompun, 20 mg/ml) was obtained from Baynet (Shawnee, KS), and ketamine (Ketal; 100 mg/ml) was obtained from Parke-Davis (Morris Plains, NJ). The competitive inhibitor of HMG–CoA reductase (compactin ML 236B) was a gift from Dr S. Silavin and Dr J. Strauss III (University of Pennsylvania, Philadelphia, PA) who had obtained this drug from Dr A. Endo (San Kyo, Ltd, Tokyo, Japan).

**Animals.** Mature female New Zealand White rabbits (3–5 kg) were obtained from Langshaw Farms (Augusta, MI) and maintained in individual cages under controlled conditions with free access to lab chow and water. Pseudopregnancy was induced with 50 i.u. hCG; the day of hCG injection was designated Day 0. To examine the effect of oestradiol during late pseudopregnancy (Day 20), rabbits were hysterectomized on Day 1 to remove any uterine luteolytic factors which would cause luteal regression. Hysterectomies were performed under sterile conditions via a mid-ventral incision, using xylazine (40 mg, i.m.) and ketamine (200 mg, i.m.) as anaesthetics. An empty Silastic implant or an implant containing 1.0 cm oestradiol-17β (Holt et al., 1975) was implanted at the base of the neck at the time of hCG injection. Blood samples were taken from the marginal ear vein beginning on Day 0 and every 4th day thereafter until the animals were killed to obtain corpora lutea for HMG–CoA reductase measurements. On each of Days 4, 8, 12, 16 and 20, 4 animals were killed by cervical dislocation.

**Treatment of blood samples.** Blood samples were taken from the marginal ear vein, allowed to clot for 24 h at 4°C and centrifuged at 800 g for 20 min at 4°C. The serum was removed and stored at −20°C until steroid determinations could be made. All serum samples from an individual rabbit were analysed in the same assay and each assay included samples from control and experimental animals.

**Serum cholesterol determination.** Cholesterol was assayed by the cholesterol oxidase method (Allain et al., 1974) using a kit sold by Sigma. Serum samples (10 μl) were assayed in duplicate and, after cholesterol reagent addition, sample absorbance was determined spectrophotometrically. Serum cholesterol was determined by comparison to known cholesterol standards and expressed in mg/dl.

**Serum oestradiol determination.** Oestradiol concentrations were measured in samples, taken on the day of HMG–CoA reductase determination, using a specific rabbit antiserum (GDN No. 930) raised against 17β-oestradiol-11-hemisuccinate conjugated to bovine serum albumin. The specificity of the antiserum has been described by England.
et al. (1974). Each sample was extracted with benzene and chromatographed on Sephadex LH-20 columns using a benzene:methanol (9:1, v/v) solvent system (Carr et al., 1971). Samples were assayed in duplicate at 4°C and incubated with antibody (final dilution of 1:12000) and [3H]oestradiol. Bound ligand was separated from free by charcoal absorption (0.25% Norit A and 0.025% dextran T70 in PBS). This assay has a sensitivity of 0.2–0.3 pg, and an average 50% inhibition point of 12–15 pg. The mass of oestradiol in each sample was corrected for procedural losses (recoveries averaged 65%) and oestradiol was expressed as pg/ml serum.

Serum progesterone determination. Progesterone concentrations were measured throughout pseudopregnancy (Days 0, 4, 8, 12, 16, 20) by the radioimmunoassay described by Day & Birnbaumer (1980). The antisera used (GDN No. 337) was raised in rabbits against progesterone-11-chloroformate conjugated to bovine serum albumin and has been characterized by Gibori et al. (1977). Each sample was extracted into hexane and assayed in duplicate with [3H]progesterone and antibody (final dilution of 1:5000). Bound ligand was separated from free by charcoal absorption (0.25% Norit A and 0.025% dextran T70). Results were calculated using the logit-log weighted-linear regression program of Rodbard et al. (1980). The inter- and intra-assay coefficients of variation were 5.9% (n = 22) and 7.4, 6.5, and 3.6% at 20, 45, and 60% binding, respectively (n = 10). Serum progesterone concentrations from each rabbit have been normalized assuming 10 CL per animal.

Determination of HMG-CoA reductase. Activity was measured by the method of Kovanen et al. (1978) with slight modifications. Rabbit ovariates were removed after cervical dislocation and placed in cold HBSS (4°C). Corpora lutea were dissected out on ice and 3–6 corpora lutea were homogenized in 1 ml buffer containing 0.3 M sucrose, 25 mM-mercaptoethanol, 5 mM-dithiothreitol, 10 mM-EDTA, and either 50 mM-NaCl or 50 mM-NaF (pH 7.0). Sodium fluoride was included in the homogenization buffer to inhibit protein phosphatase activity which activates the HMG-CoA reductase (Ingebritsen et al., 1981). Thus, corpora lutea isolated in 50 mM-NaCl buffer represents an estimation of the total HMG-CoA reductase activity whereas luteal tissue isolated in 50 mM-NaF buffer represents an estimation of the active enzyme level. The homogenization was performed at 4°C with a Dounce homogenizer using 20 strokes each with a loose and tight fitting pestle. The resulting homogenate was centrifuged at 20,000 g for 20 min to remove unbroken cells and nuclei and the supernatant was centrifuged at 100,000 g for 60 min to isolate luteal microsomes. The microsomes were resuspended in 1:0 ml buffer containing 0.1 M-potassium phosphate (pH 7.5) and 5 mM-dithiothreitol. A fraction of the resuspended microsomes (10 µl) was used to estimate protein content using the technique of Bradford (1976). HMG-CoA reductase activity in luteal microsomes was estimated by assaying 15 and 30 µg micromolar protein in 190 µl solution containing 0.1 M-potassium phosphate, 2.5 mM-NADPH, 10 mM-glucose-6-phosphate, 10 mM-sodium EDTA, and 5 mM-dithiothreitol (pH 7.5). Microsomes were preincubated in this solution for 10 min at 37°C, after which 10 µl of 3-hydroxy-3-methyl-[14C]glutaryl CoA (10,000–10,000 c.p.m.) to a final concentration of 87 µM was added to start the incubation. Incubation was carried out at 37°C for 30 min and was stopped by the addition of 30 µl 5 N-HCl. [5-3H]Mevalonic acid (2 µM; 20,000–40,000 c.p.m.) was then added to each incubate and the incubation continued for 30 min. About 500 mg sodium sulphate were added to each incubation tube and the mevalonic acid was extracted twice using ether (8 ml total). The ether was evaporated to dryness under N2 gas, the residue dissolved in 80 µl acetone containing 2 mg unlabelled mevalonic acid and applied to silica gel G–thin layer chromatography plates. Plates were developed in acetonene:benzene (1:1, v/v) and the mevalonate spots were identified by exposure to iodine vapour. Spots were scraped into scintillation vials and counted in 10 ml 3a70b scintillation fluid. The 14C radioactivity incorporated into mevalonate was calculated using [3H]mevalonate to correct for procedural loss (recovery averaged 45–50%). Enzyme activity was expressed as nmol mevalonate formed per min per mg protein.

4-Aminopyrazolo(3,4-d) pyrimidine administration. 4-Aminopyrazolo(3,4-d) pyrimidine (APP) was used to determine the effect of reduced serum cholesterol concentrations (Morrin et al., 1979) on progesterone production and HMG-CoA reductase activity in oestrogen-treated rabbits. On Days 9–11, oestrogen-treated rabbits were injected with APP (25 mg/ml in saline, 10 mg/kg, pH 2.5) or saline administered i.p. Daily blood samples were taken just before each APP injection for the determination of serum cholesterol and progesterone. Rabbits were killed on Day 12 and the corpora lutea removed for HMG-CoA reductase determination.

Dissociation of rabbit luteal tissue and incubation with compactin. Corpora lutea were dissected on Day 10 of pseudopregnancy and dissociated as previously described (McLean & Miller, 1985). The dissociation medium contained 250 U collagenase/ml, 50 µg DNase/ml, 750 µg trypsin inhibitor/ml, 20 mg BSA/ml, and 100 µg gentamicin/ml in HBSS.

Dissociated luteal cells were incubated (100,000 cells/ml) for 1 h in HBSS (6 ml) gassed with 95% O2 and 5% CO2 at 37°C and then 0–10 µg compactin/ml was added for 4 additional hours of incubation. Media fractions (1 ml) containing cells and medium were removed each hour, cells were separated by centrifugation (500 g for 5 min) and the media stored for progesterone analysis.

Statistical analysis. Results are expressed as the mean ± s.e.m. Values were compared by analysis of variance and, when significant differences were noted, groups were compared using Student–Newman–Keuls multiple comparison test (Zar, 1974). A P value of <0.05 was considered significant.
Results

Serum oestradiol, progesterone and cholesterol concentrations

Rabbits treated with a 1-0-cm oestradiol implant had significantly elevated oestradiol values when compared to animals receiving an empty implant (Table 1). Even though oestradiol concentrations were different in control and oestradiol-treated animals, serum cholesterol and progesterone did not differ in the non-hysterectomized rabbits (Table 1). Oestradiol treatment maintained elevated serum progesterone concentrations in hysterectomized animals on Days 16 and 20 of pseudopregnancy as seen previously (Miller & Keyes, 1976), but no differences in serum cholesterol values were observed (Table 1).

Table 1. The effect of oestrogen on serum sterol levels in pseudopregnant rabbits

<table>
<thead>
<tr>
<th></th>
<th>Rabbits with intact uterus</th>
<th>Hysterectomized rabbits</th>
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<tr>
<td></td>
<td>Day 4</td>
<td>Day 8</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.6 ± 1.0</td>
<td>14.4 ± 1.8</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>6.2 ± 0.9</td>
<td>12.7 ± 1.4</td>
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<tr>
<td>Cholesterol (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>68.3 ± 11.3</td>
<td>68.8 ± 8.5</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>79.9 ± 8.4</td>
<td>73.9 ± 5.6</td>
</tr>
<tr>
<td>Oestradiol (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.4</td>
<td>3.67 ± 0.5</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>15.4 ± 2.9</td>
<td>10.7 ± 1.5</td>
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Values are mean ± s.e.m. for 4 observations.

HMG-CoA reductase activity in the corpus luteum

Rabbits treated with and without oestradiol had similar levels of total HMG-CoA reductase activities on Day 4 (Fig. 1), but by Days 8 and 12 of pseudopregnancy HMG-CoA reductase activity was significantly reduced in oestradiol-treated animals. The effect of oestradiol on total HMG-CoA reductase activity was no longer observed by Day 16 of pseudopregnancy in hysterectomized rabbits. The active form of HMG-CoA reductase represented less than 20% of the total reductase enzyme activity present in luteal microsomes.

Effect of lower serum cholesterol on HMG-CoA reductase activity

Rabbits treated with APP had significantly \((P < 0.05)\) lower serum cholesterol concentrations compared to control animals \((23.8 ± 7.2 \text{ vs } 60.1 ± 18.9 \text{ mg/dl, respectively, Day 12). Since cholesterol measurements were made 24 h after APP administration, the cholesterol values in APP-treated animals represent the highest levels reached during drug treatment. There was, however, no change in the serum progesterone concentrations \((14.8 ± 3.6 \text{ vs } 17.9 ± 2.4 \text{ ng/ml in APP-treated and control rabbits, Day 12). The total activity of HMG-CoA reductase increased slightly with APP treatment, but this change was not significant \((4.28 ± 0.66 \text{ vs } 5.2 ± 0.5 \text{ nmol/min/protein). In contrast, there was a significant \((P < 0.05)\) increase in the active form of HMG-CoA reductase in luteal microsomes on Day 12 of pseudopregnancy from rabbits treated with APP \((0.90 ± 0.14 \text{ vs } 1.82 ± 0.26 \text{ nmol/min/mg/protein). Inhibition of HMG-CoA reductase in vitro

The addition of compactin \((0-001–10 \mu g/ml), a competitive inhibitor of HMG-CoA reductase (Kaneko et al., 1978; Silavin & Strauss, 1983), had no effect on progesterone secretion by dissociated
Fig. 1. Luteal cell HMG–CoA reductase activity in control (open bars) and oestradiol treated rabbits (hatched bars). Entire bars represent the total luteal reductase activity when microsomes were prepared in NaCl buffer and the black area of the bar represents the active portion of luteal reductase activity when microsomes were prepared in NaF buffer (see text). Values are mean \pm s.e.m. of 4 experiments. Hyst. = hysterectomized.

Table 2. The effect of compactin on rabbit luteal cell progesterone production (ng/ml) in vitro*

<table>
<thead>
<tr>
<th>Dose of compactin (µg/ml)</th>
<th>Hours of incubation</th>
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<tr>
<td></td>
<td>1*</td>
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<td></td>
<td>2</td>
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<td>3</td>
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<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>0 (control)</td>
<td>27.0 ± 3.9</td>
</tr>
<tr>
<td>0.001</td>
<td>21.2 ± 3.4</td>
</tr>
<tr>
<td>0.01</td>
<td>20.9 ± 3.4</td>
</tr>
<tr>
<td>0.1</td>
<td>26.7 ± 4.9</td>
</tr>
<tr>
<td>1.0</td>
<td>28.2 ± 4.9</td>
</tr>
<tr>
<td>10</td>
<td>34.1 ± 6.8</td>
</tr>
</tbody>
</table>

*Values are mean \pm s.e.m. for 5 incubations in static culture.
†During the first hour cells were exposed to HBSS only; treatment started at Hour 2.

luteal cells during 4 h of incubation (n = 5; Table 2). Progesterone concentrations in media from compactin-treated incubations were not significantly different from control incubations at any compactin concentration tested.

Discussion

It is well-established that functional luteal cells must have an efficient means of obtaining cholesterol for steriodogenesis (Strauss et al., 1981). The rabbit corpus luteum contains high levels of the rate-limiting enzyme of cholesterol synthesis, HMG–CoA reductase, which suggests that de-novo cholesterol synthesis may be the essential pathway through which rabbit luteal cells acquire cholesterol for progesterone production. Since the activity of this enzyme begins to decline after Day 5 of pregnancy (Kovanen et al., 1978) at the time when oestradiol binding to corpora lutea is first
observed (Mills & Osteen, 1977), we have examined the possibility that oestradiol regulates the activity of this enzyme during pseudopregnancy. In this study, we found that, when serum oestradiol concentrations were elevated, the total activity of luteal HMG-CoA reductase was not altered during early pseudopregnancy (Day 4) but was decreased in oestradiol-treated intact pseudopregnant rabbits on Days 8 and 12. By Day 16 in intact or Day 20 in hysterectomized pseudopregnant rabbits, oestradiol treatment no longer affected total HMG-CoA reductase activity. In contrast, the active form of HMG-CoA reductase, which is considered to represent the enzyme activity in situ (Carr et al., 1980), was unaltered by oestradiol.

De novo cholesterol synthesis did not seem to play a significant role during short-term steroidogenesis in vitro since compactin was without effect on progesterone production by rabbit luteal cells during a 4-h incubation. Although we did not directly confirm that compactin inhibited HMG-CoA reductase in our cells, the maximal dose of compactin used (10 µM) was 3–7-fold the amount required to inhibit cholesterol synthesis in human fibroblasts (Kaneko et al., 1978) and human granulosa cells (Tureck & Strauss, 1982). Similar results (i.e. failure to inhibit steroidogenesis with compactin) have been observed with hamster luteal and granulosa cells during short-term incubations (Silavin & Strauss, 1983).

HMG-CoA reductase is regulated by two separate mechanisms which involve short-term modulation of the phosphorylated/dephosphorylated enzyme state, and long-term regulation of tissue enzyme concentrations (Beg & Brewer, 1981). Since prolonged oestrogen treatment did not change the level of active HMG-CoA reductase and decreased the total (active + inactive) enzyme activity, oestrogen does not appear to regulate the phosphorylated/dephosphorylated state of the enzyme (short-term regulation) but may regulate the total content of luteal HMG-CoA reductase. These results are consistent with those of Wittmaack et al. (1986) who found that HMG-CoA reductase activity was not significantly altered after oestrogen removal in the rabbit despite a dramatic decrease in serum progesterone. In addition, McNamara & Rodwell (1972) have demonstrated that steroids can suppress HMG-CoA reductase activity solely by decreasing the quantity of reductase protein.

Morphometric analysis has shown that prolonged oestrogen treatment increases the content of luteal lipid droplets, i.e. esterified cholesterol (McLean & Miller, 1987). Since oestrogen treatment did not alter serum cholesterol concentrations at any time throughout the experiment, increased cholesterol ester stores may have suppressed HMG-CoA reductase activity through feedback inhibition (Brown et al., 1979). This hypothesis is consistent with the findings of Goldstein & Brown (1974) who showed that cholesterol ester levels were inversely related to reductase activity in cultured fibroblasts. In addition, it has been shown that the direct addition of cholesterol esters to cultured cells suppresses reductase activity more effectively than does cholesterol itself (Watson et al., 1974).

Although oestradiol appears only to exert long-term regulation of rabbit luteal HMG-CoA reductase, this enzyme is also acutely affected by changes in serum cholesterol values. When serum cholesterol concentrations were decreased by APP (Morrin et al., 1979) in oestrogen-treated rabbits, the total enzyme activity increased only slightly while the active form of this enzyme was significantly ($P < 0.05$) elevated in APP-treated animals. These results suggest that HMG-CoA reductase activity increases to compensate for the loss of exogenous cholesterol. Similar results have been reported for rat luteal tissue after treatment with APP (Christie et al., 1979; Azhar & Menon, 1981, 1982). Since HMG-CoA reductase activity increases in response to lowered serum cholesterol, it is clear that rabbit luteal tissue can utilize exogenous sources of cholesterol in situ. This suggests that despite high levels of HMG-CoA reductase in the rabbit corpus luteum, lipoprotein-derived cholesterol can also be used as a substrate for steroidogenesis. This is consistent with our previous studies which demonstrate that both low-density lipoprotein and high-density lipoprotein can stimulate progesterone production by dissociated rabbit luteal cells in vitro (McLean & Miller, 1986a). Thus, both de-novo-derived and lipoprotein-derived cholesterol appear to contribute to maintaining stable progesterone output by rabbit luteal cells.
From these results and those of our previous studies, we suggest that oestradiol indirectly controls HMG–CoA reductase by increasing cholesterol stores, thus decreasing luteal enzyme activity (long-term regulation). Short-term regulation of HMG–CoA reductase appears to be regulated by serum cholesterol concentrations. These results are consistent with the hypothesis that the luteotropic role of oestradiol in the rabbit is to maintain a constant steroidogenic pool of cholesterol rather than to regulate directly de-novo cholesterol biosynthesis.

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