Stimulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in mouse uterine epithelial cells by oestradiol-17 β

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The characteristics of 3-hydroxy-3-methylglutaryl-CoA reductase from mouse uterine epithelial cells were studied. Preliminary experiments showed that enzyme activity was stimulated approx. 10-fold 18 h after administration of 100 ng of oestradiol-17 β . This activity was associated with all particulate fractions of the uterine luminal cell. The K_m for D-3-hydroxy-3-methylglutaryl-CoA was $5.54 \pm 1.12 \mu\text{M}$. The detailed time-course of oestrogen stimulation showed two peaks of activity, 9 and 15 h after hormone treatment. The DNA content of the epithelial cells doubled between 6 and 12 h after hormone treatment, whereas the protein content increased linearly over the 18 h period. The peak of enzyme activity at 9 h is associated with early S phase of the epithelial cells; the peak at 15 h may be associated with a second S phase or with mitosis. Pretreatment with progesterone for 3 days before injection of oestradiol-17 β (a treatment which inhibits uterine epithelial DNA synthesis) reduced the oestrogenic stimulation of enzyme activity by 63%; progesterone treatment alone did not stimulate enzyme activity. These data suggest that uterine epithelial 3-hydroxy-3-methylglutaryl-CoA reductase may play an important role in the cell cycle in this tissue.

Oestradiol-17 β stimulates uterine growth in the ovariectomized rodent. During this process there is increased synthesis of most lipid classes and mobilization of lipid deposits (Beall, 1972; Nilsson, 1964). Attention has focused on two areas, the stimulation of phospholipid synthesis, particularly phosphatidylethanolamine, very soon after oestrogen stimulation (Aizawa & Mueller, 1961), and the mobilization of neutral lipid reserves linked with the oestrogen-dependent process of embryo implantation (Boshier *et al.*, 1981). In addition, significant changes in the lipid composition of the uterus occur in the oestrus cycle and during the response of the uterus of ovariectomized animals to oestrogen and progesterone treatment (Beall, 1972). Quantitative analysis has shown that, in the mouse, 27% of uterine lipid is cholesterol, and its esterification changes during the oestrus cycle (Goswami *et al.*, 1963). Further, increased uterine biosynthesis of cholesterol, as measured by the

incorporation of [1- ^{14}C]acetate *in vivo*, has also been reported after oestrogen stimulation (Emmelot & Bosch, 1954).

Recently the cholesterol biosynthetic pathway, and in particular activity of the rate-limiting enzyme, HMG-CoA reductase (EC 1.1.1.34), has been shown to be intimately linked with the cell cycle of synchronized cultured cells (Brown & Goldstein, 1980). Inhibition of this enzyme results in an inhibition of DNA synthesis and/or mitosis in a number of cell types (Cornell *et al.*, 1977; Kandutsch & Chen, 1977). Mouse uterine epithelial cells, when stimulated by a single injection of oestradiol-17 β , enter a round of DNA synthesis and mitosis with a well-characterized time course and a high degree of synchrony (Martin, 1980). We have therefore chosen to examine the activity of HMG-CoA reductase in these cells after hormone stimulation. We report an initial characterization of the enzyme, its hormone stimulation and its relationship to the cycle of the cells of the mouse uterine luminal epithelium.

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

Materials and methods

Chemicals

Reagents were purchased as follows: oestradiol-17 β , progesterone, NADP⁺, dithiothreitol, DL-HMG-CoA, glucose 6-phosphate and glucose-6-phosphate dehydrogenase from Sigma; mevalonic acid lactone from Fluka Chemicals, Switzerland; DL-[3-¹⁴C]HMG-CoA (60mCi/mmol) and DL-[2-³H]mevalonic acid lactone (1Ci/mmol) from Amersham Australia, Sydney, N.S.W., Australia. All other reagents were of analytical grade.

Treatment of animals

Female Quackenbush mice, obtained from the Central Animal Breeding House of the University of Queensland, were maintained on 12h light:12h dark cycle before use. The animals were ovariectomized when 5–6 weeks old as described by Martin & Finn (1978), and used 2–3 weeks later. Ovariectomized animals were 'primed' by administration of 200ng of oestradiol-17 β /ml of drinking water for 3 days, 5 days before the start of each experiment. Oestradiol-17 β (100ng in 0.05 ml of arachis oil) was injected subcutaneously, and the animals were killed by cervical dislocation up to 18h later. Control animals received oil only.

Animals to be treated with progesterone were 'primed' with oestrogen in the drinking water for 3 days as described above, rested for 2 days and then given 1 mg of progesterone in 0.05 ml of arachis oil subcutaneously each day for 3 days. A single injection of oestradiol-17 β (100ng in 0.05 ml of arachis oil) was given subcutaneously at the same time as the last injection of progesterone.

Preparation of mouse uterine tissue extracts

The uterine luminal epithelial cells were removed with a tissue purity of about 80% by the method of Fagg *et al.* (1979). The uterine horns from groups of 10 animals were opened longitudinally *in situ*, dissected free of mesenteric fat and placed into a buffer consisting of 0.1M-sucrose/50mM-KCl/30mM-disodium EDTA/10mM-potassium phosphate/5mM-dithiothreitol, pH7.6. Four uterine horns were shaken vigorously in a round-bottomed glass tube with five glass beads (5mm diameter) for 2min in 1 ml of buffer using a vortex shaker. The residual tissues (stroma, myometrium and glands) were removed and replaced by four more horns. The process was repeated so that up to 20 uterine horns were processed into 1 ml of buffer. Unbroken cells and large sheets of membranes were disrupted by hand homogenization in a 10ml Teflon/glass Potter-Elvehjem homogenizer. Before centrifugation the homogenate was diluted to 2ml with buffer. Removal of

the luminal epithelium was monitored by routine histology of the residues.

The extracts were centrifuged at 105000g for 60min. To obtain the particulate fraction the pellet was resuspended in 1 ml of buffer by gentle homogenization with a Teflon/glass Potter-Elvehjem homogenizer. In some experiments, the homogenate was first centrifuged at 600g for 10min and then at 15000g for 20min before the 105000g centrifugation. The pellets from these centrifugations were resuspended in 0.35ml of buffer with the Teflon/glass homogenizer as above.

Assay of HMG-CoA reductase

Assays in a final volume of 60 μ l contained 3mM-NADP⁺, 30mM-glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase, up to 66.7 μ M-DL-[3-¹⁴C]HMG-CoA (4.54 μ Ci/mmol) and approx. 140 μ g of mouse uterine epithelial cell protein. Triplicate assays were preincubated at 37°C for 15min before the reaction was started by the addition of the DL-[3-¹⁴C]HMG-CoA. After 15min incubation, the reaction was terminated with 50 μ l of 4M-HCl. DL-[methyl-³H]Mevalonic acid lactone (18 μ Ci/mmol) (0.01 ml of 0.23M) was added to monitor the efficiency of extraction of the product. The entire reaction mix was poured onto a strip of Whatman 3MM chromatography paper and thoroughly dried in an oven at 60°C for 15min. The product was eluted overnight, directly into scintillation vials, with toluene containing 2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (50mg/l), and ¹⁴C and ³H were determined in a liquid-scintillation counter. Duplicate blanks without nucleotide mix were used throughout to monitor and correct for the activity of an HMG-CoA-utilizing enzyme as previously reported for rat liver by Ness & Moffler (1979). Specific activity of the HMG-CoA reductase is expressed as nmol of mevalonate formed/min per mg of protein corrected for incorporation in blank assays.

Determination of K_m and V_{max}

Groups of 10 animals were killed 18h after oestrogen stimulation and uterine epithelial HMG-CoA reductase was assayed in quadruplicate over the range of D-HMG-CoA concentration 1–10 μ M. Hyperbolic plots were analysed by non-linear regression as described by Duggleby (1981).

Determination of protein and DNA

Protein was measured by the method of Lowry *et al.* (1951) with corrections for dithiothreitol in the samples. DNA was measured by the method of LaBarca & Paigen (1980).

Results

Initial characterization of the enzyme

In initial experiments to characterize HMG-CoA reductase activity in the uterine luminal epithelium, ovariectomized animals were killed 18 h after administration of 100 ng of oestradiol and a total particulate fraction was isolated by centrifugation at 105 000 *g* as described in the Materials and methods section. Production of mevalonate was linear up to a protein concentration of 280 μ g/assay and linear with time up to 60 min. We found little enzyme activity in the uterine epithelium from untreated ovariectomized animals, but noted that activity increased markedly 18 h after subcutaneous administration of 100 ng of oestradiol-17 β (control, 0.018 ± 0.0035 nmol of product/min per mg of protein; oestrogen-stimulated, 0.201 ± 0.025 nmol of product/min per mg of protein; means $\pm$ S.E.M. for four experiments with groups of 10 mice; $P < 0.02$).

The K_m for D-HMG-CoA was estimated to be 5.54 ± 1.12 μ M and the V_{max} 0.179 ± 0.038 nmol/min per mg of protein. These data are the means $\pm$ S.E.M. for three separate experiments each using groups of 10 oestrogen-stimulated uteri.

In an attempt to localize this activity within the uterine epithelial cell, a crude subcellular fractionation was achieved by differential centrifugation. High levels of activity were found in all the particulate fractions (Table 1) with minimal activity in the cytosol. The small amount of material available made characterization (particularly for cross-contamination) of these fractions impossible. In subsequent experiments the total particulate fraction was used for assays of enzyme activity. We attempted to characterize the enzyme in the uterine residual tissues. However, because of myofibrillar contamination, we were unable to isolate a fraction comparable with the epithelial particulate fraction. Even so we ascertained that the total activity of the residue represented 56% of the total oestrogen-stimulable uterine activity.

Since the residual tissues contain 90% of the cells of the uterus (Martin *et al.*, 1973b), the enzymic activity of this fraction on a per cell basis is no more than 10% of that of the luminal epithelial cells.

In our initial experiments we detected a high level of an HMG-CoA-utilizing enzyme. Such an enzyme has been reported in rat liver microsomal extracts by Ness & Moffler (1979). This enzyme also utilizes HMG-CoA as substrate and produces an as yet unidentified product that co-elutes with mevalonic acid lactone in some conditions, but can be distinguished in others [e.g. chromatography on Bio-Rad AG1 X8 (formate) column]. In contrast with HMG-CoA reductase, which requires NADPH for its activity, the HMG-CoA-utilizing enzyme is active in the absence of the nucleotide and inhibited by its presence. Consequently the activity of the utilizing enzyme can be detected and monitored in the absence of nucleotide mix. We found that much of the activity of this enzyme could be removed from epithelial preparations by dilution of the homogenate before centrifugation. Activity was also reduced by preincubation of the extract for 15 min in the presence of nucleotide as described by Ness & Moffler (1979). We further monitored for residual activity as described in the Materials and methods section.

Time course of hormonal induction of HMG-CoA reductase activity

The time course of changes in uterine epithelial HMG-CoA reductase activity after oestrogenic stimulation is shown in Fig. 1. The experiment was replicated in its entirety three times. At each time point in each replicate, triplicate estimations of enzyme activity were made on uterine epithelial particulate fractions pooled from groups of 10 mice.

Since overall measured levels of enzyme activity varied significantly from one replicate to another, the values from replicates 2 and 3 were normalized

Table 1. Distribution of HMG-CoA reductase activity in the mouse uterus

Preparation of subcellular fractions and HMG-CoA reductase assays are as described in the Materials and methods section. Groups of 30 animals primed 5 days beforehand were used 18 h after receiving 100 ng of oestradiol-17 β . The data are means $\pm$ S.E.M. for three replicate experiments.

Uterine fraction	Specific activity of HMG-CoA reductase (nmol/min per mg of protein)	% of total uterine HMG-CoA reductase activity
Epithelium		
Total particulate fraction	0.501 ± 0.042	42.5 ± 3.1
600 <i>g</i> pellet ('nuclear')	0.144 ± 0.023	20.7 ± 2.6
15000 <i>g</i> pellet ('mitochondria')	0.249 ± 0.063	12.1 ± 2.4
105000 <i>g</i> pellet ('microsomal')	0.108 ± 0.012	9.7 ± 4.3
Cytosol	0.002 ± 0.001	0.5 ± 0.4
Residual tissues (stroma/myometrium)	0.028 ± 0.008	56.1 ± 8.1

to those of replicate 1 by multiplying the raw data by a constant $\bar{X}_{r1}/\bar{X}_{rn}$ (where $\bar{X}_{r1}$ and $\bar{X}_{rn}$ are the overall mean values for replicates 1 and 1-3 respectively). Analysis of variance of the normal-

Fig. 1. Time course of HMG-CoA reductase activity in mouse uterine epithelial cells after a single injection of oestradiol-17 β

Animals were primed 5 days before injection of 100ng of oestradiol-17 β at time 0. The particulate fraction was isolated and HMG-CoA reductase activity was determined as described in the Materials and methods section. Each point is the mean specific activity of the particulate fraction $\pm$ S.E.M., based on the normalized data from three replicate experiments each with triplicate estimations on material pooled from 10 mouse uteri. The S.E.M. values are based on replicate means (i.e. $n = 3$).

ized data (Table 2) showed that although there was significant between-replicate variation in terms of within-group variability, the pattern of response shown in Fig. 1 was consistent and highly significant. Thus enzyme activity increased linearly from 0h to a peak at 9h ($P < 0.001$) without significant quadratic or cubic curvature (Table 2). Thereafter it fell significantly at 12h ($P < 0.01$) and subsequently rose to a second peak at 15h ($P < 0.01$) falling thereafter to 18h ($P < 0.01$). In the 18h following oestrogenic stimulation the total protein and DNA content of the particulate fraction increased by 8-fold and 2-fold respectively (Fig. 2).

Effect of progesterone pretreatment on enzyme activity

Treatment of mice with progesterone prior to the injection of oestradiol-17 β results in an inhibition of DNA synthesis in the uterine epithelial cells and the stimulation of division in the cells of the stromal layer (Martin *et al.*, 1973b). Table 3 shows the effect of pretreatment with progesterone on the stimulation of enzyme activity by oestradiol-17 β . In the uterine epithelial cells, progesterone pretreatment reduced enzyme activity to 37% of the oestrogen-treated control, while treatment with progesterone alone failed to stimulate enzyme activity.

Discussion

The results reported above are the first investigations of HMG-CoA reductase in uterine epithelial cells. The enzyme activity is stimulated by oestradiol and within the epithelial cell, is associated with the particulate fraction. In hepatocytes it is

Table 2. Analysis of variance of the data in Figure 1

** and ***, $P < 0.01$ and 0.001 respectively, tested against between-group variability; ††† $P < 0.001$ tested against within-group variability.

Source of variation	Degrees of freedom	Mean square	Variance ratio
Replicates	2		0.0
Time	(6)		
0, 3, 6, 9 h			
Linear	1	83291	28.1***
Quadratic	1	7112	2.4
Cubic	1	16	0.0
9, 12, 15, 18 h			
Linear	1	5522	1.9
Quadratic	1	3192	1.1
Cubic	1	28100	9.5**
Replicates $\times$ time interaction (between-group variability)	12	2698	7.58†††
Within-group variability	39	356	—
Missing plots	3	—	—
Total	62

Fig. 2. Time-course of the increase in DNA and protein content of the particulate fraction of mouse uterine epithelial cells. Animals were primed 5 days before injection of 100 ng of oestradiol-17 β at time 0. The particulate fraction was isolated and the protein (Δ) and DNA ($\bullet$) contents were determined as described in the Materials and methods section. The data are means $\pm$ S.E.M. for three replicate experiments each utilizing 10 animals at each time-point.

Table 3. Effect of progesterone pretreatment on uterine luminal epithelial HMG-CoA reductase

Mice, pretreated with progesterone or vehicle for 3 days, were killed 12 h after administration of 100 ng of oestradiol-17 β or vehicle. The particulate fraction was isolated and HMG-CoA reductase activity determined as described in the Materials and methods section. The data are expressed as normalized mean specific activities $\pm$ S.E.M. for four experiments with 10 animals for each treatment, or as a percentage of the activity of the corresponding oestrogen-stimulated group.

Treatment	HMG-CoA reductase activity	
	Specific activity (nmol/min per mg of protein)	% of oestrogen-treated group
Control	0.015 $\pm$ 0.006	9 $\pm$ 4.1
Oestrogen	0.188 $\pm$ 0.039	100
Progesterone	0.015 $\pm$ 0.004	8 $\pm$ 2.4
Progesterone + oestrogen	0.063 $\pm$ 0.030	37 $\pm$ 11.3

considered that HMG-CoA reductase is a microsomal enzyme. Bell *et al.* (1976) reported that in HTC cells, enzyme activity was associated with the low-speed pellet as well as the high-speed (100 000g) fraction. However they were unable to state whether this distribution was a feature of HTC cells or reflected an inability to isolate pure subcellular fractions from them. In intestinal cells the original finding that much of the HMG-CoA reductase activity was located in the mitochondrial fraction has been challenged and present evidence indicates that the enzyme is of microsomal origin (Field *et al.*, 1982). In the uterine epithelial cell we find activity in the non-microsomal fractions. However since we were unable to characterize fully these fractions, because of the small yield, the

spread of activity may result from contamination with endoplasmic reticulum.

The K_m of D-HMG-CoA for the uterine enzyme is similar to the K_m of the rat liver enzyme (3.5 μ M) recently reported by Gregg (1983) using similar methods of estimation and within the lower range of the estimates of the K_m for liver enzyme (1.01–27 μ M) reported by other authors (Langdon & Counsell, 1976; Shapiro & Rodwell, 1971).

The data displayed in Fig. 2 represent the DNA and protein content of the uterine epithelial particulate fraction. After a short lag, the protein content increased linearly to 18 h. The DNA content increased after 6 h of oestrogen stimulation and had doubled by 12 h. These measurements are consistent with the earlier finding of Martin *et al.*

(1973a) who showed that the proportion of cells in S phase first increases 6 h after oestrogen treatment, and rises steeply to a peak at 12–15 h when 60–70% of cells are in S phase, while mitoses increase from 12 h onwards to reach a peak 18 h after treatment. Fig. 1 indicates that the first peak of enzyme activity is associated with early S phase. This increase in HMG-CoA reductase may be analogous to the peak of enzyme activity which is associated with early S phase in synchronized BHK-21 cells (Quesney-Huneus *et al.*, 1979), and in platelet-derived-growth-factor-stimulated smooth muscle cells (Habernicht *et al.*, 1980). Brown & Goldstein (1980) concluded that one function of HMG-CoA reductase was to supply a non-sterol derivative of mevalonic acid necessary for the S phase of the cell cycle. Recently Quesney-Huneus *et al.* (1983) showed that cells were blocked at two distinct stages of the cell cycle, i.e. early G₁ and G₁/S, when squalene and mevalonate production respectively were inhibited. It is tempting to suggest that the pre-S phase increase in HMG-CoA reductase serves a similar function in the uterine epithelial cell, supplying compounds required for completion of the G₁/S phase of the cell cycle.

The function of the second peak is less clear. It has been argued that continued HMG-CoA reductase activity is also required for mitosis, presumably for the provision of cholesterol for new membranes (Gregg & Wilce, 1983). The peak of activity at 15 h after hormone treatment would coincide with increasing mitosis in these cells. However, Martin *et al.* (1973a) noted that oestrogen-stimulated cells enter a second round of replication over this period. It is therefore conceivable that the second peak of HMG-CoA reductase activity may be associated with this second S-phase.

Progesterone treatment of the animal results in two major changes in the response of uterine cells to oestrogen stimulation. In the epithelium, oestrogen-stimulated cell division is inhibited, whilst cell division in the connective tissue stroma is increased (Martin *et al.*, 1973b). In addition, progesterone treatment alone results in differentiation of the epithelial layer and an associated deposition of lipid in the cells (Martin & Finn, 1978). We have examined HMG-CoA reductase under these conditions. Progesterone pretreatment causes a marked inhibition of the oestrogen-stimulated increase in HMG-CoA reductase in the epithelial cells, but progesterone treatment alone had no effect. It would appear then that the stimulation of HMG-CoA reductase in the uterine epithelium is oestrogen-specific and may be associated with proliferation of the epithelial cells.

In conclusion, it would appear from these data that epithelial cell HMG-CoA reductase is particu-

larly sensitive to oestrogen stimulation, increasing over and above the well-characterized increase in protein content of the cells (a 9-fold increase in enzyme activity compared with a 2-fold increase in particulate fraction protein at 9 h; Fig. 2). We suggest that the data presented here are consistent with a link between this enzyme and the early phases of the cell cycle. Further work will be necessary to elucidate the time course of synthesis of other lipid classes and the relationship of HMG-CoA reductase and cholesterol biosynthesis to this time course.

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