Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in mouse uterine epithelial cells

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The regulation of 3-hydroxy-3-methylglutaryl-CoA reductase was studied in mouse uterine epithelium. The enzyme was rapidly inactivated during incubation with ATP/Mg²⁺ *in vitro*, and could be re-activated by incubation with partially purified rat liver phosphoprotein phosphatase. Enzyme activity was rapidly inhibited by mevalonate injection *in vivo* to approx. 30% of control. The percentage of total enzyme active *in vivo* was measured by inclusion of NaF in the isolation buffers. The percentage of enzyme active *in vivo* 18 h after stimulation by oestrogens remained at approx. 25% after inhibition of activity by mevalonate injection, cholesterol feeding or progesterone pretreatment. However, 9 h after oestrogen stimulation, cholesterol feeding inhibited enzyme activity to 57% of control, 94% of which was in the active form. We conclude that, although all components for a reversible phosphorylative regulation of 3-hydroxy-3-methylglutaryl-CoA reductase activity are present in uterine epithelial cells, a role in the rapid changes in epithelial enzyme activity has not been demonstrated.

INTRODUCTION

We previously described the oestrogenic stimulation of HMG-CoA reductase (EC 1.1.1.34) in the uterine epithelium of the ovariectomized mouse and showed that enzyme activity rose to peaks at times when epithelial cells were in early S-phase and mitosis respectively. We also showed that progesterone pretreatment, which inhibits uterine epithelial cell proliferation, also inhibits enzyme activity. On this basis we suggested involvement of the enzyme at crucial stages of the cell cycle (Wilce *et al.*, 1984).

Suggested mechanisms of regulation of HMG-CoA reductase include modulation of activity by reversible phosphorylation (Ingebritsen & Gibson, 1980), changes in rates of synthesis or degradation (Chin *et al.*, 1982; Faust *et al.*, 1982; Edwards *et al.*, 1983), or changes in the activity or degradation of the enzyme by changes in the membrane lipid environment (Orci *et al.*, 1984). Studies of peptide-hormone regulation of the enzyme have suggested that reversible phosphorylation plays an important role (Gibson *et al.*, 1981; Ingebritsen, 1983). In view of the rapid changes in enzyme activity after oestrogen stimulation (Wilce *et al.*, 1984), we have investigated the role of reversible phosphorylation in regulation of the uterine epithelial enzyme by sex hormones and other modulating factors such as dietary cholesterol.

MATERIALS AND METHODS

The sources of reagents and their quality were as described previously (Wilce *et al.*, 1984). Female Quackenbush mice were housed, ovariectomized, primed with oestradiol-17 β and treated with vehicle, oestradiol-17 β or progesterone as described previously (Wilce *et al.*, 1984). Cholesterol feeding was carried out as described

by Gregg *et al.* (1982), and mevalonic acid treatment as described by Mitropoulos *et al.* (1978).

Preparation and assay of HMG-CoA reductase

The total particulate fraction was prepared from the uterine epithelium as described previously (Wilce *et al.*, 1984), and resuspended in 0.1 M-sucrose/50 mM-KCl/30 mM-disodium EDTA/10 mM-potassium phosphate/5 mM-dithiothreitol, pH 7.6 (buffer 1).

Mouse liver microsomal fractions were prepared as follows. Livers were pooled, washed in buffer 1 and homogenized in 33% (w/v) buffer 1 with a Potter-Elvehjem Teflon/glass homogenizer. The homogenate was centrifuged at 15000 *g* for 20 min, and the resultant supernatant centrifuged at 105000 *g* for 60 min. The microsomal pellet was resuspended in buffer 1 to the original volume and centrifuged at 105000 *g* for 60 min. The washed microsomal fractions were resuspended in buffer 1. HMG-CoA reductase activity was assayed in a final volume of 60 μ l as described previously (Wilce *et al.*, 1984). Specific activity is expressed as nmol of mevalonate formed/min per mg of protein, corrected for incorporation in blank assays.

Inactivation of mouse uterine epithelial HMG-CoA reductase *in vitro*

Uterine epithelial total particulate fractions prepared in buffer 1 containing only 1 mM-disodium EDTA were divided into two equal portions. One was preincubated for 15 min at 37 °C in the presence of 4 mM-MgCl₂/2 mM-ATP/50 mM-NaF: the NaF was included to inhibit enzyme activation by endogenous phosphatases. The second acted as a control and was preincubated for 15 min at 37 °C in the absence of ATP. Both extracts were then placed on ice, and 200 mM-disodium EDTA/Tris, pH 7.6, was added to a final concentration of 30 mM-EDTA, which is sufficient to inhibit any further

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

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action of ATP/Mg²⁺. The resultant preparations were then assayed for HMG-CoA reductase activity as above.

In other experiments the epithelial extract was isolated in buffer 1 with 1 mM-disodium EDTA as above, and one portion was preincubated with 4 mM-MgCl₂/2 mM-ATP and 50 mM-NaF at 37 °C. At various times after the start of preincubation, a sample was removed, placed on ice and the concentration of EDTA adjusted to 30 mM as above. A second portion was preincubated for 5 min at 37 °C in the same medium containing 10 mM-mevalonic acid to check that apparent inactivation of HMG-CoA reductase was not an artefactual loss of [¹⁴C]mevalonate by virtue of mevalonate kinase in the presence of ATP/Mg²⁺ (Gregg & Wilce, 1983): the reaction was stopped as above, by placing the sample on ice and adding EDTA to a final concentration of 30 mM. HMG-CoA reductase activity was assayed as above.

Re-activation of HMG-CoA reductase

The enzyme was inactivated by incubation of uterine luminal epithelial total particulate fraction (3 ml) for 15 min at 37 °C in buffer containing 4 mM-MgCl₂/2 mM-ATP but no NaF. The particulate fraction was re-isolated by centrifugation at 105000 *g* for 1 h and resuspended in buffer 1 to the original volume. Re-activation was achieved by incubation of the total particulate fraction with 0.1 vol. of a partially purified preparation of rat liver phosphoprotein phosphatase (35 mg of protein/ml) in buffer 1 (Nordstrom *et al.*, 1977). Samples (50 µl) were removed at various times and HMG-CoA reductase activity was assayed immediately by the addition of cofactors and enzyme substrate.

Preparation of phosphoprotein phosphatase

A partially purified preparation of phosphoprotein phosphatase was isolated from rat liver up to the second (NH₄)₂SO₄-fractionation step by the method of Brandt *et al.* (1975). Preparations were stored in batches at -20 °C until use.

Determination of active and total HMG-CoA reductase enzyme

Uterine luminal epithelial total particulate fractions and liver microsomal fractions were prepared and assayed for HMG-CoA reductase activity in buffer 1 with and without 50 mM-NaF, which inhibits endogenous phosphatase activity.

RESULTS

It is well established that hepatic HMG-CoA reductase activity is regulated by a bicyclic cascade, in which HMG-CoA reductase is inactivated by phosphorylation by microsomal-associated HMG-CoA reductase kinase, an enzyme which in turn is activated by phosphorylation by a second protein kinase, HMG-CoA reductase kinase kinase (Ingebritsen, 1983). In addition, inactivation of HMG-CoA reductase by phosphorylation by Ca²⁺-activated and phospholipid-dependent protein kinase has been reported (Beg *et al.*, 1985). Re-activation of the enzyme is by one of several phosphatases with wide substrate specificities (Ingebritsen *et al.*, 1983). Fig. 1 shows that mouse uterine epithelial HMG-CoA reductase is inactivated by endogenous kinase activity with a half-time of inhibition of approx. 1 min. This is significantly faster than the time course of inactivation

Fig. 1. Time course of inactivation *in vitro* of uterine epithelial HMG-CoA reductase by endogenous protein kinase activity

Mice were primed 5 days before injection of 100 ng of oestradiol-17 β at zero time: 18 h later the uterine epithelial particulate fraction was isolated in buffer 1 containing 1 mM-EDTA, as described in the Materials and methods section, and preincubated at 37 °C in the presence of ATP/Mg²⁺ and NaF for various times. HMG-CoA reductase activity was then determined as described in the Materials and methods section and is expressed as the percentage (%) of control activity (no preincubation, activity = 0.253 nmol/min per mg of protein). The data are means $\pm$ S.E.M. for three independent experiments, each utilizing ten animals and triplicate assays; $\circ$ shows the inactivation of uterine luminal epithelial HMG-CoA reductase after 5 min preincubation as above, but in the presence of 10 mM-mevalonic acid.

reported for enzyme from normal liver (Gregg & Wilce, 1983). Studies *in vitro* of inactivation of HMG-CoA reductase may be complicated by removal of mevalonate by mevalonate kinase in the presence of ATP/Mg²⁺ (Ness *et al.*, 1982). However, 5 min preincubation of uterine HMG-CoA reductase preparations with ATP/Mg/NaF plus excess (10 mM) mevalonate did not alter the degree of inactivation at 5 min. Fig. 2 shows that the uterine enzyme can be re-activated by exposure to an exogenous phosphatase.

The major end product of liver HMG-CoA reductase is cholesterol, for export to other tissues. Activity of the liver enzyme has repeatedly been shown to be rapidly decreased by administration of mevalonate *in vivo* or by giving a high-cholesterol diet (Sabine, 1977). This represents classic feedback inhibition by enzyme products (cholesterol, its precursors or their metabolites). We have suggested that the increased activity of the uterine luminal epithelial HMG-CoA reductase 9 and 18 h after oestradiol stimulation may be associated with the production of materials essential for continuing DNA replication and cell division respectively. It was therefore decided to determine whether these hormone-induced peaks of enzyme activity were sensitive to the type of feedback controls which regulate the liver enzyme.

Fig. 3 shows the time course of inhibition of HMG-CoA reductase activity after intraperitoneal

Fig. 2. Time course of re-activation *in vitro* of uterine epithelial HMG-CoA reductase by an exogenous phosphoprotein phosphatase

Mice were treated as described in the legend to Fig. 1. The uterine epithelial particulate fraction was isolated in buffer 1 containing ATP/Mg²⁺ but no NaF and incubated at 37 °C for 30 min. The particulate fraction was then re-isolated in buffer 1 and incubated at 37 °C for various times with rat liver phosphoprotein phosphatase as described in the Materials and methods section. The HMG-CoA reductase activity was determined as usual, and is expressed as a percentage of control (no incubation with rat liver phosphoprotein phosphatase, activity = 0.351 nmol/min per mg of protein). The results are means $\pm$ S.E.M. for triplicate assays from an experiment utilizing ten mice. A replicate experiment gave similar results.

administration of mevalonic acid lactone (60 mg) at various times before the animals were killed 18 h after oestrogen administration. The enzyme activity in both uterus and liver was inhibited by mevalonate, with the uterine enzyme showing a higher rate of inactivation than the liver enzyme. However, in other experiments (Table 1) essentially the same degree of inhibition was obtained in both tissues 2 h after mevalonate administration. Nevertheless it is clear from Table 1 that uterine epithelial HMG-CoA activity at both 9 and 18 h after oestrogen treatment is significantly decreased by mevalonic acid pretreatment *in vivo*.

It is generally agreed that HMG-CoA reductase is activated during the extraction procedure. Inclusion of NaF in the extraction buffer prevents this and allows measurement of the proportion of enzyme that is in the active state *in vivo*. The data in Table 1 indicate that the proportion of active enzyme is unchanged at 9 h and 18 h after oestrogen and also after treatment with mevalonic acid. The percentage of active enzyme in the liver ranged from 19.7 ± 2.0 to 30 ± 3.0 (Table 2). Mevalonic acid inhibition did not change this proportion.

The data summarized in Table 2 show that, unlike the liver enzyme, uterine epithelial HMG-CoA reductase is

Fig. 3. Time course of inactivation *in vivo* of mouse uterine epithelial and mouse liver microsomal HMG-CoA reductase by mevalonic acid

Mice were primed 5 days before injection of 100 ng of oestradiol-17 β at zero time. The animals were killed 18 h later, and uterine epithelial particulate and liver microsomal fractions isolated as described in the Materials and methods section. Mevalonic acid (60 mg) was given by intraperitoneal injection in 0.25 ml of 0.9% NaCl (pH adjusted to 7.4 with KOH), at various times before killing; controls received vehicle. HMG-CoA reductase activity was determined as described in the Materials and methods section and is expressed as the percentage (%) of control activity (no mevalonate, activity = 0.28 nmol/min per mg protein). The data are means $\pm$ S.E.M. for triplicate assays of preparations from separate groups of eight mice. $\circ$, Uterine epithelium; $\bullet$, liver.

relatively resistant to inhibition by cholesterol feeding, particularly 18 h after oestrogen. There was a greater inhibition at 9 h. The percentage of active enzyme after cholesterol feeding at this time was very high. Since this diet decreased total enzyme activity by 43%, the implication is that this diet may have increased the percentage of active enzyme.

HMG-CoA reductase activity in the particulate fraction isolated from uterine epithelium of untreated ovariectomized mice is so low that it is not practical to measure accurately the proportion of active enzyme by comparing the specific activities of such preparations isolated in the presence or absence of NaF. It therefore remained possible that the extremely low activities obtained in such fraction isolated in the absence of NaF resulted from a lack of endogenous phosphatase activity. We therefore isolated total particulate fraction from the uterine epithelium of a group of 20 untreated ovariectomized mice and incubated samples with rat liver phosphoprotein phosphatase at 37 °C for 20 min, before determining HMG-CoA reductase activity. There was no increase in specific activity as a result of preincubation (controls 0.029 ± 0.001 nmol/min per mg of protein; preincubated 0.023 ± 0.002 nmol/min per mg of protein; mean $\pm$ S.E.M. for triplicate assay). It seems therefore that the activity in the unstimulated uterus epithelium is due not to a deficiency of phosphoprotein phosphatase but to low amounts of enzyme. A similar experiment was

Table 1. Effect of pretreatment with mevalonic acid *in vivo* on HMG-CoA reductase activity in uterine epithelium and liver

Mice primed 5 days beforehand were killed 9 or 18 h after administration of 100 ng of oestradiol-17 β . They were given 60 mg of mevalonic acid intraperitoneally or vehicle as described in Fig. 3, 2 h before death. Uterine epithelial particulate and liver microsomal fractions were isolated in the presence or absence of NaF, as described in the Materials and methods section. The results are means $\pm$ S.E.M. for three to five replicate experiments each with triplicate assays on preparations from six to eight mice.

	HMG-CoA reductase activity		
	Total sp. activity		% active
	(nmol/min per mg of protein)	(% of control)	
Expt. 1: 9 h after oestradiol			
Uterine epithelium			
Control	0.130 $\pm$ 0.010	—	34.0 $\pm$ 4
Mevalonic acid	0.048 $\pm$ 0.026	37.0 $\pm$ 5.3	28.0 $\pm$ 3
Expt. 2: 18 h after oestradiol			
Uterine epithelium			
Control	0.197 $\pm$ 0.047	—	25.4 $\pm$ 1.8
Mevalonic acid	0.063 $\pm$ 0.027	32.0 $\pm$ 3.4	27.9 $\pm$ 1.3
Liver microsomal fractions			
Control	0.215 $\pm$ 0.019	—	19.7 $\pm$ 2.0
Mevalonic acid	0.030 $\pm$ 0.003	15.7 $\pm$ 1.8	16.7 $\pm$ 1.4

Table 2. Effects of cholesterol feeding on HMG-CoA reductase activity in uterine epithelium and liver

Mice primed 5 days beforehand were killed 9 or 18 h after administration of 100 ng of oestradiol-17 β , having been fed on normal or high-cholesterol diet over the previous 3 days. Uterine epithelial total particulate and liver microsomal fractions were isolated in the presence or absence of NaF as described in the Materials and methods section. The results are means $\pm$ S.E.M. from two to four replicate experiments each with triplicate assays on preparations from six to eight mice.

	HMG-CoA reductase activity		
	Total sp. activity		% active
	(nmol/min per mg of protein)	(% of control)	
Uterine epithelium			
9 h after oestradiol			
Control	0.063 $\pm$ 0.007	—	31 $\pm$ 1
Cholesterol-fed	0.031 $\pm$ 0.004	57.3 $\pm$ 2.0	94 $\pm$ 7
18 h after oestradiol			
Control	0.139 $\pm$ 0.050	—	28 $\pm$ 6
Cholesterol-fed	0.112 $\pm$ 0.040	87.9 $\pm$ 2.1	14 $\pm$ 6
Liver microsomal fractions			
9 h after oestradiol			
Control	0.112 $\pm$ 0.035	—	22 $\pm$ 5
Cholesterol-fed	0.011 $\pm$ 0.001	12.2 $\pm$ 2.5	28 $\pm$ 6
18 h after oestradiol			
Control	0.089 $\pm$ 0.025	—	30 $\pm$ 3
Cholesterol-fed	0.008 $\pm$ 0.002	10.4 $\pm$ 0.3	—

carried out to determine if the decrease in HMG-CoA reductase activity produced by progesterone pretreatment was due to inhibition of enzyme activation rather than enzyme synthesis. Uterine epithelial particulate fractions were isolated in the presence and absence of NaF from vehicle- and progesterone-pretreated mice 9 h after the administration of 100 ng of oestradiol. Progesterone pretreatment markedly decreased total activity, but did not increase the proportion of inactive enzyme (Table 3). In this experiment, samples of the particulate fractions isolated without NaF were also

preincubated for 20 min with rat liver phosphoprotein phosphatase. There was no increase in HMG-CoA reductase activity. Thus it seems that, in samples isolated in the absence of NaF, we measure total available enzyme activity.

DISCUSSION

The reversible phosphorylation of HMG-CoA reductase isolated from several tissues has been reported (reviewed by Gibson *et al.*, 1981). The inactivation of

Table 3. Effect of pretreatment with progesterone *in vivo* on HMG-CoA reductase activity in uterine epithelium

Mice primed 2 days beforehand were treated for 3 days with 0.1 ml of arachis oil or with 0.1 ml of arachis oil containing 1 mg of progesterone. On day 3 of treatment they received 100 ng of oestradiol-17 β and were killed 9 h later. Uterine luminal epithelial total particulate fractions were isolated in the presence or absence of NaF, and HMG-CoA reductase activity was determined as described in the Materials and methods section. The results are means $\pm$ S.E.M. for triplicate assays on preparations from ten animals. A replicate experiment gave similar results.

	HMG-CoA reductase activity			
	Total sp. activity			% active
	(nmol/min per mg of protein)		(% of control)	
	+ NaF	– NaF		
No progesterone	0.136 ± 0.003	0.558 ± 0.010	—	24.4
Progesterone pretreatment	0.018 ± 0.001	0.079 ± 0.001	14.2	22.8

microsomal HMG-CoA reductase by ATP/Mg²⁺ is mediated by an HMG-CoA reductase kinase present in both cytosol and microsomal fractions. The kinase appears to have some specificity for HMG-CoA reductase, but will phosphorylate other substrates (Beg *et al.*, 1979). The removal of the phosphate from the protein *in vitro* can be achieved by non-specific phosphoprotein phosphatases. The data reported in the present paper clearly demonstrate that the essential features of this system are also present in the uterine epithelial particulate fraction. The particulate enzyme was inactivated by endogenous kinase activity, the implication being that this kinase is analogous to the HMG-CoA reductase kinase described in the liver. The inclusion of excess mevalonate and careful choice of assay conditions eliminate the possibility that this inactivation is due to conversion of mevalonate into phosphomevalonate (Ness *et al.*, 1982).

Uterine epithelial HMG-CoA reductase was re-activated by a partially purified preparation of rat liver phosphoprotein phosphatase. We were unable to obtain sufficient phosphatase activity from uterine epithelial supernatant to conduct re-activation with this system. However, since enzyme activity is decreased by approx. 70% if the particulate fraction is isolated in the presence of NaF, an endogenous phosphoprotein phosphatase acting on HMG-CoA reductase in this tissue is implicated.

We previously reported that, under oestrogen stimulation, HMG-CoA reductase activity increases rapidly by approx. 7-fold at 9 h, falls to half this value within a further 3 h, only to double again after a further 3 h. Furthermore, mevalonate rapidly inhibits enzyme activity by approx. 65% within 2 h (Fig. 3 and Table 1). These rapid changes could be consistent with modulation of enzyme through reversible phosphorylation. We were, however, unable to demonstrate any change in the proportion of active enzyme under these conditions. In particular, we have excluded any role for this mechanism for the activation of enzyme in the uterine response to oestrogen. In the liver a constant ratio of active to inactive HMG-CoA reductase under conditions where total activity is markedly different has been reported (Brown *et al.*, 1979; Kleinsek *et al.*, 1980). Phosphorylation may be important in the initial inhibition by mevalonate, followed by loss of enzyme protein (Arebello

et al., 1980, 1981, 1982; Beg & Brewer, 1981; Erickson *et al.*, 1980; Parker *et al.*, 1983). In contrast, other workers using similar methodology found no evidence for inactivation and suggested that the decrease could be explained by loss of enzyme protein (Dugan *et al.*, 1982; Jenke *et al.*, 1982). It appears that the precise role of the phosphorylation system in liver is still obscure. However, it may be that subtle changes in, for example, viscosity of the supporting membrane or phosphorylation of the enzyme may precipitate a rapid degradation of the enzyme. The enzyme in the uterine epithelium is extremely labile, with rapid changes in activity in both activation and inhibition. The function of phosphorylation in uterine epithelium may be to induce a rapid loss of enzyme protein by proteolytic degradation (as suggested for the hepatic enzyme by Parker *et al.*, 1984).

For the most part, manipulation of the uterine enzyme activity with hormones and feedback inhibition failed to alter the percentage of active enzyme. The exception was the response of the uterine enzyme 9 h after oestrogen stimulation in cholesterol-fed animals. Here the total enzyme content was decreased, but the proportion of active enzyme increased, so that 'expressed' activity was actually higher than in normal fed animals. Comparable activation was not observed in the liver enzyme or the uterine enzyme 18 h after oestrogen stimulation. At 9 h after oestrogen stimulation a majority of uterine epithelial cells are committed to DNA replication, a process for which products of HMG-CoA reductase activity appear to be essential (Quesney-Huneus *et al.*, 1983). It is tempting to speculate that commitment of the cell to the critical process of DNA replication leads to the expression of regulatory mechanisms which can compensate for fluctuations in enzyme activity arising from the cholesterol feedback pathway.

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