Role of reversible phosphorylation in mediating changes in the activity of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase during pregnancy and lactation in the rat

Richard A. EASOM* and Victor A. ZAMMIT†

Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

1. The expressed and total (completely dephosphorylated) activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase were measured in microsomal fractions isolated from cold-clamped liver samples from female rats in various stages of the reproductive cycle. 2. There was little change in total HMG-CoA reductase activity during pregnancy and early lactation, but after 2 days *post partum* there was a marked increase in total activity. 3. The expressed/total activity ratio of HMG-CoA reductase showed a profound decrease during the last 2 days of pregnancy. The fraction of the enzyme in the active form increased progressively during the first 2 days of lactation. 4. The combined effect of these changes was that the expressed activity of HMG-CoA reductase changed in parallel with the known changes in the hepatic rate of cholesterol synthesis during pregnancy and lactation *in vivo*.

INTRODUCTION

In pregnancy and lactation, there are major changes in hepatic lipid metabolism (see Williamson, 1980; Zammit, 1985). These are related to altered dietary intake and the requirement of peripheral tissues, particularly the lactating mammary gland, for lipid substrates. The secreting mammary gland requires exogenous fatty acids, which it obtains through lipoprotein lipase-dependent hydrolysis of triacylglycerols associated with very-low-density lipoproteins and chylomicrons (see Robinson & Wing, 1970; Zinder *et al.*, 1976) and exogenous cholesterol, which is transferred as cholesterol ester (associated with these lipoproteins), during lipoprotein lipase action (Chajek-Shaul *et al.*, 1981). Gibbons *et al.* (1983) have suggested that cholesterol ester uptake from very-low-density lipoproteins may make a substantial contribution towards exogenous cholesterol requirement of the rat mammary gland during lactation, because mammary endogenous synthesis of the sterol accounts for less than 40% of the rate of cholesterol secretion in rat milk. Similarly, dietary cholesterol appears to make a minor contribution towards the rate of milk cholesterol secretion in this species (Gibbons *et al.*, 1983).

The involvement of the liver in the supply of cholesterol for both bile and very-low-density lipoprotein secretion during pregnancy and lactation is likely to reflect both the longer-term changes in the hormonal environment associated with the transition from the unmated to the pregnant and lactating states, as well as shorter-term changes associated with acute phenomena such as food intake. In this respect, parturition may be expected to involve both types of mechanism, because of its relatively short duration as a physiological event, and of its effect on other functions, including food intake.

The reaction catalysed by 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is considered to be a major

locus at which regulation of the rate of hepatic cholesterol synthesis is exerted (see Brown & Goldstein, 1980). The activity of the enzyme is controlled by changes in the amount of enzyme protein present (i.e. the total activity) and by modulation of the fraction of the enzyme in the active form (Gibson, 1985; Kennelly & Rodwell, 1985). The latter mechanism occurs through reversible phosphorylation of the enzyme, the phosphorylated enzyme being largely inactive (Zammit & Easom, 1987). Modulation of the phosphorylation state of HMG-CoA reductase in the liver results in large variations in the effective or 'expressed' activity of the enzyme *in vivo* (see Easom & Zammit, 1984b, 1985, 1987; Zammit & Easom, 1987). These changes occur in response to normal hormonal stimuli resulting from changes in the physiological state of the animals. Although the interrelationship between the two mechanisms of modulation of hepatic HMG-CoA reductase activity has been studied for a variety of patho-physiological conditions (see Zammit & Easom, 1987), no such studies have been performed on pregnant and lactating animals, in which, as discussed above, there are likely to be a multiplicity of factors involved in the control of hepatic metabolism during the physiological transitions involved. In previous attempts to obtain such information (Leoni *et al.*, 1984), the activities of enzymes that are likely to alter the phosphorylation state of the enzyme (i.e. protein kinases and phosphatases) were inadequately inhibited during preparation of liver samples (see Zammit & Easom, 1987). Consequently, the assertion that changes in phosphorylation state of HMG-CoA reductase may not be involved in the control of the activity of the enzyme during late pregnancy (Leoni *et al.*, 1984) may require re-evaluation.

In the present study, therefore, we have sampled rat liver by the cold-clamping technique, in order to preserve the phosphorylation state of HMG-CoA reductase and enable its assay *in vitro* (Easom & Zammit, 1984a). This

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

* Present address: Department of Biochemistry, University of Texas Health Science Centre, San Antonio, TX 78284, U.S.A.

† To whom correspondence and reprint requests should be addressed.

enabled us to study the relationship between the expressed and total activities of the enzyme during different stages of the reproductive cycle, and particularly around parturition. The results suggest that post-translational covalent modification of HMG-CoA reductase may play a major part in effecting the changes in expressed activities that occur in the liver and may be involved in determining the rate of cholesterol synthesis in these physiological states.

MATERIALS AND METHODS

Animals

These were female Wistar rats purchased from A. Tuck and Son (Battlesbridge, Essex, U.K.) that were mated 2 weeks after arrival, and moved into a reversed-light-cycle room (lights on from 19:00 h to 07:00 h) during the first 3 days of pregnancy. They were maintained on Oxoid Breeding Diet (Oxoid, Basingstoke, Hants., U.K.). They weighed 280–300 g at day 12 of pregnancy, and 230–270 g and 340–370 g on day 1 and day 12 of lactation respectively. The day of conception, determined by the appearance of vaginal plugs, was considered to be day 0 of pregnancy, and animals littered on day 21. All litters of lactating rats were adjusted to eight pups. Animals used to study the effects of premature weaning had their pups removed on day 11 after parturition, 24 h before being killed. Animals were trained individually to handling for several minutes each day for 4 days before being killed, in order to minimize the effects of stress on the day of the experiment.

Liver sampling and preparation of microsomes

Animals were anaesthetized, 4 h into the dark phase of the light–dark cycle, by intraperitoneal injection of pentobarbital (60 mg/kg body wt., except for late-pregnant animals, which received 75 mg/kg). After 20 min, the abdominal cavity was opened and a portion (approx. 1 g) of liver was cold-clamped as described previously (Easom & Zammit, 1984a). The homogenization medium contained 50 mM-sucrose, 100 mM-KF, 10 mM-Tris/HCl, 10 mM-EDTA, 1 mM-dithiothreitol and 0.5 mM-phenylmethanesulphonyl fluoride (pH 7.4 at 0 °C). Further treatment of the homogenate, in order to obtain expressed and total activities of HMG-CoA reductase, was as described previously (Easom & Zammit, 1984b).

Chemicals

[¹⁴C]HMG-CoA was synthesized as described by Williamson & Rodwell (1981), starting with 3-hydroxy-3-methyl[3-¹⁴C]glutaric acid purchased from Amersham International (Amersham, Bucks., U.K.). Phenylmethanesulphonyl fluoride and other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

The total activity (1.40–1.50 nmol/min per mg of protein at 37 °C) and expressed/total activity ratio (60–70 %) for HMG-CoA reductase in microsomes isolated from female unmated rats were both slightly lower than the corresponding parameters found in microsomes from normal male rats (2.0–3.2 nmol/min per mg of protein and 80 % respectively) at the same time

point in the light–dark cycle (Easom & Zammit, 1984b, 1985, 1987). The reason for such a difference between the sexes is not known. The choice of 4 h into the dark phase was made in view of previous results which indicated that this was the time of maximal fractional activation of the enzyme (Easom & Zammit, 1984b). The diurnal pattern of food intake is maintained in pregnancy and lactation, although the ‘baseline’ of intake is considerably increased during lactation (Munday & Williamson, 1983). Similarly, the diurnal cycle in HMG-CoA reductase activity observed in male rats (Rodwell *et al.*, 1976; Easom & Zammit, 1984b) is also retained in lactating female rats (Walker & Hahn, 1981; Gibbons *et al.*, 1983).

The total activity of HMG-CoA reductase activity tended to decrease progressively during pregnancy and in the immediate period *post partum*, such that the activity in 2-day-lactating animals was lower, by about 25 %, than in unmated animals (Fig. 1a), although the difference was not statistically significant. In contrast with the results obtained by Leoni *et al.* (1984), we did not observe any sharp decreases in total (fully dephosphorylated) reductase activity on the last day of pregnancy. This apparent discrepancy between the two sets of data may have arisen from the fact that those authors did not attempt to prevent dephosphorylation of HMG-CoA reductase during liver sampling and homogenization. During late pregnancy, the enzyme becomes very highly phosphorylated (see below), and it is possible that in the experiments of Leoni *et al.* (1984) apparently much lower ‘total’ HMG-CoA reductase activities were observed during this period, owing to unequal degrees of dephosphorylation by endogenous phosphatases.

The total activity of HMG-CoA reductase increased markedly between 2 and 4 days *post partum*, such that the activity reached a value approx. 60 % higher (per mg of microsomal protein) than in unmated animals (Fig. 1a). A similar increase was reported by Walker & Hahn (1981) for reductase activity assayed in the middle of the light phase of the light–dark cycle. In the present study, however, the induction of the reductase activity occurred somewhat later than reported by those authors. During the subsequent 8-day period of lactation there was no significant change in total reductase activity. After removal of the pups from the mother for 24 h, however, the total activity of HMG-CoA reductase was at the value present in unmated animals (Fig. 1a).

The effects of pregnancy and lactation on the expressed/total activity ratio of HMG-CoA reductase were different from those observed for total activity of the enzyme. The ratio remained at around 60 % throughout the first 20 days of pregnancy, but showed a profound decrease, to approx. 20 %, on the day of parturition (Fig. 1b). Subsequently, during the first 4 days *post partum*, the ratio increased steadily to reach values that were not significantly different from those observed for unmated or pregnant rats. However, the ratio tended to decline during lactation and reached a mean value of 40 % on day 12 of lactation. Removal of the pups from the mother had no significant effect on this value (Fig. 1b).

The resultant effect of these changes in the total activity of HMG-CoA reductase and in the fraction of the enzyme in the active form was that the expressed activity of reductase in liver microsomes underwent a marked decline around parturition, which was already apparent on day 20 of pregnancy and was most

Fig. 1. Changes in (a) total, (b) expressed/total activity ratio and (c) expressed activity of HMG-CoA reductase in liver microsomes isolated from unmated, pregnant, lactating and weaned rats

See the Materials and methods section for experimental details. Values are means $\pm$ S.E.M. for three or four separate determinations. Key: V, virgin; P, parturition; W, weaned.

pronounced during the first 2 days of lactation (Fig. 1c). Subsequently, the expressed activity increased to values that were not significantly higher than those observed during mid-pregnancy, in spite of the higher total specific activity of the enzyme (Fig. 1c). It is noteworthy that the period around parturition is associated with diminished food intake as well as poorly established lactation

(during the first 2 days *post partum*). Consequently it may represent a phase in the reproductive cycle during which the demands on the liver for secretion of bile acid and very-low-density lipoprotein may be at their lowest. It is instructive to attempt to relate changes in reductase expressed activity with possible hormonal stimuli. The marked increase in the phosphorylation state of the enzyme during the last 2 days of pregnancy occurs at a time when the insulin concentration declines precipitously (by 75%; Lorenzo *et al.*, 1983). In view of the dependence of the phosphorylation state of reductase on insulin (Easom & Zammit, 1985), it would be plausible to suggest that the fall in circulatory insulin concentration during late pregnancy is a major factor in mediating the increased phosphorylation of the enzyme at this stage. However, other observations suggest that other factors are likely to be involved in the regulation of HMG-CoA reductase activity. Thus, although the total activity of reductase is marginally decreased, it does not show a marked decline, although insulin is known to induce HMG-CoA reductase (total) activity (see Easom & Zammit, 1985). Similarly, the increases in total activity and expressed/total activity ratio observed 2 days *post partum* occurred in spite of sustained low circulating insulin concentrations (Flint *et al.*, 1979). Consequently, other hormones may be involved; for example, late pregnancy in the rat is associated with low concentrations of progesterone, whereas prolactin secretion increases markedly on parturition (Flint *et al.*, 1979). A putative insulin-antagonizing factor may also operate *in vivo* on removal of the pups. If insulin were the sole determinant of reductase activity, it would have been expected that the rise in insulin concentration that accompanies weaning (Agius *et al.*, 1979) would have resulted in increased hepatic reductase activity (expressed and/or total). However, this did not occur, suggesting that another factor, possibly the rapid decrease in circulating prolactin concentrations (Agius *et al.*, 1979), may antagonize the effect of increased insulin concentration.

The present results demonstrate the role that modulation of the phosphorylation state of hepatic HMG-CoA reductase may play in determining the rate of hepatic cholesterogenesis *in vivo*. The decreased rate of cholesterol synthesis observed for liver slices obtained from late-pregnant rats (Leoni *et al.*, 1984) did not appear to be accompanied by a substantial acute decrease in total activity of reductase. By contrast, there was a very marked decrease in the fraction of the enzyme in the active form, owing to increased phosphorylation of the enzyme (i.e. reversible by protein phosphatase treatment *in vitro*). Another indication of the role of reductase phosphorylation is evident from the similarity between the rates of hepatic cholesterogenesis, measured *in vivo*, in unmated and lactating rats (Gibbons *et al.*, 1983), in spite of the markedly different total specific activity of HMG-CoA reductase between the two physiological states (Walker & Hahn, 1981; Gibbons *et al.*, 1983; Feingold & Moser, 1985; the present paper).

In our previous studies on the role of changes in phosphorylation state of HMG-CoA reductase *in vivo*, we have observed that the expressed/total activity ratio and the total activity of the enzyme tend to change in the same direction. For example, the diurnal cycles of the two parameters, although separated by a small phase difference, are basically similar (Easom & Zammit, 1984b), and increased phosphorylation is followed by

decreased total activity, and vice versa. Also, food deprivation and insulin deficiency decrease chronically both the expressed/total activity ratio and the total activity of the enzyme (Easom & Zammit, 1985, 1987). These parallel changes have been claimed as support for the suggestions that increased phosphorylation of reductase predisposes it to an increased rate of degradation both *in vitro* (Parker *et al.*, 1984) and in intact hepatocytes (Marrero *et al.*, 1986). The observation, in the present study, that the marked increase in phosphorylation state of reductase during late pregnancy is not accompanied by an equally marked decrease in total activity suggests that any putative relationship between increased phosphorylation and degradation of the enzyme may be counteracted by other mechanisms during late pregnancy. One possibility is that there is a simultaneously increased rate of synthesis of reductase, during late pregnancy and early lactation, thus resulting in an effective increase in the turnover of the enzyme.

We thank Miss A. Caldwell for excellent assistance. R. A. E. was the recipient of a studentship funded by the Nutrition Consultative Panel of the U.K. Dairy Association.

REFERENCES

- Agius, L., Robinson, A. M., Girard, J. R. & Williamson, D. H. (1979) *Biochem. J.* **180**, 689–692
- Brown, M. S. & Goldstein, J. L. (1980) *J. Lipid Res.* **21**, 505–517
- Chajek-Shaul, T., Friedman, G., Halperin, G., Stein, O. & Stein, Y. (1981) *Biochim. Biophys. Acta* **666**, 216–222
- Easom, R. A. & Zammit, V. A. (1984a) *Biochem. J.* **220**, 733–738
- Easom, R. A. & Zammit, V. A. (1984b) *Biochem. J.* **220**, 739–745
- Easom, R. A. & Zammit, V. A. (1985) *Biochem. J.* **230**, 747–752
- Easom, R. A. & Zammit, V. A. (1987) *Biochem. J.* **241**, 183–188
- Feingold, K. R. & Moser, A. H. (1985) *Am. J. Physiol.* **249**, G203–G208
- Flint, D. J., Sinnett-Smith, P. A., Clegg, R. A. & Vernon, R. G. (1979) *Biochem. J.* **182**, 421–427
- Gibbons, G. F., Pullinger, C. R., Munday, M. R. & Williamson, D. H. (1983) *Biochem. J.* **212**, 843–848
- Gibson, D. M. (1985) in *Regulation of HMG-CoA Reductase* (Preiss, B., ed.), pp. 79–131, Academic Press, New York
- Kennelly, P. J. & Rodwell, V. W. (1985) *J. Lipid Res.* **26**, 903–913
- Leoni, S., Spagnuolo, S., Conti Devirgilis, L., Mangiantini, M. T. & Trentalancia, M. (1984) *Experientia* **40**, 703–704
- Lorenzo, M., Benito, M., Caldes, T. & Medina, J. (1983) *Biochem. J.* **216**, 695–699
- Marrero, P. F., Haro, D. & Hegardt, F. G. (1986) *FEBS Lett.* **197**, 183–186
- Munday, M. R. & Williamson, D. H. (1983) *Biochem. J.* **214**, 183–187
- Parker, R. A., Miller, S. J. & Gibson, D. M. (1984) *Biochem. Biophys. Res. Commun.* **125**, 629–635
- Robinson, D. S. & Wing, D. R. (1970) *Horm. Metab. Res. Suppl.* **2**, 41–47
- Rodwell, V. W., Nordstrom, J. L. & Mitschelen, J. J. (1976) *Adv. Lipid Res.* **14**, 1–74
- Walker, B. L. & Hahn, P. (1981) *Can. J. Biochem.* **59**, 829–892
- Williamson, D. H. (1980) *FEBS Lett.* **117**, K93–K105
- Williamson, I. R. & Rodwell, V. W. (1981) *J. Lipid Res.* **22**, 184–187
- Zammit, V. A. (1985) *Biochem. Soc. Trans.* **13**, 831–833
- Zammit, V. A. & Easom, R. A. (1987) *Biochim. Biophys. Acta* **927**, 223–228
- Zinder, O., Mendelson, C. R., Blanchette-Mackie, E. J. & Scow, R. O. (1976) *Biochim. Biophys. Acta* **431**, 426–437

Received 10 July 1987/20 October 1987; accepted 21 October 1987