Diurnal changes in the fraction of 3-hydroxy-3-methylglutaryl-CoA reductase in the active form in rat liver microsomal fractions

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(Received 9 January 1984/Accepted 1 March 1984)

1. 'Initial' and 'total' activities of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) were measured in cold-clamped samples of liver from rats at 2 h intervals throughout the 24 h light/dark cycle. Initial activities were obtained in microsomes (microsomal fractions) isolated and assayed in the presence of 100 mM-KF, whereas 'total' activities were measured in microsomes prepared from the same homogenates but washed free of KF and incubated with exogenous partially purified rat liver protein phosphatase. 2. The initial/total-activity ratio for HMG-CoA reductase underwent a diurnal cycle, which had a nadir 4 h into the light phase (when initial activity was 28% of total activity) and a peak 12 h later, i.e. 4 h into the dark phase (when initial activity was 80% of total activity). These low and high points of the cycle were separated by gradual steady changes in the ratio. 3. The characteristics of this diurnal cycle were different from those of the cycle observed for total activity, which had a plateau of high activity between 2 and 10 h into the dark cycle preceded and succeeded by a very rapid increase and decrease, respectively, in the total activity of HMG-CoA reductase. 4. The combination of the two cycles resulted in the dampening of the resultant cycle for the initial or effective activity of HMG-CoA reductase, such that the changes in initial activity around the beginning and end of the dark phase were more gradual than would otherwise have been the case if the initial/total-activity ratio for HMG-CoA reductase were constant throughout the diurnal cycle. 5. The physiological implications of the observed diurnal variation in the fraction of hepatic HMG-CoA reductase in the active form are discussed.

A paradoxical situation exists in the literature with respect to the regulation of the activity of hepatic HMG-CoA reductase (EC 1.1.1.34) by covalent modification *in vivo*. It is well established that the purified enzyme (Beg & Brewer, 1981; Font *et al.*, 1982) and the enzyme in isolated microsomal membranes (Beg *et al.*, 1973, 1978; Nordstrom *et al.*, 1977) can undergo reversible changes in its phosphorylation status under the influence of protein kinase or phosphatase (exogenous or endogenous to the microsomes) with concomitant inhibition or activation, respectively (Beg *et al.*, 1978; Beg & Brewer, 1981, 1982). Similar changes in the fraction of the enzyme in the active form have been shown to occur in isolated hepatocytes incubated with insulin or glucagon, which induce an increase and a decrease, respec-

tively, in the 'expressed' activity of HMG-CoA reductase with respect to the total enzyme activity obtained after complete activation by protein phosphatase (Ingebritsen *et al.*, 1979). However, the occurrence of such changes in the fraction of HMG-CoA reductase in the active form *in vivo* in the rat liver has proved capable of demonstration only when animals have been exposed to acute, non-physiological, experimental conditions (Higgins & Rudney, 1973; Higgins *et al.*, 1974; Arebalo *et al.*, 1980, 1981; Beg *et al.*, 1980; Beg & Brewer, 1982). Attempts at detecting such changes in the fraction of the enzyme in the active form in the liver *in vivo* in response to more physiological hormonal or nutritional changes, e.g. such as would occur in the rat during a diurnal cycle of feeding and short-term starvation, have been unsuccessful. Thus studies by Nordstrom *et al.* (1977), Brown *et al.* (1979) and Dugan *et al.* (1982)

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

led to the conclusion that under normal physiological conditions HMG-CoA reductase is always largely (about 90%) inhibited in spite of the large range of the rates of hepatic cholesterol synthesis *in vivo* (see, e.g., Rodwell *et al.*, 1976). Unfortunately, the notion that modulation of HMG-CoA reductase activity through changes in the fraction of the enzyme in the active form does not occur *in vivo* in response to normal physiological stimuli has persisted (see, e.g., Berndt *et al.*, 1976; Hardgrave *et al.*, 1979; Kliensek *et al.*, 1980; Jenke *et al.*, 1981; Dugan *et al.*, 1982; Liscum *et al.*, 1983; Gregg & Wilce, 1983; Ness, 1983), in spite of some evidence to the contrary (Edwards *et al.*, 1980).

By analogy with the physiologically induced changes in the activity state *in vivo* of other enzymes regulated through a phosphorylation-dephosphorylation mechanism (see, e.g., McNeillie & Zammit, 1982; Zammit & Corstorphine, 1982*a,b*; for review, see Cohen, 1982) it is highly unlikely that the bicyclic protein phosphorylation mechanism which has been described for the regulation of HMG-CoA reductase activity (see, e.g., Beg & Brewer, 1981, 1982) goes largely unused *in vivo*. Therefore the questions arise as to whether (i) the modest changes in the fraction of the enzyme in the active form that are observed in livers of animals challenged acutely with unphysiological concentrations of hormones or dietary manipulation (see above) may be remnants of much larger responses, and (ii) whether the putative, more gradual changes in this parameter induced by physiological stimuli (e.g. diurnal cycle) may have been overlooked.

As recognized by Ingebritsen *et al.* (1979), a major difference between studies *in vitro* and *in vivo* involving measurement of HMG-CoA reductase activity is the ease and rapidity with which the cells (*in vitro*) or tissue (*in situ*) can be harvested or sampled, respectively, and homogenized in an appropriate medium in order to arrest or slow down the metabolic changes which may otherwise alter the phosphorylation status of an enzyme such as HMG-CoA reductase. With isolated cells this is easily achieved (Ingebritsen *et al.*, 1979; Zammit & Corstorphine, 1982*b*). In the liver *in situ*, previous studies have made use of the freeze-clamping technique (see, e.g., Zammit & Corstorphine, 1982*a*) in which the liver of the anaesthetized rat is frozen between metal blocks cooled to liquid-N₂ temperature to give an instantaneous value for the 'initial' and 'total' activities of the enzyme (e.g. acetyl-CoA carboxylase: Stansbie *et al.*, 1976; Zammit & Corstorphine, 1982*a*). However, this technique cannot be used in studies on hepatic HMG-CoA reductase, because freezing and subsequent thawing of the liver sample results in lysis of the mitochondria and the release of HMG-CoA

lyase (EC 4.1.3.4) activity, which is many-fold higher than that of HMG-CoA reductase in liver (Clinkenbeard *et al.*, 1975); the activity of HMG-CoA lyase interferes with the subsequent measurement of HMG-CoA reductase activity. In addition, the freeze-clamping technique makes it impossible to obtain microsomal membranes of sufficient purity to enable the valid measurement of HMG-CoA reductase activity per unit of microsomal-membrane protein. Consequently, in studies on hepatic HMG-CoA reductase the activity of the enzyme is measured in microsomes isolated from the liver by differential centrifugation. As conventionally performed, this involves the excision of the liver from the animal, fine mincing of the tissue and mild homogenization. This procedure necessarily introduces time delays between the cessation of circulation through the liver sample and the inhibition of protein phosphatase and kinase activities in the tissues by homogenization in an appropriate medium. The problems arising from such delays are likely to be compounded by the difficulty in achieving rapid and uniform cooling of the liver sample during mincing of the tissue.

To minimize these difficulties, we have developed a technique which allows rapid (<5 s) sampling and cooling of liver samples obtained from anaesthetized animals and subsequent homogenization with retention of mitochondrial integrity (Easom & Zammit, 1984). In the present study we have used this technique to obtain microsomes from livers of rats at different times during the diurnal light/dark cycle. This technique has enabled us to obtain 'initial' and 'total' values for the activity of hepatic HMG-CoA reductase (see above) and to establish that changes in the initial/total-activity ratio do occur during the 24 h cycle.

Materials and methods

Animals

Male Wistar rats were housed from birth in a light-controlled room (dark phase 03:30–15:30 h) and otherwise maintained as described previously (Zammit, 1980, 1981) on Oxoid rat breeding diet (Oxoid, Basingstoke, Hants, U.K.) fed *ad libitum*. The animals were used within a weight range of 160–200 g (i.e. 5–6 weeks old). They were trained (i.e. handled for 5 min daily) for several days before being killed.

Chemicals and enzymes

These were as described by Easom & Zammit (1984).

Sampling of liver, preparation of liver homogenates and separation of microsomes

Anaesthesia was induced in animals by an intra-peritoneal injection of pentobarbital (60 mg/kg body wt.). After 15–20 min the body cavity was opened and the left lateral lobe of the liver sampled by the cold-clamping technique described by Easom & Zammit (1984). By this technique sampling and homogenization of the liver were routinely achieved in 5–10 s. The clamped liver sample was homogenized by three passes in a Teflon/glass homogenizer (radial clearance 0.20–0.25 mm) driven by a constant-torque motor operated at 1000 rev./min. The ice-cooled homogenization medium (Buffer A) contained 50 mM-sucrose, 10 mM-Tris/HCl (pH 7.4), 100 mM-KF, 10 mM-EDTA, 1 mM-dithiothreitol and 0.5 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged at 5000 *g* for 10 min; the supernatant was centrifuged again at this speed to ensure removal of intact mitochondria. The final supernatant was divided into two parts, and both were centrifuged at 100 000 *g* for 45 min. One of the resulting microsomal pellets was resuspended in a buffer containing 50 mM-KH₂PO₄, pH 7.25, 50 mM-KCl, 100 mM-KF and 1 mM-EDTA, and the other in a similar buffer in which the KF had been replaced by an equivalent concentration of KCl. After a further centrifugation at 100 000 *g* for 45 min, the pellets were resuspended in the respective buffers to a concentration of approx. 8 mg of microsomal protein/ml and used for the assay of HMG-CoA reductase.

Assay of HMG-CoA reductase

HMG-CoA reductase was assayed essentially as described by Shapiro *et al.* (1974), except that the assay volume was 75 μ l. The reaction was started by the addition of 25 μ l of cofactor solution containing 30 000 d.p.m. of [³H]mevalonolactone as internal standard. The reaction was performed at 37°C and was stopped after 15 min by the addition of 25 μ l of 5 M-HCl. After lactonization, samples (75 μ l) were applied to activated t.l.c. plates (silica gel G; 0.5 mm thickness) and chromatographed vertically in toluene/acetone (1:1, v/v). The product (located by visual detection of a concentrated mevalonolactone standard marker run in parallel on the same plates) was recovered from the plates by extraction with 2 vol. (7.5 ml) of diethyl ether. The combined extracts were dried down at 40°C and the residue was dissolved in water (400 μ l), to which 10 ml of scintillant (Scintillator 299; Packard) was added. The amount of radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter and the results were corrected for the efficiency of extraction, which was found routinely to be 70–80%. In preliminary

experiments it was ascertained that formation of mevalonolactone product was linear with time of assay and with the amount of microsomal protein used.

Activation of HMG-CoA reductase by protein phosphatase to obtain total activity

Dephosphorylation and activation of the enzyme was achieved by incubating microsomes (140–160 μ g of protein) with 4 units of liver protein phosphatase as described previously (Zammit & Corstorphine, 1982a). Controls from which protein phosphatase was omitted, but in which 100 mM-KF was included, were run concurrently. (The incubation media for phosphatase-treated and non-phosphatase-treated microsomes were equalized with respect to KF concentration before assay of HMG-CoA reductase activity.)

Protein concentration was measured as described by Lowry *et al.* (1951). Bovine serum albumin was used as standard.

Expression of results

By analogy with terminology used for other enzymes that are regulated by covalent modification, the activity of HMG-CoA reductase obtained before full activation with phosphatase treatment is referred to as the 'initial' activity, whereas that of the fully activated enzyme is designated 'total' activity. The fraction of the enzyme in the active form is expressed as initial/total-activity ratio. Absolute values of HMG-CoA reductase for both initial and total activities are expressed as nmol/min per mg of microsomal protein at 37°C.

Results and discussion

The use of the cold-clamping technique enabled us to obtain initial and total activities for HMG-CoA reductase in microsomes prepared from livers of rats at various stages (2 h intervals) of the light/dark cycle. A typical progress curve of the phosphatase-induced activation of HMG-CoA reductase activity in microsomes in the absence of KF is shown in Fig. 1. Routinely, two measurements of activity of HMG-CoA reductase in microsomes after suitable intervals of treatment with protein phosphatase were performed to ascertain that maximal dephosphorylation and activation of HMG-CoA reductase had been achieved. In the presence of KF no activation of HMG-CoA reductase activity was induced by protein phosphatase treatment (Fig. 1). In this way initial/total-activity ratios could be obtained for HMG-CoA reductase activity similar to those obtained for other enzymes regulated by

Fig. 1. Time course of activation of HMG-CoA reductase in rat liver microsomes by exogenous partially purified liver protein phosphatase

Microsomes prepared from the liver of a rat killed by cervical dislocation 6h into the dark phase were incubated with protein phosphatase (approx. 4 units) in the presence of 100mM-KCl (●) or 100mM-KF (▲). At the intervals shown, samples of the incubation mixtures were assayed for activity of HMG-CoA reductase. Routinely, microsomes were incubated for 10 and 15 min with phosphatase to ascertain the attainment of maximal activation in the measurement of total reductase activity (see the text).

phosphorylation-dephosphorylation (e.g. acetyl-CoA carboxylase; Zammit & Corstorphine, 1982a).

The total activity of HMG-CoA reductase showed the well-documented diurnal variation (Rodwell *et al.*, 1973, 1976), which has been shown by previous workers to be associated with changes in the amount of immunotitratable enzyme in the liver (Higgins & Rudney, 1973) and to be brought about specifically by changes in the rate of synthesis of the enzyme (Higgins & Rudney, 1973; Higgins *et al.*, 1974). In the present study the total activity of HMG-CoA reductase in microsomes obtained from rats sampled at the mid-dark time point (D6) was approx. 6-fold higher than in microsomes from rats sampled in the middle of the light phase (L6). This high activity at D6 was part of a plateau of activity which lasted for 8h of the dark phase, i.e. from D2 to D10 (see above). The rise of total HMG-CoA reductase activity to the value obtained during this plateau was very rapid and occurred primarily during the first 2h of the dark period, i.e. between the light/dark transition (L-D) and D2. A similarly rapid decrease in total HMG-CoA reductase activity occurred between

Fig. 2. Diurnal variation in the initial (○) and total (●) activities of HMG-CoA reductase in rat liver microsomes

Values are means $\pm$ S.E.M. for three to seven determinations performed on microsomal preparations obtained from separate rats, except for L2 and L4 values, which are averages of two separate determinations which gave very similar results. L-D and D-L refer to the light/dark and dark/light transitions respectively.

D10 and the D-L transition. As a result of these rapid increases and decreases in total reductase activity at the beginning and at the end of the dark period, respectively, the diurnal peak of total HMG-CoA reductase activity was more or less symmetrical around the plateau of activity between D2 and D10 (Fig. 2).

The most significant observation of the present study was that the degree of activation of HMG-CoA reductase by protein phosphatase varied markedly depending on the phase of the light/dark cycle at which the microsomes were obtained from the rats. Thus the enzyme in microsomes from animals the livers of which were sampled 4h into the light period (L4) was activated 3.5-fold after phosphatase treatment, whereas HMG-CoA reductase in microsomes prepared from livers of D4 animals was activated only 1.2-fold. These observations contrasted with those made by Brown *et al.* (1979) and by Dugan *et al.* (1982), who reported that protein phosphatase treatment of rat liver microsomes always resulted in an approx. 7-fold activation of HMG-CoA reductase activity irrespective of the phase in the light/dark cycle at which the animals were sampled.

As a consequence of the different extent to which HMG-CoA reductase was activated by protein phosphatase, the ratio between the initial activity (obtained before activation by phos-

phatase) and of total activity (after phosphatase treatment) varied markedly in a diurnal pattern between 28% and 80% at the nadir and peak, respectively, of the cycle (Fig. 3). This diurnal cyclical variation in initial/total-activity ratio had several important differences from the diurnal pattern of variation observed for total activity alone, as follows. (i) The initial/total-activity ratio from HMG-CoA reductase had a pronounced well-defined peak at D4, in contrast with the much broader (8h-long) plateau of high total activity between D2 and D10. (ii) The increase to, and decline from, this peak in initial/total-activity ratio were much more gradual than the corresponding phases of the cycle of the total activity alone. Thus the initial/total-activity ratio increased virtually linearly with time over the 10h period between L6 and D4. The decrease in initial/total-activity ratio after the D4 peak was even more gradual, such that the fraction of the enzyme in the active form was still over 50% at the D-L transition. (iii) The peak and nadir of the diurnal variation in initial/total-activity ratio did not coincide with those for the diurnal cycle for total activity (D6 and L6). The peak for the ratio occurred at D4 and the nadir occurred at the corresponding time point in the light cycle 12 h later, i.e. at L4. Therefore there was an apparent 2h phase difference between the two diurnal cycles, in addition to the other differences described above. These observations suggested that the diurnal variations in the two parameters (i.e. total activity of HMG-CoA reductase and the fraction of the enzyme in the active form *in vivo*) were controlled, at least partly, by different, possibly hormonal, diurnally variable stimuli *in vivo*.

The variation in the fraction of HMG-CoA reductase in the active form through the light/dark cycle had several effects on the resultant diurnal cycle in initial activity of the enzyme (Fig. 3), which is likely to be the best estimate of the activity of HMG-CoA reductase *in vivo* at the time of sampling of the liver (cf. Stansbie *et al.*, 1976; Zammit & Corstorphine, 1982a). Because of the increase in the initial/total ratio, concomitantly with the increase in total activity of HMG-CoA reductase during the dark period, the initial activity of the enzyme increased 12-fold, compared with only a 6-fold increase in total activity. In addition, the effect of the relatively slow, steady, increase in initial/total-activity ratio between L6 and D4 resulted in a more gradual increase in the initial activity of HMG-CoA reductase than in its total activity, such that the actual rate of cholesterologenesis *in vivo* would increase much more gradually than the increase in total activity alone would suggest. In this respect, and in view of the previous observation that the diurnal pattern of

Fig. 3. Diurnal variation in the fraction of HMG-CoA reductase in the active form in rat liver microsomes. Microsomes prepared from cold-clamped livers were used to obtain 'initial' and 'total' activities of HMG-CoA reductase (see the Materials and methods section) at 2h intervals of the light/dark cycle in which the rats were maintained. The fraction of HMG-CoA reductase in the active form is expressed as the initial/total activity (per cent). Values are means $\pm$ S.E.M. for three to seven determinations performed on microsomal preparations obtained from separate rats, except for L2 and L4 values, which are averages of two separate determinations which gave very similar results.

HMG-CoA reductase activity is associated most closely with feeding activity (Dugan *et al.*, 1972), it is noteworthy that the feeding pattern of rats shows a gradual increase towards the end of the light period which is continued into the dark period, i.e. more similar to the increase in initial HMG-CoA reductase activity rather than the sudden increase in total activity (Peret *et al.*, 1973; Bruckdorfer *et al.*, 1974; Munday & Williamson, 1983). Hence the combined effects of the difference in the diurnal cycles for the initial/total ratio and for total activity of HMG-CoA reductase were to produce a diurnal cycle in initial activity of the enzyme which may more closely approximate to the feeding behaviour of the animals. A similar dampening effect on the diurnal cycle of the initial activity was evident for the latter part of the dark phase, when HMG-CoA reductase activity was decreasing.

From the above, it emerges that the role of the changes in the fraction of the HMG-CoA reductase in the active form in response to entirely physiological stimuli may be the achievement of a more flexible response of the cholesterol-synthetic

capacity of the liver (as far as it is determined by the initial activity of HMG-CoA-reductase) than would otherwise occur if the activity of the enzyme *in vivo* were determined solely by the amount of enzyme in the liver at any one particular time point during the diurnal cycle. It is also evident that other stimuli, which are capable of affecting the initial/total-activity ratio through the bicyclic mechanism of phosphorylation-dephosphorylation described by Ingebritsen *et al.* (1978), will also add to this flexibility of response of the enzyme *in vivo*. Thus it is plausible to suggest that, for example, adrenergic stimulation of the liver could result in rapid changes in the initial/total-activity ratio for the enzyme by affecting the relative activities of the protein kinase(s) and/or phosphatase(s) involved. In this respect it was interesting to observe that when liver samples were obtained from acutely stressed rats (e.g. animals stunned by a blow on the head, or sufficiently overdosed with anaesthetic to produce respiratory stress) the fraction of HMG-CoA reductase in the active form was invariably very low (about 10%; see, e.g., Fig. 1), i.e. much lower than the ratio for the enzyme in the liver of the unstressed animal even at the nadir of the cycle of the initial/total-activity ratio. It is possible that the rapid inactivation of hepatic HMG-CoA reductase in response to acute stress may have been an additional contributory factor towards the persistently low initial/total-activity ratios obtained by other workers who obtained liver samples from untrained rats killed by cervical dislocation (Brown *et al.*, 1979; Dugan *et al.*, 1982).

In conclusion we suggest: (i) that changes in the fraction of HMG-CoA reductase in the active form do occur in the liver *in vivo* in response to normal physiological conditions such as occur during the diurnal light/dark cycle; (ii) that these changes have the role of producing more gradual transitions in the rates of hepatic cholesterogenesis, thus co-ordinating the cholesterogenic capacity of the liver more effectively with the feeding pattern of the animals; and (iii) that we have identified at least two possible factors (namely the lack of rapidity of liver sampling and the stressing of animals) as possible contributory factors towards the artefactual results obtained previously.

We thank Dr. W. W. Christie for interest and encouragement. R. A. E. was the recipient of a studentship funded by the Joint Committee of the Nutritional Consultative Panel, U.K.

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