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Direct demonstration that increased phosphorylation of 3-hydroxy-3-methylglutaryl-CoA reductase does not increase its rate of degradation in isolated rat hepatocytes

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Increased phosphorylation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase has been suggested to target the protein towards an increased rate of degradation. Our previous observations [Zammit & Caldwell (1990) Biochem. J. **269**, 373–379] suggested that, although Ca^{2+}-mobilizing hormones and other effectors can alter both the phosphorylation state of the enzyme and its total activity in isolated rat hepatocytes, there appears to be no causal correlation between the two parameters. In the present paper we set out to make direct measurements of the specific rate of degradation of ^{35}S-labelled HMG-CoA reductase in hepatocytes treated with agents that produced very marked and prolonged increases in the degree of phosphorylation of the protein, through different mechanisms. Okadaic acid (which inhibits phosphatases 1 and 2A), fructose (which increases cellular AMP through its metabolism to fructose 1-phosphate) and the Ca^{2+} ionophore A23187 (which also raises cellular AMP through an unknown mechanism) were all unable to alter the rate of HMG-CoA reductase degradation. We conclude that the basal rate of degradation of HMG-CoA reductase is unaffected by its phosphorylation state and that a transiently increased degree of phosphorylation cannot be the mechanism through which mevalonate increases the rate of degradation of the enzyme in rat hepatocytes and other cell types.

INTRODUCTION

The expression of the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in the liver is controlled very closely through modulation of the turnover rate of the protein and by phosphorylation-mediated inhibition. In a variety of cell lines, it has been shown that the rate of synthesis of the protein is controlled by oxysterols acting at both transcriptional and translational levels [1,2], although in a recent publication [3] it was reported that cholesterol feeding of rats resulted in a marked decrease of reductase protein in the absence of a decrease in the reductase mRNA in the liver. Non-sterol products derived from mevalonate metabolism inhibit translation of reductase mRNA [4,5]. At a post-translational level, three available mechanisms for the control of the expression of the protein are observed: these include the following. (i) The modulation of the rate of protein synthesis at initiation. We have shown [6,7] that Ca^{2+} mobilization in isolated rat hepatocytes produces a rapid and marked inhibition of synthesis of HMG-CoA reductase protein as part of a general inhibition of peptide-chain initiation [8,9]. For a protein whose rate of degradation is very fast, this has a near-instantaneous and profound effect on the activity [6,10]. (ii) Phosphorylation of HMG-CoA reductase, which inhibits its catalytic activity. This phenomenon is known to occur *in vivo* in response to normal physiological stimuli [11–13], as well as in isolated hepatocytes in response to hormones that mobilize Ca^{2+} [6]. *In vitro* the enzyme is phosphorylated by at least three different protein kinases [14–16], which all phosphorylate the same serine residue close to the *C*-terminal end [17]. (iii) Modulation of the rate of degradation of HMG-CoA reductase has been shown to occur in response to exposure to mevalonate, low-density lipoprotein and oxysterols in hepatocytes and in various other cell types [18,19].

Because mevalonate induces both an increase in degree of phosphorylation of HMG-CoA reductase and an increased rate of degradation of the protein [19], it has been suggested that phosphorylation targets HMG-CoA reductase towards degradation [19–21]. This hypothesis received support from the observation that phosphorylation of HMG-CoA reductase in isolated liver microsomes renders it more susceptible to proteolysis by the Ca^{2+}-dependent proteinase calpain II [22]. Recent work has also shown that a neutral Ca^{2+}-dependent proteinase may be responsible for mevalonate-induced degradation in intact CHO cells [23].

In our previous work on the modulation of HMG-CoA reductase activity by Ca^{2+} mobilization in hepatocytes, we observed a dissociation between the induction of increased phosphorylation by hormones, e.g. by vasopressin or phenylephrine in the presence of glucagon, and the ability of these hormones to induce the loss of total HMG-CoA reductase activity [6]. In subsequent work we showed that this dissociation is due to the fact that these hormones exert their influence solely through changes in the rate of synthesis of the protein [7]. Two pieces of indirect evidence, however, further indicated that increased phosphorylation is also dissociated from an enhanced rate of degradation. Firstly, the transiently increased phosphorylation induced by hormones was not followed by enhanced degradation [7], in contrast with the observations on the effects of mevalonate [19]. Although this was a reproducible result, it had the drawback of involving very transient increases in phosphorylation such that any sustained effects of this covalent modification could not be ascertained. Secondly, incubation of hepatocytes with okadaic acid (a specific inhibitor of type-1 and -2A phosphatases) resulted in a profound and long-lasting increase in phosphorylation of the enzyme without a decrease in the total activity of HMG-CoA reductase [6]. However, because in those studies we could not distinguish between the effects on synthesis and/or degradation, no definitive conclusions could be reached about the relationship between phosphorylation and degradation.

In order to provide direct experimental evidence bearing on the relationship between HMG-CoA reductase phosphorylation and degradation, in the present study we have studied three conditions which produce sustained and pronounced increases in the phosphorylation of HMG-CoA reductase in hepatocytes. We have measured directly the specific rate of degradation of ^{35}S-labelled HMG-CoA reductase protein in rat hepatocytes treated with these agents. The data demonstrate that prolonged phosphorylation does not alter the rate of HMG-CoA reductase degradation in hepatocytes.

MATERIALS AND METHODS

Animals

These were female Wistar rats (200–220 g) which were fed on a rat chow diet (3% fat, 56% carbohydrate, 19% protein; Special Diet Services, Edinburgh, U.K.) *ad libitum*. Hepatocytes that were to be labelled with [^{35}S]methionine were prepared from animals that were additionally given 2% cholestyramine and 0.5% simvastatin for 4 and 2 days respectively, before being used. All animals were kept under a 12 h-light/12 h-dark regime and were used 4 h into the dark period.

Preparation and incubation of hepatocytes

Hepatocytes were prepared as described previously [6] and incubated at 37 °C, with shaking, for 20 min before making any additions. Ionophore A23187, fructose and okadaic acid were added as portions of concentrated solutions in Krebs medium (for fructose) or dimethyl sulphoxide. The final concentration of the latter never exceeded 0.1 % of total volume. Controls received solvent only. In experiments involving the measurement of HMG-CoA reductase activity, the incubations were terminated by cold-quenching and centrifugation [6]. HMG-CoA reductase was assayed as in [24]. The AMP content of cells was measured (as in [10]) in $HClO_4$ extracts obtained after rapid centrifugation through a silicone-oil layer [17]. Hepatocytes were labelled with [^{35}S]methionine by bulk incubation of hepatocytes [(4–5) $\times 10^7$ cells] in 15 ml of Krebs medium containing 0.7 mCi of carrier-free [^{35}S]methionine (1000 Ci/mmol). After 20 min, the cells were washed twice with Krebs medium containing 2 mM-methionine and resuspended at the same density in separate flasks (1.5 ml each). The amount of [^{35}S]methionine present in HMG-CoA reductase at zero time was obtained by harvesting cells at the time of addition of the effectors (routinely about 10 min after exposing the cells to 2 mM-methionine). At the indicated times, the cells were harvested by light centrifugation and extracted in detergent-containing medium [7]. The amount of radioactivity still associated with HMG-CoA reductase (97 kDa band) was quantified after specific immunoprecipitation and autoradiography as described in [7].

Chemicals

The sources of most of these were as described previously [7, 10]. In addition, fructose was from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

Effects of okadaic acid

We have previously shown that the specific type-1 and type-2A phosphatase inhibitor okadaic acid (1 μM) when added to hepatocytes induces a rapid and prolonged decrease in the expressed activity of HMG-CoA reductase (i.e. that obtained after extraction of cells in fluoride-containing medium; see [6]), without changing the total activity of the enzyme (obtained in phosphatase-treated microsomes isolated from Cl^--extracted cells; see [6]). Even after 40 min of incubation with okadaic acid we observed no decrease in total reductase activity, in spite of continued high phosphorylation of the enzyme (as evidenced by the depressed expressed/total activity ratio, which stayed around 0.20). This observation could have resulted either from unaltered rates of synthesis and degradation of reductase or from an accelerated rate of degradation that was matched by an equal enhancement of the rate of synthesis. Because of previous suggestions (see the Introduction) that increased phosphorylation of reductase leads to an increased rate of degradation, it was important to establish by direct experimentation whether the rate

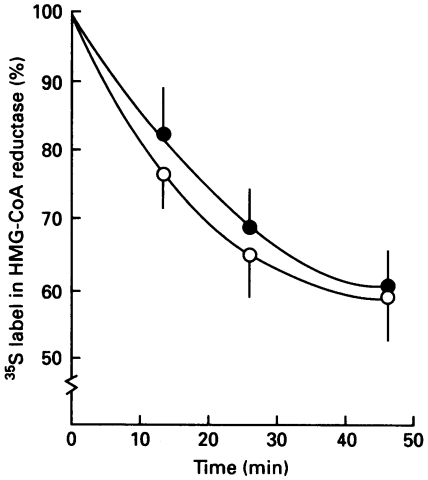

Fig. 1. Effect of okadaic acid on the degradation of ^{35}S-labelled HMG-CoA reductase in isolated rat hepatocytes

Cells were labelled with [^{35}S]methionine, washed and incubated in fresh medium (containing 2 mM unlabelled methionine) in the absence (○) or presence (●) of 1 μM-okadaic acid. Values are means (±S.E.M.) for three separate cell preparations.

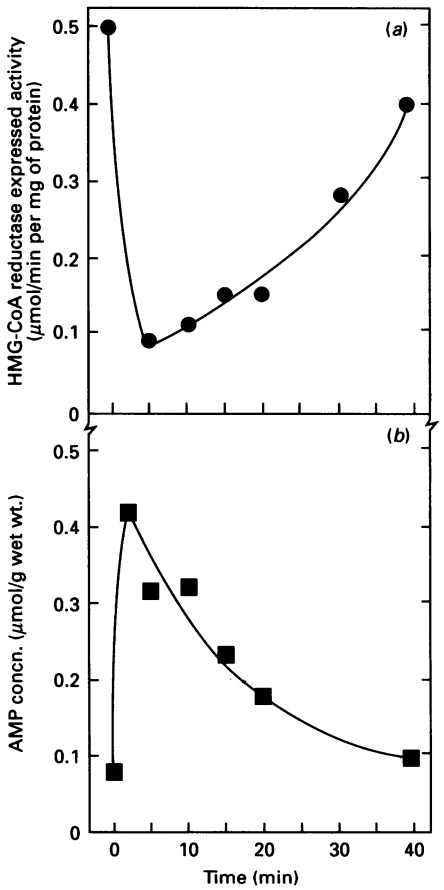

Fig. 2. Time course of the effects of fructose (10 mM) on the expressed activity of HMG-CoA reductase (*a*) and on the AMP content of isolated hepatocytes (*b*)

Hepatocytes were incubated in oxygenated medium for 20 min before addition of 10 mM-fructose at zero time. The activity of HMG-CoA reductase was obtained at the indicated times in microsomes obtained from cold-quenched cells extracted in fluoride-containing medium (see the Materials and methods section). AMP content of cells was obtained after rapid filtration of the hepatocytes through a silicone-oil layer into $HClO_4$. Results from a representative experiment are shown.

of degradation of HMG-CoA reductase is affected under these conditions. As shown in Fig. 1, incubation of cells with okadaic acid did not result in any change in the rate of disappearance of ^{35}S label associated with newly synthesized HMG-CoA reductase in the hepatocytes.

Effects of fructose and A23187

Okadaic acid has profound and multiple effects on intact cells, as, by necessity, it results in the increased phosphorylation of many proteins. We therefore decided to induce the phosphorylation of HMG-CoA reductase more specifically by raising the cellular concentration of AMP. We have previously obtained evidence [10] that the AMP-activated kinase [25–27] is involved in the action of ionophore A23187 to increase the degree of phosphorylation of HMG-CoA reductase in intact hepatocytes. The reductase is an excellent substrate for the AMP-activated kinase *in vitro* [16,17]. Consequently, we explored the possibility that the increase in AMP induced in hepatocytes by metabolism of fructose [28,29] may result in the prolonged phosphorylation of HMG-CoA reductase. Figs. 2 and 3 show that this was indeed the case, with a close correlation being evident between cellular AMP concentrations and expressed HMG-CoA reductase activity. Fructose had no effect on total reductase activity (results not shown). In the light of these observations, we tested the effect of incubation of cells with 10 mM-fructose, on the rate of degradation of the reductase. As with okadaic acid, we found no effect on the rate of degradation of HMG-CoA reductase (Fig. 4). The addition of the Ca^{2+} ionophore A23187 to hepatocyte suspensions was shown by us to result in the prolonged phosphorylation of HMG-CoA reductase [6,10]. As shown in Fig. 5, addition of ionophore to hepatocytes under the same conditions used previously [6,10] had no significant effect on the rate of degradation of HMG-CoA reductase, although there was a tendency to an increased rate. If this effect was genuine, it was relatively minor and could have resulted from direct effects

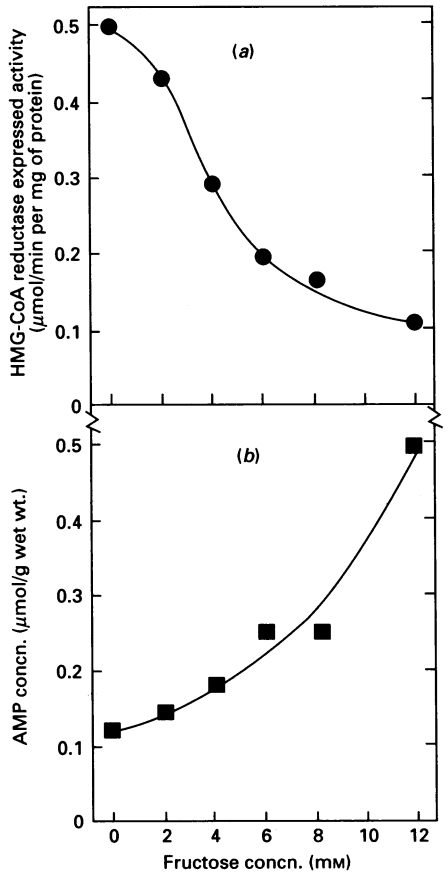

Fig. 3. Effects of increasing concentrations of fructose on the expressed activity of HMG-CoA reductase (*a*) and cellular AMP content (*b*) in isolated rat hepatocytes

Hepatocytes were incubated for 20 min before addition of the indicated concentrations of fructose. Microsomal expressed HMG-CoA reductase activity and AMP content of the cells were measured 10 min after addition of fructose. Results from a representative experiment are shown.

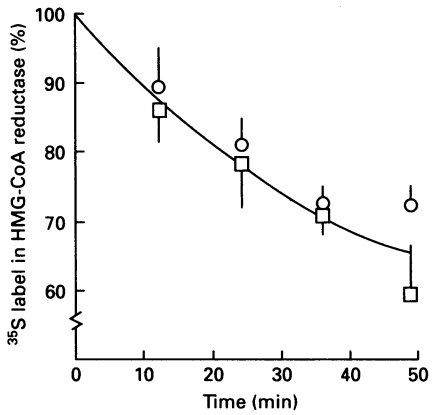

Fig. 4. Increased phosphorylation of HMG-CoA reductase induced by fructose-mediated increase in cellular AMP concentration does not affect the rate of degradation of HMG-CoA reductase

Cells were labelled with [^{35}S]methionine, washed and resuspended in fresh medium containing 2 mM-methionine and further incubated in the absence (□) or presence (○) of 10 mM-fructose. Values are means (±S.E.M.) for three separate cell preparations.

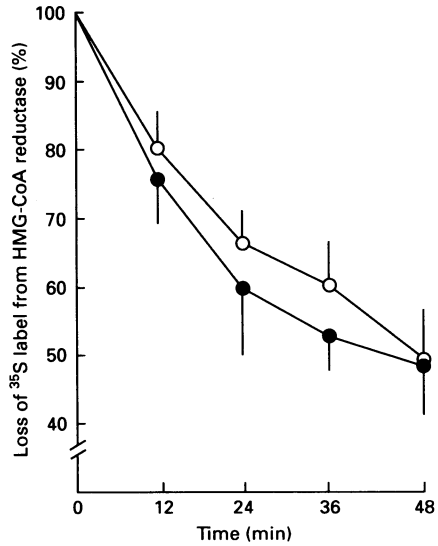

Fig. 5. Effects of incubation of hepatocytes with the Ca^{2+} ionophore A23187 on the rate of degradation of HMG-CoA reductase

Cells that were pre-labelled with [^{35}S]methionine (see legends to Figs. 1 and 4) were incubated in the absence (○) or presence (●) of 10 μM ionophore A23187. The cells were harvested at the indicated times, and the amount of ^{35}S label associated with immunoprecipitated HMG-CoA reductase protein was determined (see the Materials and methods section). Absolute rates of degradation were more variable in this series of experiments than in those shown in Figs. 1 and 4.

which disruption of the Ca^{2+} status of the cell may have on degradation rates [23].

Conclusion

We have tested three conditions that raise the degree of phosphorylation of HMG-CoA reductase for prolonged periods through at least two different mechanisms. In none of these instances have we been able to detect any effect as the specific rate of degradation of ^{35}S-labelled HMG-CoA reductase. We are therefore driven to the conclusion that, whereas increased phosphorylation of the enzyme makes it more susceptible to proteolysis by the neutral Ca^{2+}-dependent proteinase calpain II *in vitro* [21,22], increased phosphorylation, by itself, does not predispose the enzyme to enhanced degradation in the intact cell. This does not rule out the possibility, however, that agents that alter the rate of degradation of HMG-CoA reductase *in vivo* (e.g. mevalonate) could act by increasing the rate of Ca^{2+}-dependent proteolysis, as suggested by the studies of [23].

The conclusions of the present paper would appear to concur with other recent observations. Thus it has been shown [17] that the only phosphorylation site on the enzyme occurs near the *C*-terminus, within the catalytically active M_r-53000 fragment released by calpain II-induced proteolysis [19]. Moreover, expression *in vivo* of a cDNA for this M_r-53000 fragment has been shown to yield a stable protein which does not respond to mevalonate exposure of the cells by increased degradation [30], even though it would be expected that the phosphorylation site in this soluble protein would be available as a substrate for the various protein kinases known to act on it.

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