CONVERSION OF INACTIVE 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE TO ITS ACTIVE ENZYME DURING CULTURE OF RAT HEPATOCYTES

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1. Introduction

Primary cultures of rat liver cells have been found to retain many in vivo physiological functions and have been used as experimental models for various studies of enzyme regulation [1–4]. We have reported [5] that HMG-CoA reductase (EC 1.1.1.34), the key regulatory enzyme in hepatic cholesterol biosynthesis, approached a steady level of activity after rat liver cells had been cultured as monolayers for 48–72 h. A steady level of this enzyme activity was obtained regardless of the nutritional state of the rats or the time when the rats were sacrificed. Cultured hepatocytes appear to obviate the residual effects of endogenous inducers and suppressors, and this enzyme activity becomes free from the influence of diurnal variation.

Hepatic HMG-CoA reductase was reported inactivated in vitro when unwashed microsomes were incubated with ATP and magnesium [6]. This inactivated enzyme was found to be reactivated by incubation with a cytosolic factor from liver [7]. This cytosolic activating factor was identified as a protein phosphatase [8]. The reactivation of HMG-CoA reductase can be prevented by NaF. When microsomes of rat liver were prepared in the presence of 50 mM NaF [7] the reductase activity was also only 15% of those microsomes prepared without NaF. Thus, it was suggested [7] that 85% of the HMG-CoA reductase in vivo was in the inactive form. We have observed that the inactive form of HMG-CoA reductase gradually disappears with time in cultured rat hepatocytes. After 48 h in culture, HMG-CoA reductase of liver cells become fully activated, spontaneously. The active reductase can still be inactivated and reactivated. Thus cultured hepatocytes can be used to study HMG-CoA reductase induction [5] and also activation–inactivation.

2. Materials and methods

All chemical reagents were of reagent grade and obtained commercially. Leibovitz L-15 medium and fetal calf serum were purchased from Grand Island Biological Company.

Adult male Sprague-Dawley rats (250–350 g) were used for cell preparations. Rats were maintained on light cycles of 6 a.m.–6 p.m. and sacrificed at 10 a.m. on the day of cell isolation. When indicated, rats were starved for 72 h. Liver parenchymal cells were isolated and cultured in collagen-coated Petri dishes as in [9]. Culture medium was Leibovitz L-15 containing 28 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*2-ethane-sulfonic acid) (pH 7.4), 100 unit/ml penicillin and 100 μg/ml streptomycin. Fetal calf serum (10%) was included for the first 24 h of culture to obtain a better monolayer of cells. Incubation medium was changed once, 24 h after plating, and shifted to serum-free L-15.

To harvest cells at indicated times, cell monolayers were washed twice with 0.9% saline before they were gently scraped from the dishes and collected in a test tube in cold buffer solution containing 0.25 M sucrose, 2.5 mM EDTA, 25 mM reduced glutathione and 75 mM nicotinamide (pH 7.0). Cells from 3–5 Petri dishes were pooled together for 1 test sample. Microsomes were prepared as in [5] and stored at −70°C until used. NaF (50 mM) was added to the buffer when it was intended to prevent activation of reductase during microsomal preparation. Microsomal HMG-CoA reductase activity was assayed according

to [10]. Cytosolic protein phosphate, an activating factor for HMG-CoA reductase, was partially purified according to [11].

To determine cellular ATP concentration, cells were extracted with 48% ethanol containing 5 mM EDTA and 0.07 M NaCl at 0°C for 15 min followed by heating at 72°C for 15 min. After denatured proteins were removed by centrifugation, the remaining supernatant was used for ATP determination that utilized the coupling actions of hexokinase and glucose-6-phosphate dehydrogenase.

3. Results and discussion

Figure 1 shows the time course of HMG-CoA reductase activity in cultured rat hepatocytes. The result is essentially that in [5], except cells have been cultured in the presence of 10% fetal calf serum for the first 24 h, while those reported before were cultured in serum-free medium throughout the experiments. The addition of fetal calf serum does not eliminate the increase of HMG-CoA reductase activity in rat liver cells cultured for 24 h as it does to cultured human fibroblasts [12]. This insensitivity of hepatic HMG-CoA reductase activity to fetal calf serum is in agreement with [13].

The ratio of reductase activity, when microsomes are prepared with (+) or without (–) NaF, is 0.5 for fresh isolated liver cells (fig.1). The ratio (+NaF/–NaF) gradually increases with culture time and reaches 1.0 after 48 h as shown in fig.2. This ratio in fresh isolated liver cells is significantly higher than that in whole liver (0.2 : 0.3), suggesting that partial activation of in vivo HMG-CoA reductase may have occured during the cell isolation process.

To be sure that microsomal HMG-CoA reductase activity of cultured liver cells is fully activated, microsomes of 72 h cultured cells were incubated with partially purified protein phosphatase prior to the assay of reductase activity. Table 1 shows that protein phosphatase readily reactivates fresh liver microsomes prepared in the presence of NaF, but has no effect on reductase activity of microsomes prepared without NaF. Moreover, it does not further enhance microsomal HMG-CoA reductase activity of 72 h cultured

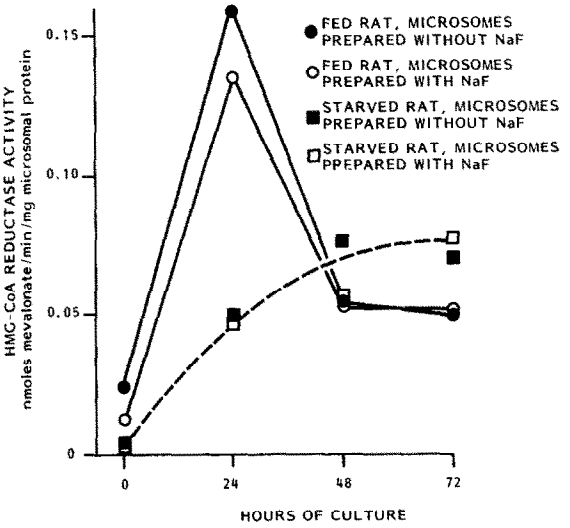

Fig.1. Time course of HMG-CoA reductase activity in cultured rat hepatocytes. Liver cells are isolated at the low point of the diurnal rhythm of HMG-CoA reductase activity. Fetal calf serum (10%) is added to culture medium (L-15) for the first 24 h only. Cells are maintained in serum-free L-15 medium after 24 h. Microsomes are prepared each day with buffer as in section 2. When indicated, 50 mM NaF is added to microsomal buffer during microsome preparation. Results represent average of 2 rats.

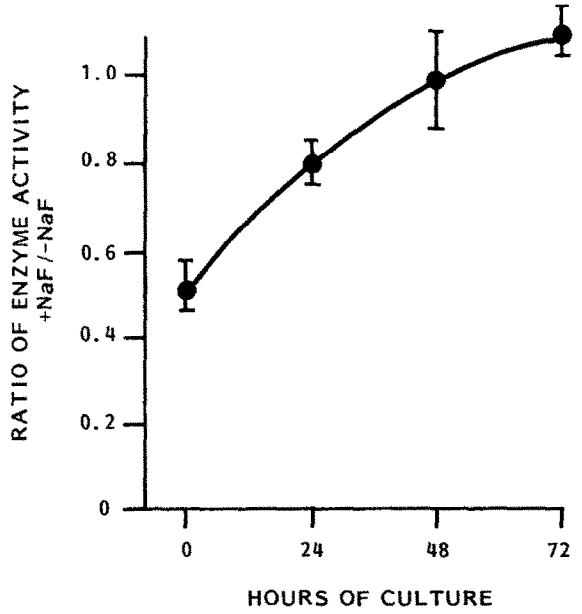

Fig.2. Ratios of HMG-CoA reductase activity in cultured rat hepatocyes when microsomes are prepared in the presence and the absence of NaF. Liver cells were isolated from fed rats and cultured as described in fig.1. The data represent means of 5 expt. Brackets indicate ± SEM. NaF used in microsomal preparation is 50 mM.

Table 1
Effects of activating factor on microsomal HMG-CoA reductase activity of fresh liver and cultured liver cells

	Microsomes prepared with	Protein[a] phosphatase	SEA[b]
Fresh liver	50 mM NaF	–	0.032
	50 mM NaF	+	0.108
	No NaF	–	0.103
	No NaF	+	0.118
72 h Cultured rat liver cells	50 mM NaF	–	0.121
	50 mM NaF	+	0.129
	No NaF	–	0.140
	No NaF	+	0.127

[a] (+) Partially purified protein phosphatase (0.11 mg protein) has been preincubated with microsomes (0.05–0.06 mg protein) for 30 min at 37°C prior to enzyme assay. (–) Microsomes have been preincubated with buffer only
[b] Specific enzyme activity, nmol mavalonate . mg microsomal protein^{-1} . min^{-1}

liver cells whether NaF has been added or not during microsome isolation. Therefore, in 72 h cultured cells, all HMG-CoA reductase is in the active form. When unwashed microsomes of 72 h cultured cells are incubated with ATP and $MgCl_2$, HMG-CoA reductase decreases to 36% of control microsomes (table 2). Table 3 shows that MgATP-inactivated microsomes of cultured cells can be fully reactivated by cytosolic protein phosphatase. Therefore, HMG-CoA reductase activity in 72 h cultured rat hepatocytes can be inactivated or activated reversibly as that in fresh rat liver [7].

Since both ATP and magnesium are required for the inactivation of reductase, the disappearance of its inactive enzyme form may be due to diminishing cellular ATP during the culture process. We find that our cultured liver cells can restore ATP and maintain a level of 7–8 nmol ATP/mg cellular protein. Although the ATP level falls significantly during cell preparation, table 4 shows that the cells restore the level to that of cultured cells from regenerated rat liver [1].

Table 2
Effects of ATP and $MgCl_2$ on microsomal HMG-CoA reductase activity of cultured rat liver cells

Sample	Treatment	SEA[b]
1	None	0.159
2	Microsomes incubated with imidazole buffer[a] at 37°C for 30 min	0.169
3	Microsomes incubated with imidazole buffer[a] containing 4 mM $MgCl_2$ and 2 mM ATP, 37°C for 20 min	0.060

[a] 50 mM imidazole (pH 7.4) + 0.5 mM dithiothreitol
[b] Specific enzyme activity, nmol mavalonate . mg microsomal protein^{-1} . min^{-1}

Microsomes from 72 h cultured rat liver cells were prepared in the presence of 50 mM NaF

Table 3
Reactivation of MgATP-inactivated HMG-CoA reductase activity of cultured rat liver cells

Sample	Protein phosphatase	SEA[a]
Control microsomes	–	0.059
	+	0.062
MgATP-inactivated microsomes	–	0.012
	+	0.058

[a] Specific enzyme activity, nmol mavalonate . mg protein^{-1} min^{-1}

Microsomes from 72 h cultured rat liver cells were prepared without NaF. Inactivated microsomes had been treated with 4 mM $MgCl_2$ and 2 mM ATP in 50 mM imidazole buffer (pH 7.4) for 20 min at 37°C. Control microsomes had been treated with imidazole buffer only. When indicated (+), 0.106 mg microsomal proteins were preincubated with 0.269 mg partially purified protein phosphatase for 30 min at 37°C prior to enzyme assay

Table 4
ATP concentration in freshly isolated and cultured rat hepatocytes

Expt	Hepatocytes	Day of culture	nmol ATP / mg cellular protein
1	Freshly isolated	0	3.75
	Cultured	1	6.91
	Cultured	2	8.88
	Cultured	3	6.83
2	Freshly isolated	0	3.30
	Cultured	1	6.05
	Cultured	2	7.40
	Cultured	3	6.61

Small volume of 48% ethanol containing 5 mM EDTA and 70 mM NaCl was added to washed monolayers of cells. Cells were then scraped off Petri dishes with a rubber policeman. The suspension was kept at 0°C for 15 min followed by heating at 72°C for 15 min, then centrifuged at 10 000 × g for 15 min. Supernatant was used for ATP determination

A bicyclic regulatory scheme for activation and inactivation of the reductase has been proposed [14]. It states that in the presence of magnesium and ATP, reductase kinase catalyzes phosphorylation of HMG-CoA reductase. Thus a phosphorylated and inactive form of enzyme is produced. On the other side of this scheme, a phosphoprotein phosphatase catalyzes dephosphorylation of reductase and converts it into active form. The activation (dephosphorylation) does not require MgATP. Our results show that after hepatocytes have been cultured as monolayers for 48–72 h, HMG-CoA reductase becomes 100% active. The cellular inactivation system appears to be intact in cultured cells because MgATP can inactivate reductase activity of unwashed microsomes of cultured cells, and the MgATP-inactivated reductase can be reactivated by phosphatase. The diminishing of the inactive reductase form is not due to diminishing cellular ATP level. To be consistent with the scheme in [14], an increasing level (or activity) of phosphoprotein phosphatase during cell culture would increase the active dephosphorylated form of HMG-CoA reductase. Further investigation is needed to verify this point.

The physiological significance of phosphorylation–dephosphorylation of HMG-CoA reductase is not established, as most of this work has been carried out in vitro. It was found in [15] that 75–90% of reductase under all physiological conditions was in a phosphorylated (inactive) form at the time the homogenates were prepared. The proportion of phosphorylated enzyme remained constant under conditions in which total reductase activity and the rate of cholesterol synthesis varied as much as 50-fold. They raise the question whether lower activity observed in the presence of fluoride represents the true level of enzymatic activity within the intact liver cell. They suggest a rapid phosphorylation reaction might occur while the animal is being killed and the liver is being removed. The achievement of a steady level of HMG-CoA reductase activity, of total activation and of reversible inactivation of this enzyme form in cultured liver cells suggests that this experimental model is ideal for study of enzyme activation–inactivation as well as of induction [5].

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