A STEADY STATE OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE ACTIVITY IN CULTURED RAT LIVER CELLS AND THE INCREASE OF ITS ACTIVITY BY DEXAMETHASONE

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1. Introduction

Many studies have shown that HMG-CoA reductase (EC 1.1.1.34) is the key regulatory enzyme in hepatic cholesterol biosynthesis [1]. In rat liver, this enzyme activity shows a circadian rhythm [2] related to the feeding cycle and is regulated by the cholesterol content of the diet [3], starvation [4] and various hormones [5,6]. Because of these factors, in vivo studies have been difficult to interpret and the relative importance of various regulators remains uncertain [1]. We believe that a better understanding of the control of HMG-CoA reductase activity in liver cells can be achieved by studies with a cell culture system, an approach which has proven successful in studying this enzyme in human fibroblasts [7]. By culturing isolated rat hepatocytes in serum-free medium, we have been able to obtain a steady-state level of HMG-CoA reductase activity. This paper reports the conditions for achieving this steady-state activity and the effects of cholesterol, its derivatives, and of some hormones on HMG-CoA reductase activity in cultured rat liver cells.

2. Materials and methods

Adult male Sprague-Dawley rats (250–350 g) were used for cell preparations. Materials used in this study were obtained from the following sources: Leibovitz L-15 medium and fetal calf serum, Grand Island Biological, Grand Island, NY; DL-3-hydroxymethyl-[3-^{14}C]glutaryl coenzyme A, New England Nuclear, Boston, MA; glucagon, insulin and 20α-hydroxycholesterol, Sigma Chemical, St Louis, MO; cholesterol, ICN Life Science Group, Cleveland, OH; 7-ketocholesterol, Steraloids, Wilton, NH; pure dexamethasone, a gift from Merck, Sharp and Dohme, West Point, PA; hydrocortisone sodium succinate, Upjohn, Kalamazoo, MI. After it was purified by repeated crystallization in ethanol, the preparation showed only one spot. 7-Ketocholesterol showed only trace contamination with cholesterol. Commercial cholesterol was contaminated with unidentified sterols by thin layer chromatography.

Rats were maintained on light cycles of 6 a.m. to 6 p.m. or 4 p.m. to 4 a.m. for at least two weeks, and were sacrificed at 10 a.m. on the day of cell isolation. Liver parenchymal cells were isolated and cultured in collagen-coated petri dishes [8]. Culture medium was serum-free Lebovitz' L-15 containing 28 mM Hepes (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) pH 7.4, 100 units/ml penicillin and 100 μg/ml streptomycin. Incubation medium was changed once, 24 h after plating. Sterile steroid or hormone solutions were added to the medium after 48 h incubation or when otherwise indicated.

Cell monolayers were washed twice with 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 were homogenized with a Polytron for 5 s at top speed. Cell homogenates were centrifuged at 10 000 × *g*

for 10 min. The supernatant was then recentrifuged at 100 000 × *g* for 60 min. Microsomal pellets were stored at −70°C for no more than 1 week before assay of the enzyme. Microsomal HMG-CoA reductase activity was assayed according to Shapiro et al. [9]. Tyrosine transaminase (TAT) activity in cell homogenate was assayed according to Spencer and Gelehrter [10]. Protein was measured by the method of Lowry et al. [11]. One unit (U) of activity of HMG-CoA reductase is defined as the formation of one nanomole of [^{14}C]mevalonate from ^{14}C-labelled HMG-CoA in one minute per milligram of microsomal protein.

3. Results and discussion

Figure 1 shows the effect of time on HMG-CoA reductase activity of rat liver cells cultured in serum-free, L-15 medium. When cell isolation was performed at the middle of the light cycle, enzyme activity was initially 0.052 ± 0.011 (SEM) nmol/min/mg. It increased three-fold after 24 h in culture, decreased to 0.081 ± 0.012 nmol/min/mg by 48 h and remained at this level through 72 h. On the other hand, liver cells isolated at the middle of the dark cycle (peak of circadian rhythm) exhibited an activity of 0.481 ± 0.185 nmol/min/mg. When incubated, the enzyme activity decreased rapidly in the first 48 h and reached a steady level between 48 h and 72 h of 0.098 ± 0.041 nmol/min/mg. Fresh liver cells isolated from rats starved for 72 h showed an almost undetectable activity which increased gradually over 48 h in culture to reach a steady level of 0.084 ± 0.008 nmol/min/mg until 96 h. Attainment of a steady-state activity of HMG-CoA reductase in monolayer cultures of liver cells in serum-free medium is the major finding of this report. Thus this critical enzyme of cholesterol synthesis in liver, which is subject in vivo to a circadian variation and to many control factors, can now be studied in a condition free of background variations. The reasons why this steady-state develops in vitro are conjectural at present. Breslow [12] did not obtain a steady state of HMG-CoA reductase activity in monolayer rat liver cells, cultured in serum-free L-15 medium. The major difference in his methodology from ours is that the rats underwent a partial

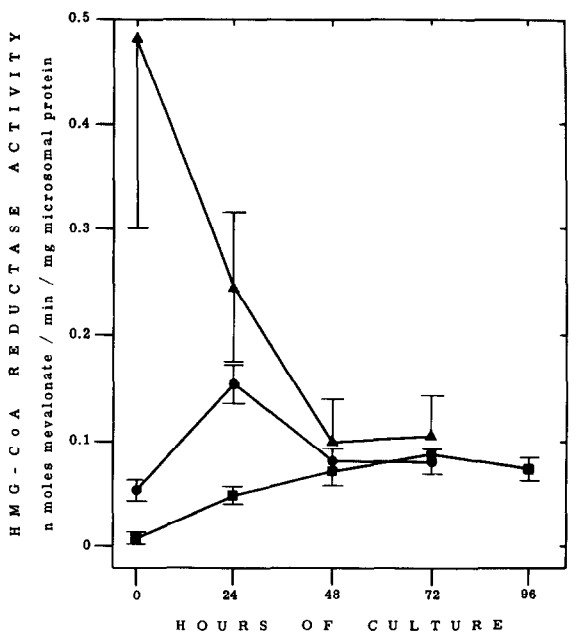

Fig.1. Liver cells are cultured in serum-free L-15 medium as described in Materials and methods. Medium was changed once at 24 h. Cells isolated from rats on light cycle (•), dark cycle (▲), or after 72 h starvation (■). Brackets indicate ± SEM of the means of 4–15 donor animals.

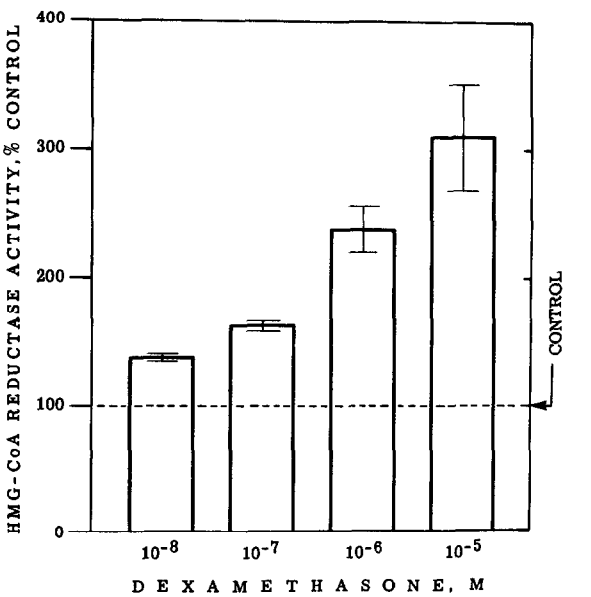

Fig.2. The results are expressed as % control (control = 100%). Brackets indicate ± SEM of the means of 8 donor animals. Dexamethasone was added to culture medium at 48 h. Culture liver cells were harvested at 72 h.

hepatectomy four days before liver cell isolation, whereas ours were isolated from non-regenerated livers.

While studying the effects of certain hormones on the steady state activity of HMG-CoA reductase in cultured liver cells, we found that dexamethasone progressively increased the activity, by 1.4-fold at 10^{-8} M to 3.1-fold at 10^{-5} M (fig.2). Hydrocortisone sodium succinate at 10^{-5} M increased the activity 1.6-fold. Previous studies injecting adrenal corticoids into whole rats showed no effect [13] on the liver enzyme (a result we have confirmed) or a suppression of reductase activity [6]. Why there is a discrepancy between the effects of dexamethasone and other adrenal corticoids in vivo versus in vitro is not understood at present. One other group has reported that dexamethasone increased reductase activity in cultured (HeLa) cells [14]. TAT activity was used as a positive control enzyme in our liver cell cultures, and it increased 3-fold at 10^{-8} dexamethasone.

The increase of enzyme activity with dexamethasone was prevented by 10 μM cycloheximide and by 1 μM actinomycin D, as shown in table 1. Previous studies with low doses of cycloheximide in vivo [15] suggested that it blocks new enzyme synthesis but does not change the rate of enzyme degradation. Our results, a fall in activity of 60% in 6 h, are consistent with a half-life of 4.2 h derived from in vivo studies with cycloheximide [15]. The block in dexamethasone stimulation by actinomycin D is only presumptive evidence that DNA-directed RNA synthesis is required for induction to occur. To prove that dexamethasone increases reductase synthesis will require studies of both synthesis and degradation rates using a radioactive amino acid incorporation method, such as that used to study the diurnal cycle [16].

To test whether cultured liver cells would show enzyme suppression by certain oxy-derivatives of cholesterol as occurs in other cultured cells [17,18], we added 7-ketocholesterol and 20α-hydroxycholesterol (50 μg/ml) to the serum-free medium and obtained suppression to 22% and 33% of control, steady-state levels, respectively. Purified cholesterol (50 μg/ml) showed no effect while unpurified cholesterol suppressed the reductase to 39% of control, a finding in agreement with those in cultured fetal mouse liver cells [18]. Controls were treated with the

Table 1
Evidence for dexamethasone induction of HMG-CoA reductase in cultured liver cells

Additions to culture medium[a]	Concentration	HMG-CoA reductase activity (% Control)
None	–	100
Actinomycin-D	10^{-6} M	76
Cycloheximide	10^{-5} M	41
Dexamethasone	10^{-5} M	212
Dexamethasone + Actinomycin D	10^{-5} M 10^{-6} M	92
Dexamethasone + Cycloheximide	10^{-5} M 10^{-5} M	35
Hydrocortisone-sodium succinate	10^{-5} M	160

[a] 6 hours incubation

Compounds were added to culture medium after 48 h in culture, and cells were harvested at 54 h

Table 2
Suppression of HMG-CoA reductase by cholesterol and its derivatives in cultured liver cells

Additions to culture medium[a]	Concentration	HMG-CoA reductase activity (% Control)[b]
None		100
7-Ketocholesterol	50 μg/ml	22
20α-Hydroxy-cholesterol	50 μg/ml	33
Purified cholesterol	50 μg/ml	102
Impure cholesterol	50 μg/ml	39

[a] 24 h incubation, 48–72 h
[b] Average of three experiments

Steroids were dissolved in ethanol before adding to the culture medium
Control contained 0.68% ethanol as the tests

same amount of ethanol (0.68%) which was used as a solvent for the steroids.

Glucagon, 10^{-6} M, added twice in 24 h during the steady state, decreased activity by 20%, somewhat less than its inhibitory effect in vivo [6].

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