Radioimmune Precipitation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase from Chinese Hamster Fibroblasts

EFFECT OF 25-HYDROXYCHOLESTEROL

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Antibody prepared against 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of rat liver can be shown to inhibit this enzyme in extracts prepared from cultured Chinese hamster ovary (CHO-K1) cells. The molecular weight (53,000) of the HMG-CoA reductase subunits of rat liver and Chinese hamster liver is identical with a [35S]-methionine-labeled polypeptide that can be precipitated from CHO-K1 lysates by this antibody used in conjunction with protein A Sepharose. It is shown that 25-hydroxycholesterol which lowers HMG-CoA reductase activity in cultured fibroblasts blocks the incorporation of labeled methionine into this polypeptide. Furthermore, the antibody immune precipitates two other polypeptides with molecular weights of 127,000 and 60,000. The latter polypeptide responds to 25-hydroxycholesterol in the same fashion as the 53,000-dalton polypeptide. In a dominant 25-hydroxycholesterol-resistant mutant of the CHO-K1 cell, 25-hydroxycholesterol did not inhibit incorporation of labeled methionine into either the 53,000- or 60,000-dalton polypeptides.

The rate of cholesterol biosynthesis in cultured mammalian cells in the presence of serum is largely determined by the number of receptors for the low density lipoprotein. As has been shown by the studies of Brown and Goldstein (1), this protein, which is the major cholesterol carrier in serum, enters cells which possess receptors and after hydrolysis releases free sterol which in turn inhibits cholesterol biosynthesis. In tissue culture systems (2), it can be shown that even cells which lack the low density lipoprotein receptor will be inhibited in cholesterol biosynthesis. In tissue culture systems (2), it can be shown that even cells which lack the low density lipoprotein receptor will be inhibited in cholesterol biosynthesis. In mammalian cells has not previously been directly demonstrated, although experiments with inhibitors of protein biosynthesis and RNA biosynthesis have been shown to prevent the full expression of HMG-CoA reductase when regulatory sterols are removed from culture medium (4), and immune titration experiments (9) have suggested that these sterols as well as other regulatory effectors (10, 11) can affect both the number and type of HMG-CoA reductase molecules. The object of this report is to present data obtained by direct radioimmune precipitation analysis of HMG-CoA reductase in a cultured fibroblast line, the CHO-K1 cell. These results show the effects of 25-hydroxycholesterol on the biosynthesis of HMG-CoA reductase in these cells. We have chosen to examine a cultured Chinese hamster fibroblast line as a model because of the increasing interest in utilizing somatic cell mutants of such cells (4-6) in analyzing the mechanism of regulation of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Medium and Cells—The CHO-K1 cell (12) was used in these experiments. Cells were grown on Ham's F12 medium (13) supplemented with 8% newborn calf serum or 2% delipidized serum (14). 25-Hydroxycholesterol (Steraloids, Wilton, NH) was added to cultures in ethanol and made up immediately prior to use.

Assays and Antisera—The antibody (ammonium sulfate-fractionated (28) IgG) used in this study was prepared in rabbits against rat liver HMG-CoA reductase. This reductase antibody was prepared against enzyme with a specific activity of 21,000 nmol of NADPH/min/mg of protein and monospecificity was demonstrated by Ouchterlony double diffusion and immunoelectrophoresis (10). Nonspecific IgG was also prepared from normal rabbit serum by ammonium sulfate fractionation. Both specific and nonspecific IgG were kept at a concentration of 10 mg of protein/ml in 0.15 M NaCl, pH 8.4, and 0.01 M boric acid buffer at 4°C. HMG-CoA reductase activity was assayed in CHO-K1 cell extracts prepared by freeze-thawing in KVO EOB as has been previously described (4). Extracts prepared in this fashion are stable for at least 18 h at room temperature.

Radioimmune Precipitation Analysis of HMG-CoA Reductase from Chinese Hamster Ovary Cells—Cells (6 × 10⁶) were plated on to each of six 150-mm Petri plates and incubated for 24 h. The medium is then changed to F12 supplemented with 2% delipidated calf serum

The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate.
Radioimmune Precipitation of HMG-CoA Reductase

Incubation conditions

| Incubation conditions | HMG-CoA reductase |
|-----------------------|-------------------|
|                       | nmol mevalonate/ h/mg protein |
| A 1 h, methionine-free | 26.9 ± 4.9 |
| F12, 3 h, methionine-free | 22.0 ± 2.7 |
| F12, plus 4 × 10^7 M methionine | 43.3 ± 4.8 |
| C 1 h, methionine-free | 8.1 ± 0.3 |
| F12, 3 h, methionine-free | 8.1 ± 0.3 |
| F12, plus 4 × 10^7 M methionine and 0.5 pg/mg of 25-hydroxysterol | 8.1 ± 0.3 |

* Results are shown ± S. D.

The effect of incubation in medium containing low methionine levels on the HMG-CoA reductase activity of CHO-K1 cells.

Cells (1 × 10^9) were inoculated into medium F12 supplemented with 8% newborn calf serum and incubated for 24 h. The medium over the cells was then changed to F12 supplemented with 2% delipidized serum and the cells incubated for another 16 h. This was followed by another incubation of 4 h, with 2% delipidized serum, under the conditions indicated, and then the cells were harvested and assayed for HMG-CoA reductase activity. Each measurement was done in triplicate. The concentration of methionine in F12 is 3 × 10^−8 m.

Effect of 25-hydroxycholesterol on the incorporation of [35S]methionine

The incorporation of [35S]methionine (8 pCi/dish, with 8% newborn calf serum) was monitored in CHO-K1 cells. The cells were then incubated in methionine-free medium followed by labeling with [35S]methionine (8 μCi/dish, 4 × 10^−3 M) for 3 h. The cells were then harvested by scraping into 600 ml of 0.05 M Tris, pH 7.4, in 0.15 M NaCl and broken by sonication. Fifty ml of sample was set aside for a protein determination, and 5 ml was added to 900 ml of cold 7% trichloroacetic acid. After 5 min on ice, the samples were collected on Millipore filters and washed one time with 2 ml of cold 7% trichloroacetic acid followed by two washes with 5 ml of 7% trichloroacetic acid. The filters were then counted by liquid scintillation.

Effect of 25-hydroxycholesterol on the incorporation of [35S]methionine into trichloroacetic acid-precipitable material in CHO-K1 cells

CHO-K1 cells (8.0 × 10^9) were inoculated into medium F12 supplemented with 8% newborn calf serum in 60-mm Petri dishes and incubated for 24 h. The medium was then changed to F12 supplemented with 2% delipidized serum and the cells incubated for another 16 h. This was followed by another incubation of 4 h, with 2% delipidized serum, under the conditions indicated, and then the cells were harvested and assayed for HMG-CoA reductase activity. Each measurement was done in triplicate. The concentration of methionine in F12 is 3 × 10^−8 m.

Incubation conditions

| Incubation conditions | [35S]Methionine incorporation |
|-----------------------|-----------------------------|
|                       | dpm/mg                      |
| Without 25-hydroxycholesterol | 5.59 (±0.27) × 10^6 |
| With 25-hydroxycholesterol (0.5 μg/ml) | 5.79 (±0.35) × 10^6 |

* Results are expressed ± S. D. of the mean.
Radioimmune Precipitation of HMG-CoA Reductase

Fig. 1. The time course of HMG-CoA reductase activity in cells incubated in the absence of sterols. CHO-K1 cells (5 x 10^6/150-mm plate) were incubated for 24 h after inoculation in normal growth medium. The medium was changed at zero time to F12 supplemented with 2% delipidized newborn calf serum with (○) or without (×) 0.5 μg/ml of 25-hydroxycholesterol. Cells were harvested at the times shown and assayed for HMG-CoA reductase activity. The results shown are the average of two determinations.

Fig. 2. Loss of HMG-CoA reductase activity in 25-hydroxycholesterol-treated cells. CHO-K1 cells (5 x 10^6/150-mm plate) were incubated for 24 h after inoculation in normal growth medium. The medium was changed to F12 supplemented with 2% delipidized newborn calf serum and incubated for another 18 h. At this time, 25-hydroxycholesterol (0.5 μg/ml) was added to each plate and cells harvested in duplicate and assayed for HMG-CoA reductase activity at the time shown. We have previously reported a half-life of HMG-CoA reductase activity determined with cycloheximide of 2.3 h in CHO-K1 cells (4, 23).

results are in accord with data that have been published by other workers (6, 15) and suggest that observation of the incorporation of radioactive amino acids into HMG-CoA reductase after 16 h of incubation of CHO-K1 cells in delipidized serum would be of interest.

Radioimmune Precipitation Analysis of the Incorporation of [35S]Methionine into Cultured Chinese Hamster Fibroblast HMG-CoA Reductase—We have found that it is difficult to isolate large quantities of HMG-CoA reductase from Chinese hamster liver by published procedures (10) although preparation of small amounts of purified enzyme by these methods is possible. This is due to a combination of the small size of Chinese hamsters and an order of magnitude lower specific activity (and, hence, presumably enzyme protein) in the livers of these animals as opposed to rat liver. However, purified enzyme from Chinese hamster liver has a similar subunit molecular weight to that of rat liver (Fig. 3) of 53,000, suggesting that the hamster enzyme might be cross-reactive with the rat enzyme antibody. This has in fact proven to be the case (Fig. 4). This antibody was used in a radioimmune
precipitation protocol to analyze the effect of 25-hydroxycholesterol on the incorporation of radioactive methionine into CHO-K1 cell HMG-CoA reductase. Cells were incubated for 16 h in delipidized serum in order to maximize HMG-CoA reductase activity. At this time, the cells were starved for methionine for 1 h and then labeled with [3S]methionine for 3 h. In samples treated with 25-hydroxycholesterol, this compound was added at the beginning of the incubation period with delipidized serum and was present at all times until the cells were harvested. Under these conditions, cells treated with 25-hydroxycholesterol show a substantial decrease in measured HMG-CoA reductase activity of about an order of magnitude. The HMG-CoA reductase was solubilized by treating whole cells with the detergent Kyro EOB and membranes removed from the preparation by ultracentrifugation at 100,000 × g for 1 h. An aliquot of 100,000 × g supernatant was removed and assayed for enzyme activity to ensure comparable solubilization in treated and control samples. These extracts were immune precipitated with antibody to rat liver HMG-CoA reductase and protein A Sepharose. After washing the protein A Sepharose beads thoroughly to remove as much nonspecific binding material as possible, the immune-precipitated material was solubilized in SDS sample buffer and applied to a 5–20% SDS-polyacrylamide gel for electrophoresis. The results (Fig. 5) show that treatment with 25-hydroxycholesterol substantially decreases the incorporation of radioactive methionine into polypeptides with molecular weights of 53,000 and 60,000. We also observed that the antibody seems to precipitate a polypeptide with molecular weight of approximately 127,000.

A similar result was obtained (Fig. 6) when microsomes were prepared from cells after methionine labeling, HMG-CoA reductase activity solubilized, and the microsomal ex-

![Fig. 5. The [3S]methionine-labeled polypeptides precipitable from CHO-K1 extracts by HMG-CoA reductase antiserum.](image)

![Fig. 6. Radioimmune precipitation of HMG-CoA reductase solubilized from CHO-K1 microsomes.](image)
tracts subjected to immune precipitation. Cytosols (100,000 x g supernatant) of cells broken by homogenization showed no specifically immune-precipitable bands. Because of these results, all subsequent experiments were performed on extracts prepared from whole cells.

The decrease of an immune-precipitable polypeptide of molecular weight identical with that of purified HMG-CoA reductase concomitant with a decrease in enzyme activity brought about by 25-hydroxycholesterol supports the contention that this compound produces its effects on enzyme activity by decreasing the number of enzyme molecules present in the cell. We next sought to determine whether this was at least in part due to an effect of 25-hydroxycholesterol on HMG-CoA reductase synthesis.

To examine the effect of 25-hydroxycholesterol on HMG-CoA reductase synthesis, cells were incubated with delipidized serum and labeled with methionine as described above except that 25-hydroxycholesterol was added to the cultures simultaneously with the labeled methionine. Under these conditions, the cells treated with 25-hydroxycholesterol show little difference in activity of HMG-CoA reductase from untreated cells (see "Experimental Procedures"). After solubilization of HMG-CoA reductase and removal of insoluble material by ultracentrifugation at 100,000 x g for 1 h, an aliquot of the soluble extract was assayed for enzyme activity. Matched amounts of enzyme activity could thus be immune precipitated and analyzed by SDS-polyacrylamide gel electrophoresis. The results of such experiments (Fig. 7) show that treatment with 25-hydroxycholesterol again results in decreased incorporation of label into the bands at 53,000 and 60,000.

In order to verify the physiological relevance of these results, we have performed an identical experiment on a dominant 25-hydroxycholesterol-resistant somatic cell mutant (CR1) which has been previously described in detail (7). Prior measurement of the half-life of enzyme activity in cyclohexi-
mide-treated wild type and 25-hydroxycholesterol-resistant somatic cell mutant cells suggested that this mutant would be defective in the regulation of synthesis of HMG-CoA reductase by 25-hydroxycholesterol (4). The results of an "activity-matched" immune precipitation experiment (Fig. 8) show no effect of 25-hydroxycholesterol on incorporation of labeled methionine into either the 53,000- or 60,000-dalton polypeptides.

**DISCUSSION**

The antibody which was used in these studies has previously been demonstrated to be monospecific in Ouchterlony double diffusion and immunoelectrophoresis experiments (10) with crude microsomal extracts. Yet, quite clearly, at least three microsomal polypeptides (Fig. 6) can be recognized by this antibody, only one of which corresponds in molecular weight to the subunit of purified HMG-CoA reductase from animal liver. This polypeptide, with a molecular weight of 53,000, seems to be affected by 25-hydroxycholesterol in a fashion which corresponds well with the effects of this compound on the activity of HMG-CoA reductase, and it is our observations on this polypeptide that lead us to conclude that the synthesis of HMG-CoA reductase is affected by oxygenated sterols. However, the polypeptide with a molecular weight of 60,000 is also affected by 25-hydroxycholesterol in a fashion consistent with the effects of this compound on HMG-CoA reductase. No definite conclusions about the relationship between this polypeptide and HMG-CoA reductase can be drawn from the studies in this report. We have noted, however, when we have compared samples with variable solubilization of enzyme activity that the band at 53,000 has become relatively more intense with increasing solubilization of activity. The gel shown in Fig. 7 is an example with 50% solubilization of both 25-hydroxycholesterol-treated and untreated samples. The pattern shown in Fig. 6 (lane C) is an example of 25% solubilization. Furthermore, in a somatic cell mutant which we have recently isolated which has an order of magnitude greater HMG-CoA reductase activity than the wild type CHO-K1 cell, both the 60,000 and 53,000 bands are increased. This mutant will be the subject of a subsequent report. Likewise, as described in this report, neither the 60,000 nor the 53,000 bands are affected by 25-hydroxycholesterol in a dominant 25-hydroxycholesterol-resistant mutant.

The role of the polypeptide with a molecular weight of 127,000 in understanding the mechanism of regulation of HMG-CoA reductase is not clarified by this report, although labeling of this band relative to untreated controls does not seem to be reduced by 25-hydroxycholesterol treatment of cells.

A variety of studies have been performed in a number of laboratories over the last few years on the mechanism of regulation of HMG-CoA reductase by oxygenated sterols. Prior to this report, all such studies have been indirect, utilizing either immune titration experiments (9) or the analysis of the rates of disappearance of enzyme activity in the absence of protein synthesis by means of treatment with cycloheximide (4, 16). These studies have been performed on several cell types and taken together support the contentions that oxygenated sterols affect both the number and type of HMG-CoA reductase molecules and enhance the rate of disappearance of enzyme activity in the absence of protein biosynthesis. A similar conclusion has been reached regarding the mechanism of other regulatory effectors of HMG-CoA reductase by immune titration experiments (10, 11). A considerable amount of literature on the effects of phosphorylation on HMG-CoA reductase (17, 18) and the physiological conditions which alter the degree of phosphorylation (19-21) provides a plausible mechanism for regulation based on changing the catalytic efficiency of this enzyme.

Because of the indirect nature of prior attempts to determine whether the number of HMG-CoA reductase molecules in cells is altered by treatment with oxygenated sterols, and particularly, because of the potential difficulties in interpreting data obtained with cycloheximide (22), the role of enzyme turnover in the regulation of this enzyme has remained somewhat ambiguous. The direct demonstration of effects on synthesis reported above and current studies on degradation should help eliminate this ambiguity. Furthermore, radioimmune precipitation analysis is essential in carrying forward studies on the mechanism by which HMG-CoA reductase synthesis and degradation are regulated, particularly when performed on somatic cell mutants defective in these processes (23).

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