Evidence Indicating that Inactivation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase by Low Density Lipoprotein or by 25-Hydroxycholesterol Requires Mediator Protein(s) with Rapid Turnover Rate

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The half-life ($t_{1/2}$) of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of Chinese hamster ovary cells grown in fetal calf serum medium is approximately 2 h. When cells are switched to grow in delipidated serum medium (Del-M) for more than 24 h, the $t_{1/2}$ of the enzyme is found to be drastically altered to approximately 13 h. Exposure of low density lipoprotein (LDL) (100 µg of protein/ml) or 25-hydroxycholesterol (1 µg/ml) to cells grown in Del-M suppresses reductase activity more rapidly than would be expected solely if reductase synthesis were suppressed, showing that inactivation of reductase activity by sterols, previously demonstrated using only analogs of cholesterol, is a normal mechanism for regulation of HMG-CoA reductase activity by the physiologically important sterol source (LDL). This inactivation effect by LDL or by 25-hydroxycholesterol is shown to be at least in part due to acceleration of reductase degradation rate. Furthermore, the inactivation effect by sterols is shown to be largely abolished if cycloheximide (250 µg/ml) is added simultaneously to the growth medium, indicating that continuous synthesis of a class of mediator protein(s) is necessary in mediating the effect of LDL or 25-hydroxycholesterol. Two different protein synthesis inhibitors (emetine and puromycin) were used and gave essentially identical results. Preincubation of cell culture with cycloheximide for 2 h essentially completely abolishes the effect of 25-hydroxycholesterol, indicating that the mediator protein(s) turns over rapidly, with $t_{1/2}$ less than 3 or 4 h.

It has been established that a key-regulated reaction in the biosynthesis of cholesterol from acetyl units is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate (for reviews, see Refs. 1–3), catalyzed by the enzyme HMG-CoA reductase (EC 1.1.1.34). The activity of this enzyme appears to be regulated by cholesterol through complex feedback mechanisms by which sterols modulate the reductase activity in various biological systems, and there is evidence that this is operational in this cell line. The Abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; Del-M, F-12 medium + 10% delipidated fetal calf serum; FCS-M, F-12 medium + 10% fetal calf serum; 25-OH cholesterol, 25-hydroxycholesterol; MeSO, dimethyl sulfoxide; LDL, low density lipoprotein; TLC, thin layer chromatography; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid suppressions. Currently, it is believed that several regulatory mechanisms of this enzyme by which sterols modulate the reductase activity in various biological systems probably exist, e.g., earlier studies by Higgins and Rudney (4, 5) suggested that cholesterol feeding lowered rat hepatic HMG-CoA reductase activity by two mechanisms, an immediate inactivation of the enzyme and a longer term reduction of enzyme synthesis. The work by Edwards and Gould (6) is also consistent with the concept that the initial cholesterol-enhanced diurnal decline of reductase activity in rat liver may be attributed either to direct enzyme inactivation or to an increased rate of enzyme degradation. In cultured human fibroblast cells and other cultured mammalian cells, removal of lipoproteins from the growth medium causes a large increase in reductase activity, which has been shown to be dependent on continuous protein synthesis (7). Adding low density lipoprotein (LDL) to the growth medium specifically and rapidly suppresses reductase activity (8); results of analyses of the first order kinetics of the decline of reductase activity in the presence of LDL or cycloheximide are consistent with the idea that the LDL-derivived cholesterol acts solely by decreasing the rate of reductase enzyme synthesis (8). The work by Kirsten and Watson (9) using unfractionated lipoproteins and hepatoma tissue culture cells is largely consistent with this view. In contrast to these results with LDL, certain oxygenated analogs of cholesterol, including 7-ketocholesterol and 25-hydroxycholesterol (25-OH cholesterol) (10), lower reductase activities in several tissue culture cell lines (11–13) more rapidly than would be expected solely if reductase synthesis were suppressed, suggesting that these sterols may act by inactivating the enzyme by acceleration of enzyme degradation or some other inactivation mechanism(s). Whether this inactivation mechanism operates using LDL as the sterol source has not been extensively studied. The possibility that sterols may act through the phosphorylation-dephosphorylation cycle to modify the reductase protein has been suggested (14–16); however, at present, there is no evidence that this mechanism may play a significant role in tissue culture cells (17, 18).

In this report, we have chosen to use Chinese hamster ovary (CHO) cells as a model system to examine the role of LDL or 25-OH cholesterol in suppressing HMG-CoA reductase activities. It has been previously shown (18–20) that the LDL pathway for regulation of cholesterol metabolism (21) is operational in this cell line.

**EXPERIMENTAL PROCEDURES**

**Materials**

Radioactive chemicals were from New England Nuclear. Biochemicals were from Sigma. 25-OH cholesterol was from Steraloids. 25-OH cholesterol acts solely by decreasing the rate of reductase enzyme synthesis (8). The work by Kirsten and Watson (9) using unfractionated lipoproteins and hepatoma tissue culture cells is largely consistent with this view. In contrast to these results with LDL, certain oxygenated analogs of cholesterol, including 7-ketocholesterol and 25-hydroxycholesterol (25-OH cholesterol) (10), lower reductase activities in several tissue culture cell lines (11–13) more rapidly than would be expected solely if reductase synthesis were suppressed, suggesting that these sterols may act by inactivating the enzyme by acceleration of enzyme degradation or some other inactivation mechanism(s). Whether this inactivation mechanism operates using LDL as the sterol source has not been extensively studied. The possibility that sterols may act through the phosphorylation-dephosphorylation cycle to modify the reductase protein has been suggested (14–16); however, at present, there is no evidence that this mechanism may play a significant role in tissue culture cells (17, 18).

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Drs.

[24-'H]cholesterol was synthesized chemically as previously described (20). Purities of radiochemical and non-radioactive 25-OH cholesterol were greater than 95% as previously reported (20). 25-OH cholesterol-3β-oleate was synthesized and purified according to Goodman (22) by Drs. J. Nelson and T. Spencer at the Chemistry Department of Dartmouth College. Its structure was unambiguously established by IR and NMR analyses. Purity of this compound as analyzed by three different thin layer chromatographic (TLC) solvent systems was found to be greater than 98%. Human LDL was prepared according to published procedure (23) as previously reported (20). 25-OH cholesterol IR and NMR analyses. Purity of this compound as analyzed by three different TLC solvent systems was found to be greater than 98%. Human LDL was prepared according to published procedures (20). Purities of radioactive and nonradioactive 25-OH cholesterol reported (20). Purities of radioactive and nonradioactive 25-OH cholesterol recovered from Microbiological Associates. All other chemicals were of analytical grades.

**Methods**

**Cells—** Monolayer cultures of normal (wild type) CHO cells (20, 24) were grown in 25-cm² Falcon flasks or 60-mm Falcon dishes in F-12 medium (hormone and non-hormone added) or in M-199. The cells were grown in F-12 medium containing 10% FCS, 5% sodium bicarbonate, and 0.2 medium without added hormones, with or without added hormones. The cells were cultured in F-12 medium containing 10% FCS, 5% sodium bicarbonate, and 0.2% of the media were seeded at 0.5% or less than 0.3% as previously described (20). LDL, cycloheximide, emetine, or puromycin was added to the growth medium immediately before use from a 50-fold or 100-fold stock solution in sterile saline. All experiments reported in this paper involving cell culture were carried out using the following format. Stock flasks of cells grown at high cell density in 100-cm² flasks or 75-cm² Flask or 50-mm dish in 3 ml of FCS-M and allowed to grow for 63 h at 37 °C in a 5% CO₂ incubator. Cells at this stage are designated as starting cultures. Afterward, the medium was changed and cells were allowed to grow in various media as indicated in specific figure legends and tables. Media were refreshed frequently during cell growth. We found that frequent medium changes produce cells grown in FCS-M and Del-M with higher in vitro reductase activities, as well as many other enzyme activities. Cells were harvested during log phase growth by visual examination. For all experiments reported here, duplicate cultures were used for each assay point. Variation of measurement between duplicate cultures was within 5 to 7%

**Cell Homogenization and HMG-CoA Reductase Assay—** Cells were harvested by scraping and efficiently homogenized in our laboratory and reported in detail elsewhere (26). Briefly, cells monolayers were rapidly washed with 3 to 5 ml of PBS and 1 to 5 ml of hypotonic buffer (Buffer K) (1 × 10⁷ Tris-HCl, 1 mM EDTA, and 1 mM MgCl₂, pH 7.6) at 4 °C and exposed to 50 µl of Buffer K at room temperature for 2 min. The buffer was drained, 94 µl/dish of Buffer K at room temperature was added, and the swollen cells were rapidly scraped into a corner by a metal scraper fitted with a silicone rubber blade (Belco Co., Catalog No. 7731-22000). Cell homogenization took place during scraping. The extent of cell breakage was greater than 95% by microscopic examination. Usually, 100 µl of the cell homogenate was used for each assay of 100 µl of rat liver homogenate. The homogenate contained 5% imidazole and 25 mM concentration of dithiothreitol, pH 7.4) and preincubated at 37 °C for 10 min for activation. It is known that this step provides the complete dephosphorylation and activation of the reductase by the endogenous phosphatase activity present in the liver. In a previous experiment, the buffer used during the activation step contained 50 mM phosphate (27) which might have inhibited the phosphatase activity. After preincubation, duplicate aliquots (usually 40 µl/ aliquot, containing 15 to 40 µg of protein) of cell extracts were taken and assayed for HMG-CoA reductase activity as described previously (18, 20, 24) with minor modifications. The complete assay system contained in 80 µl: 0.245 mM of NADPP⁺, 10.4 µM of Nα-L-[³H]HMG-CoA (90,000 dpm), 1.78 µM of glucose 6-phosphate, 0.073 unit of glucose 6-phosphate dehydrogenase, 2.84 µM of potassium phosphate (pH 7.4), 0.064 µM of dithiothreitol, 4 µM of potassium chloride, 1.78 µM of EDTA, and 800 µM of imidazole (pH 7.4). There were also small amounts (20 µM each) of MgCl₂, EGTA, and Tris-HCl.

**RESULTS**

Effect of Cycloheximide and/or 25-OH Cholesterol on HMG-CoA Reductase Activity of CHO Cells—As shown in Fig. 1B, in the presence of the protein synthesis inhibitor cycloheximide, the HMG-CoA reductase activity of log phase cells grown in Del-M decreased with first order kinetics in four separate experiments, its average half-life (t₁/₂) was found to be 12.8 ± 1.7 (S. E.) h, a value much higher than any other reported t₁/₂ value for this enzyme in the literature (for a review, see Ref. 1). Addition of 25-OH cholesterol to the...
Effects of cycloheximide and/or LDL on HMG-CoA Reductase Activity—An experiment using LDL as the sterol source was next carried out. As shown in Fig. 2, the rate of suppression of reductase activity by LDL is also faster than its normal degradation rate, and its effect is almost completely abolished by the simultaneous presence of cycloheximide. The data shown in Fig. 1 and Fig. 2 reinforce the concept that 25-OH cholesterol is an intracellular analog of LDL-derived cholesterol (20, 30). They also indicate that inactivation of reductase by sterol(s), previously demonstrated using only analogs of cholesterol (11–13), is a normal mechanism for regulation of HMG-CoA reductase activity by the physiologically important sterol source (LDL). In this experiment, the \( t_{1/2} \) of the reductase activity in cells grown in parallel in FCS-M was determined and found to be 1.8 h, which is in fair agreement with previously reported \( t_{1/2} \) values by other investigators in various systems (see Ref. 1 for a review). Since the specific activity of reductase in zero time culture grown in FCS-M or in DeL-M was found to be 0.17 or 2.29 nmol·min\(^{-1} \cdot\)mg\(^{-1}\), respectively, assuming cycloheximide causes no significant change in enzyme degradation, it follows that the decrease in rate of degradation of this enzyme (a 6.3-fold change) constitutes an important mechanism accounting for the observed increase in reductase specific activity (a 13.5-fold change) upon removal of serum lipids from the growth medium.

### Table 1

| Time of preculture with cycloheximide | Amount of [\(^{3}H\)]25-OH cholesterol incorporated into cells | \% esterification of cell-bound [\(^{3}H\)]25-OH cholesterol |
|----------------------------------------|---------------------------------------------------------------|-------------------------------------------------------------|
| 6                                       | 2.0 ± 0.05                                                   | 18 ± 1.2                                                   |
| 1                                       | 2.4 ± 0.01                                                   | 25 ± 0.1                                                   |
| 2                                       | 2.6 ± 0.03                                                   | 36 ± 0.2                                                   |
| 3                                       | 2.7 ± 0.03                                                   | 27 ± 0.2                                                   |
| 4                                       | 2.8 ± 0.03                                                   | 25 ± 0.3                                                   |

\(^3\) In experiments not shown, we found that after a 3-h or a 6-h incubation with the cells, the concentration for 25-OH cholesterol to exert its maximal effect in suppressing the reductase activity was at 1 to 5 \( \mu \)g/ml; the concentration needed to cause half-maximal suppression was between 0.1 and 0.2 \( \mu \)g/ml.

\(^4\) The same result was seen in at least three separate experiments.
Effects of Other Specific Protein Synthesis Inhibitors on 25-OH Cholesterol Action(s)—Emetine and puromycin are two well known protein synthesis inhibitors. The mode of action of either agent is known to be different from that of cycloheximide (31, 32). As shown in Fig. 3, A and B, the results using emetine as the inhibitor confirm the results using cycloheximide. The fact that emetine is somewhat more effective in the 47-h grown culture (Fig. 3B) than in the 24-h grown culture (Fig. 3A) is consistent with the idea that the pre-existing mediator protein(s) in cells before exposure to 25-OH cholesterol may be present in higher concentration in the 24-h grown culture than in the 47-h grown culture. It is possible that the mediator protein(s) is present in very high concentrations in FCS-M grown culture and is rapidly depleted when cells are switched to grow in DeL-M. Further investigations are needed to explore this possibility.

The experiment using puromycin as the inhibitor was not completely satisfactory, since the cells treated with puromycin for 8 h or longer started to detach from the surface of the tissue culture flask; approximately 20 to 30% of the cells were lost into the medium at the end of the 10-h period. Nevertheless, the result shown in Fig. 3C qualitatively confirms the data shown in Fig. 1B and Fig. 3B. As a control experiment, the efficiency of these three different protein synthesis inhibitors in intact CHO cells was measured and found to be very high in each case (Table II). Taken together, these data rule out the possibility that the result shown in Fig. 1B might have been due to some unknown property of cycloheximide other than its ability to inhibit protein synthesis.

Effect of Preincubation of LDL or 25-OH Cholesterol on HMG-CoA Reductase Degradation Rate—If a major effect of LDL or 25-OH cholesterol on inactivation of reductase activity is to accelerate its rate of degradation, then preincubation of cells with LDL or 25-OH cholesterol followed by cycloheximide exposure should provide t_{1/2} values shorter than the value with cycloheximide alone. This has been shown in Fig. 4. Preincubation of LDL for 3 h accelerates the reductase degradation rate by 2.7-fold; similar treatment with 25-OH cholesterol accelerates the reductase degradation rate by 3.3-fold. These experiments suggest that an important mechanism of reductase inactivation by LDL or by 25-OH cholesterol is to accelerate the reductase degradation rate. Furthermore, these results are consistent with the following working...
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Starting cultures in 60-mm dishes as described under “Methods” were rinsed with 1 x 5 ml of PBS and allowed to grow for another 24 h in 3 ml of fresh Del-M. Afterward, cells were exposed to 1.0 ml of fresh Del-M with or without various inhibitors at the indicated final concentration for 30 min at 37°C. This treatment was followed by adding 10 µCi/dish of L-[4,5-3H]leucine (50 Ci/mmol in H2O) to cell culture for 1 h at 37°C. Afterward, cells were washed with 5 x 5 ml of PBS at 4°C, followed by 5 x 5 ml of 5% trichloroacetic acid at room temperature. The trichloroacetic acid-insoluble material was digested by 1.0 ml/dish of 0.5 M NaOH at room temperature for 30 min. Aliquots were taken for cellular protein content and trichloroacetic acid-insoluble radioactivity determinations. Duplicate dishes were used for each measurement. Variation between duplicates was within 5% from the mean.

Table II

| Inhibitor Used | % Inhibition of Protein Synthesis in Intact Cells* |
|----------------|---------------------------------------------------|
| Cycloheximide (0.89 mM) | 99 |
| Erucamide (0.005 mM) | 97 |
| Puromycin (0.17 mM) | 96 |

* Determined by disintegrations per minute of trichloroacetic acid-insoluble counts per mg of protein in treated culture versus that found in the control culture. The control culture contained 9.59 x 10^6 dpm/mg of protein.

Fig. 4. Acceleration of HMG-CoA reductase degradation rate by preincubation of cells with LDL or 25-OH cholesterol. Starting cultures were switched to grow in Del-M for 47 h as described in Fig. 3, B and C. Afterward zero time flasks were harvested and assayed; the remaining cells were fed with 5 ml/flask of fresh Del-M containing no addition (O), 0.89 mM concentration of cycloheximide (x), 100 µg of protein/ml of LDL (A), or 1 µg/ml of 25-OH cholesterol (D). Three h later, certain flasks of cells exposed to LDL or 25-OH cholesterol were further exposed to cycloheximide at 0.89 mM. At the indicated time, cells were harvested and assayed for HMG-CoA reductase activities.

Fig. 5. Preincubation of cells with cycloheximide abolishes the effect of 25-OH cholesterol on inactivation of HMG-CoA reductase. Starting cultures were rinsed with 1 x 5 ml of PBS and switched to grow in 4 ml of Del-M for 23 h. Duplicate flasks were harvested and assayed for zero time determinations. The remaining flasks were exposed to 0.3% Me2SO (O), 0.89 mM concentration of cycloheximide (x), 0.89 mM concentration of cycloheximide and 1 µg/ml of 25-OH cholesterol (E), or 1 µg/ml of 25-OH cholesterol (E). Certain flasks of cells exposing to cycloheximide for 2, 4, or 6 h were further exposed to 1 µg/ml of 25-OH cholesterol for 8 h (D), 6 h (G), or 4 h (V). At the indicated time, cells were harvested and assayed for HMG-CoA reductase activities.

relative to the noncycloheximide-treated cells in the presence of LDL, but not so for the 25-OH cholesterol-treated cells (Fig. 4), may be due to the fact that the time lag for the LDL action(s) was shown to be approximately 3 h longer (Figs. 2 and 4) than that for 25-OH cholesterol; i.e. during the preincubation period (3 h), the sensitization process with LDL may be less complete than with 25-OH cholesterol.

Effect of Preincubation of Cycloheximide on the Effect of 25-OH Cholesterol in Reductase Inactivation—The data presented in Figs. 1 to 3 strongly suggest that the mediator protein(s) turns over rapidly. This is confirmed by the preincubation of cells with cycloheximide before exposure to 25-OH cholesterol. As shown in Fig. 5, preincubation of cycloheximide for 2 h essentially completely abolishes the effect of the subsequent exposure to 25-OH cholesterol; the 4-h and the 6-h preincubation data confirm the 2-h preincubation data. Since a time lag of approximately 1 to 2 h for the 25-OH cholesterol action(s) has always been seen (as typically shown in Fig. 5), the t1/2 of the mediator protein(s) cannot be accurately measured from this type of experiment, but can be estimated to be less than 3 to 4 h.

Discussion

Data presented in this report show that in CHO cells, the decrease in rate of degradation of HMG-CoA reductase constitutes an important mechanism accounting for the observed increase in specific activity of the enzyme upon removal of serum lipids from the growth medium. Whether this is a general phenomenon for other cell types grown in similar conditions remains to be investigated. The t1/2 values of this enzyme from cells grown in Del-M determined in different
experiments range from 9 to 17 h (see Figs. 1 to 4) regardless of whether cycloheximide or emetine is used as the protein synthesis inhibitor. We do not yet fully understand why this value fluctuates over such a large range; it seems to be related to the freshness and/or quantity of the growth medium and to cell density during the experiment. It is known that alteration of nutrients in the growth medium can alter rates of protein degradation in tissue culture cells (33). This possibility remains to be clarified by future experiments. Evidence is presented (Figs. 1–4) which strongly suggests that both 25-OH cholesterol and LDL accelerate the normal degradation rate of this enzyme by at least 3- to 4-fold (Figs. 1–4), which is consistent with the concept that 25-OH cholesterol is an intracellular analog of LDL-derived cholesterol (11–13). These data also indicate that inactivation of reductase activity, at least in part via acceleration of degradation rate, is a normal mechanism by which LDL exerts its action in suppressing the HMG-CoA reductase activity in log phase CHO cells. Previously, evidence provided by Brown et al. (8) suggested that the major suppression effect by LDL on reductase activity in confluent human fibroblast cells was due to inhibition of specific enzyme synthesis. The difference in results between our study and ours may be due to the fact that the different cells at different stages of growth were used. It is known that control of enzyme synthesis may be as well as control of enzyme degradation in cultured cells may vary with different growth conditions (33, 34). It is likely that inhibition of reductase synthesis by LDL also occurs under our condition; however, further experiments are needed before it can be unambiguously demonstrated. The pre-existing data (7–13) and the data presented here support the conclusion that regulation of reductase activity by exogenous cholesterol occurs at the level of specific enzyme degradation and specific enzyme synthesis. In addition, it is entirely possible that the LDL-cholesterol or 25-OH cholesterol may cause a direct inactivation of reductase via specific allosteric inhibition; this type of inhibition can not be demonstrated by data presented in this report and remains to be explored.

An unexpected finding demonstrated in this report discloses the need for a class of mediator protein(s) to mediate the intracellular effect of 25-OH cholesterol or LDL in accelerating the reductase inactivation." The mediator protein(s) is shown to depend on protein synthesis to maintain its competency; the synthesis of the mediator protein(s) may be inducible by serum lipids, by LDL-derived cholesterol, or by the cholesterol analog (25-OH cholesterol) present in the growth medium. Alternatively, it is possible that the mediator protein(s) is constitutive in the cell and may only require an exogenous signal generated from the incoming sterol to manifest its regulatory effect(s) on reductase. The turnover rate of the functional mediator protein(s) in cells grown in Del-M is shown to be very rapid, with t½ estimated to be less than 3 or 4 h. Recently, Randutsch and co-workers (36, 37) have described two interesting classes of 25-OH cholesterol-binding proteins in various tissue culture cell lines. It is unlikely that these binding proteins bear any functional relationship to the mediator protein(s) reported here since these binding proteins all possess very long half-lives (37). The nature of the mediator protein(s) is unknown at present. It may be a specific binding protein for cholesterol (or its analogs) or may be an enzyme modifying cholesterol into an active suppressor. Other possibilities are not excluded at present. The possibility that it may

play a role in controlling the permeability of 25-OH cholesterol into cells has been eliminated (Table 1). It is important to realize that the experiment shown in Table I merely indicates that the difference in effects of 25-OH cholesterol with or without cycloheximide cannot be accounted for by the differences observed in percent cellular saponification of this particular sterol. This experiment does not eliminate the possibility that the mediator protein(s) may play a role in mediating the effect of 25-OH cholesterol or LDL-derived cholesterol in stimulating the activity of the enzyme acyl CoA:cholesterol acyltransferase (30). It is also possible that the mediator protein(s) may play a role in mediating the suppression effects of LDL-derived cholesterol or its oxygenated analog (25-OH cholesterol) on other cholesterogenic enzymes (20). Experiments designed to explore these possibilities are currently in progress in this laboratory.

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