Sterol-independent Regulation of 3-Hydroxy-3-methylglutaryl-CoA Reductase by Mevalonate in Chinese Hamster Ovary Cells

MAGNITUDE AND SPECIFICITY*

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In this paper, we assess the relative degree of regulation of the rate-limiting enzyme of isoprenoid biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, by sterol and nonsterol products of mevalonate by utilizing cultured Chinese hamster ovary cells blocked in sterol synthesis. We also examine the two other enzymes of mevalonate biosynthesis, acetoacetyl-CoA thiolase and HMG-CoA synthase, for regulation by mevalonate supplements. These studies indicate that in proliferating fibroblasts, treatment with mevalonic acid can produce a suppression of HMG-CoA reductase activity similar to magnitude to that caused by oxygenated sterols. In contrast, HMG-CoA synthase and acetoacetyl-CoA thiolase are only weakly regulated by mevalonate when compared with 25-hydroxycolesterol. Furthermore, neither HMG-CoA synthase nor acetoacetyl-CoA thiolase exhibits the multivalent control response by sterol and mevalonate supplements in the absence of endogenous mevalonate synthesis which is characteristic of nonsterol regulation of HMG-CoA reductase. These observations suggest that nonsterol regulation of HMG-CoA reductase is specific to that enzyme in contrast to the pleiotropic regulation of enzymes of sterol biosynthesis observed with oxygenated sterols. In Chinese hamster ovary cells supplemented with mevalonate at concentrations that are inhibitory to reductase activity, at least 80% of the inhibition appears to be mediated by nonsterol products of mevalonate. In addition, feedback regulation of HMG-CoA reductase by endogenously synthesized nonsterol isoprenoids in the absence of exogenous sterol or mevalonate supplements also produces a 70% inhibition of the enzyme activity.

Mevalonic acid has been shown to be the first committed precursor for the biosynthesis of isoprenoids (1). In cholesterogenic tissues such as mammalian liver, the major metabolic fate of endogenously synthesized mevalonic acid is conversion to cholesterol. In normal culture media supplemented with various forms of serum lipoproteins, uptake of cholesterol through the low density lipoprotein receptor pathway satisfies the cholesterol needs of the cell, leading to suppression of enzymes of cholesterogenesis (2). A similar physiological situation pertains in a number of animal species in which the bulk of cholesterol synthesis appears to be hepatic, and extrahepatic cholesterol requirements are met through receptor-mediated endocytosis of lipoproteins (3). However, it has been pointed out that in proliferating cells, other products of isoprenoid metabolism might be required for cell growth besides cholesterol (4). In cell culture, such a requirement for nonsterol derivatives of mevalonate can be readily illustrated through the isolation of somatic cell mutants auxotrophic for mevalonate (5, 6). The growth requirements of such mutants cannot be met by cholesterol alone but rather have a specific demand for mevalonate supplements.

The enzyme that catalyzes the formation of mevalonic acid in all biological systems studied to date is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (1, 2). This enzyme has a well-characterized set of regulatory responses to exogenously supplied sterol supplements in mammalian cells (7), including transcriptional control of the synthesis of the enzyme (8). In addition, it has been reported that reductase has a distinct regulatory response to one or more nonsterol mevalonate-derived products which also involves the synthesis of the enzyme protein (9–11). However, in contrast to the transcriptional control of the synthesis of this enzyme by sterols, the regulation of HMG-CoA reductase synthesis by nonsterols appears to operate primarily at the level of translation (10, 11).

It has been known for some time that in the absence of exogenous cholesterol supplements, treatment with mevalonate can down-regulate HMG-CoA reductase activity both in hepatic tissue (which is primarily a population of quiescent cells) and in proliferating cells in culture (12, 13). Since mevalonate metabolism can produce both sterol and nonsterol regulators, it has become of interest to examine the capacity of mevalonate to act as a regulator in the absence of endogenous synthesis of sterols. Such studies can provide us with a better quantitative understanding of the relative role of each type of regulator during treatment of cells with mevalonate. Another important question derives from observations that suggest that sterol regulation of enzymes of mevalonate biosynthesis and indeed of other enzymes of sterol biosynthesis is pleiotropic at the transcriptional level (13, 14). The activities of both acetoacetyl-CoA thiolase and HMG-CoA synthase can be suppressed by supplementation of culture media with either oxysterols or low density lipoproteins (15). The mechanism of such down-regulation appears to have much in

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1 The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; TMD, 4,4,10-trimethyl-trans-decal-3-ol; HPLC, high performance liquid chromatography.
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common with the one by which sterols regulate HMG-CoA reductase synthesis. Although regulation of HMG-CoA reductase activity by mevalonate supplements in the absence of exogenous sterols has been widely reported, similar studies have not been performed for acetooxyl-CoA thiolase and HMG-CoA synthase.

That mevalonate could regulate HMG-CoA reductase by a nonsterol-mediated mechanism was suggested by the observation that inhibition of mevalonate biosynthesis in cultured cells produced a loss of regulation of HMG-CoA reductase activity by sterols (4, 9). Supplementation of culture media with small amounts of mevalonate restored the full extent of suppression, suggesting the involvement of products of sterol and nonsterol branches of the isoprenoid biosynthetic pathway in the down-regulation of a common enzyme. This phenomenon was referred to as the multivalent feedback regulation of HMG-CoA reductase (4). It is, however, possible that the endogenous regulator generated from mevalonate could also be a sterol whose biological action is synergistic with that of exogenous sterols (16). We examine this possibility in the current work.

We also investigate the possibility that HMG-CoA synthase and acetooxyl-CoA thiolase may be subject to multivalent control. It is known that the inhibition of synthesis of HMG-CoA synthase by sterols in cultured cells occurs by a transcriptional control mechanism analogous to the one for HMG-CoA reductase (17). In the current report, we add new information to our understanding of the nonsterol regulatory mechanism for HMG-CoA reductase by comparing the regulatory response of this enzyme to mevalonate with that of HMG-CoA synthase and acetooxyl-CoA thiolase under various culture conditions. We also determine the relative roles of sterol and nonsterol regulatory products derived from mevalonate in the regulation of HMG-CoA reductase in CHO cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Cells were grown routinely in Ham’s F-12 (18) supplemented with 5% (v/v) fetal calf serum (F12FC5). The cell lines utilized were CHO-K1(19), MeV-1 (20), and mutant 215 (20, 21). The latter two cell lines are somatic cell mutants of the CHO-K1 cell. Mve-1 is a mevalonate auxotroph lacking HMG-CoA synthase activity. Mutant 215 is a cholesterol auxotroph defective in demethylation of lanosterol. This mutant line was kindly provided by Dr. T. Y. Chang, Dept. of Biochemistry, Dartmouth Medical School, Hanover, NH. Cells were cultured for 3 days in the stachyochelose synthetic medium described previously (17). In the current report, we add new information to our understanding of the nonsterol regulatory mechanism for HMG-CoA reductase by comparing the regulatory response of this enzyme to mevalonate with that of HMG-CoA synthase and acetooxyl-CoA thiolase under various culture conditions. We also determine the relative roles of sterol and nonsterol regulatory products derived from mevalonate in the regulation of HMG-CoA reductase in CHO cells.

Enzyme Assays—The activities of acetooxyl-CoA thiolase and HMG-CoA synthase were assayed in the cytosolic fraction of cells as described previously (51). For the assay of HMG-CoA reductase, cells from each 60-mm culture dish were scraped into 200 ml of harvest buffer containing 0.25% (v/v) Kyro-EOB detergent (Proctor & Gamble) in 20 mM imidazole-HCl, pH 7.4, 5 mM dithiothreitol. The suspension was incubated at 37 °C for 15 min and briefly centrifuged at 5000 g for 3 min. Aliquots of the supernatant extract (30–50 µl) were incubated for enzyme activity at 37 °C for 60 min in a final volume of 80 µl in 35 mM potassium phosphate buffer, pH 7.4, 22.5 mM EDTA, 50 mM KCl, 10 mM dithiothreitol, 2.5 mM NADPH, 30 mM glucose 6-phosphate, 3 units/ml glucose 6-phosphate dehydrogenase, 130 µM [1-3H]HMG-CoA (12,000 dpm/nmol), and 1000 µCi [3H]mevalonolactone. The reaction was stopped by the addition of 200 µl of 6 N HCl, and the samples were incubated at 37 °C for an additional 30 min. After centrifugation at 11,000 × g for 1 min in a Sorvall Microspin 24S centrifuge, 50 µl of clear supernatant liquid was applied to Whatman LKGD silica gel thin layer chromatographic plates. The chromatograms were developed in benzene/acetonitrile (1:1, v/v), and the mevalonolactone bands were visualized by autoradiography. After spraying the plate with water, silica gel from the area corresponding to [1-3H]mevalonolactone was scraped into counting vials containing Scintiverse BD for the determination of radioactivity in a Beckman LS1801 liquid scintillation counter. Specific activities were expressed as the mean ± S.E. of triplicate cultures. The data were fitted by polynomial least squares regression analysis using the SigmaPlot program (Jandel Scientific, Sausalito, CA). Protein determinations were carried out by the method of Schacterle and Pollack (22) using bovine serum albumin as the standard.

Conversion of [1-3H]Acetate and [3H]Mevalonolactone to Products—Labeling with [1-3H]acetate was used to monitor the overall rate of sterol biosynthesis. CHO-K1 cells (6 × 105 cells/60-mm culture dish) were grown for 24 h in F12FC5. The medium was then changed to F12DIF5 with or without 20 µg/ml TMD for 16 h. At this time, the cells were pulsed for 5 h with 33.3 µCi of [3-3H]acetate. At the end of the labeling period, cells were harvested by scraping into phosphate-buffered saline, and total lipids were extracted by the method of Bligh and Dyer (27). Neutral and polar lipids were separated on silic acid columns by stepwise elution (30 column volumes of CHCl3 for neutral lipids and 20 column volumes of methanol for polar lipids) as described by Dittmer and Wells (28). The neutral lipids were saponified, and the C-27 sterols were precipitated with digitonin (28). The ratio of incorporation of [3H] into digitonin-precipitable material to that into polar lipids were compared in the presence or the absence of TMD. By this criterion, TMD (20 µg/ml) caused a >99% inhibition of synthesis of digitonin-precipitable sterols in the presence of a wide range (0–15 µM) of mevalonate concentrations.

Conversion of [3H]mevalonate to nonsaponifiable lipids by CHO-K1 and mutant 215 cells was determined by growing cells (2.5 × 105 cells/60-mm culture dish) for 24 h in F12FC5. The medium was then changed to F12DIF5 containing either no addition or 20 µg/ml TMD or 20 µg/ml TMD + 30 µM ketoconazole. After 3 h, mevalonate (0–15 mM) was added together with 20 µCi of [5-3H]mevalonate. After 18 h of labeling, monolayers were rinsed with saline, and total cellular lipids were extracted into hexane/isopropryl alcohol (3:2, v/v) as described previously (29, 30). The lipid extract was evaporated under N2, and the residue was saponified at 75 °C for 30 min in 1 ml of 1 N KOH in methanol/benzene (4:1, v/v) containing 20 µg/ml pyrogallol. After cooling to room temperature, the mixture was diluted with 0.6 ml of water, and nonsaponifiable lipids were extracted twice with 4 ml of hexane. The combined hexane extracts were backwashed once each with 50% saturated NaHCO3, water, and 70% (v/v) ethanol and filtered through an Acro LCS0 filter (Gelman Sciences, Inc., Ann Arbor, MI). After evaporation of hexane under N2, the residue was dissolved in ethanol for analysis by reverse phase HPLC on a Zorbax ODS column (30 cm × 3.9 cm) (Du Pont-New England Chemical Co., Deerfield, IL) using acetonitrile/water (92:8, v/v) as the mobile phase at 2 ml/min. The Beckman HPLC system consisted of two Altex llOB pumps, 421A controller, model 165 variable wavelength detector, and a Beckman LS1801 liquid scintillation counter. Specific enzyme activities are expressed as the mean ± S.E. of triplicate cultures. The data were fitted by polynomial least squares regression analysis using the SigmaPlot program (Jandel Scientific, Sausalito, CA). Protein determinations were carried out by the method of Schacterle and Pollack (22) using bovine serum albumin as the standard.

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with the authentic standards by normal phase HPLC analysis on a SupelcoLC Si (4.6 mm x 15 cm) silica column (Supelco, Inc., Bellefonte, PA). The mobile phase (1 ml/min) was 0.5% (v/v) isopropanol in hexane for the first 10 min and was increased to 2.5% (v/v) isopropanol alcohol over the next 5 min.

**RESULTS**

Comparison of Multivalent Control by Mevalonate and Oxidogenated Sterols of Acetoacetyl-CoA Thiolase, HMG-CoA Synthase, and HMG-CoA Reductase—Treatment of CHO cells in culture with inhibitors of HMG-CoA reductase such as compactin or mevinolin causes an abolition of mevalonate biosynthesis (4, 5). The concentration of mevalonate required to support the growth of such cells in delipidized media is at least 10 mM in the absence of added cholesterol (5) and at least 0.1 mM in the presence of sterol supplements (4). We therefore examined the effect of acute inhibition of mevalonate biosynthesis by mevinolin treatment on the regulation of acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase by 25-hydroxycholesterol in CHO cells. We also examined the effect of low (0.4 mM) and high (13.5 mM) levels of mevalonate supplements on such regulation. The results (Table I) indicate that at a concentration of mevalonate (13.5 mM) which can support cell growth, neither HMG-CoA synthase nor acetoacetyl-CoA thiolase is down-regulated as effectively as is HMG-CoA reductase. On the other hand, both synthase and thiolase are effectively regulated by 25-hydroxycholesterol in the absence of endogenous mevalonate biosynthesis (caused by mevinolin treatment). Furthermore, no additional down-regulation of these enzymes is produced by treatment with the combination of 25-hydroxycholesterol and mevalonate over that produced by 25-hydroxycholesterol alone as is observed for HMG-CoA reductase activity. Thus, these findings provide no evidence of multivalent control by mevalonate and sterols of acetoacetyl-CoA thiolase or HMG-CoA synthase.

Possible multivalent control of acetoacetyl-CoA thiolase can also be examined under other conditions. We have described previously the somatic cell mutant, Mev-1 (5), which is auxotrophic for mevalonate due to a lack of detectable HMG-CoA synthase activity. These cells exhibit multivalent control of HMG-CoA reductase similar to that seen in the wild-type cells treated with mevinolin (9, 10). Maximal suppression of HMG-CoA reductase activity of Mev-1 cells by 25-hydroxycholesterol is observed only in the presence of mevalonate supplements (Table II). However, when we tested Mev-1 cells for a similar requirement of mevalonate for down-regulation of acetoacetyl-CoA thiolase activity by 25-hydroxycholesterol, we did not observe any evidence of multivalent control.

We were surprised by the weak regulation of acetoacetyl-CoA thiolase and HMG-CoA synthase relative to that of HMG-CoA reductase by high concentrations of mevalonic acid (Tables I and II). It has generally been assumed that at such concentrations of mevalonate, most of the down-regulation of enzymes of sterol biosynthesis occurs through generation of endogenous sterol regulators. It has been reported that in cultured fibroblasts, HMG-CoA reductase and HMG-CoA synthase activities exhibit similar inhibitory dose-response curves to exogenous sterols (15). Another example of such a comparison is shown in Fig. 1.

We thus decided to examine the dose-response curve of HMG-CoA synthase and reductase activities to mevalonate in more detail. The results (Fig. 2) show that the high concentration of mevalonate used in Table I does produce a near maximal inhibition (~2-fold) of HMG-CoA synthase activity. However, it is clear that the degree of this inhibition is considerably smaller than that observed for HMG-CoA reductase (~20-fold). Since HMG-CoA synthase and HMG-CoA reductase activities are suppressed to a similar extent by sterol supplements (Fig. 1) but differ in their susceptibility to mevalonate supplements (Fig. 2), it is therefore possible that a substantial amount of the observed down-regulation of reductase activity by mevalonate supplements occurs through a conversion to nonsterol regulators. If conversion of mevalonate to sterol regulators were entirely responsible for the effects of this compound on HMG-CoA synthase and HMG-CoA reductase, the comparable response of these two enzymes to exogenous sterols would predict a similar response to exogenous mevalonate. Obviously, this is not the case.

**Regulation of HMG-CoA Reductase Activity in CHO Cells Blocked in Sterol Biosynthesis**—In order to examine further the extent to which mevalonate acts as a regulator of HMG-CoA reductase activity in CHO cells through conversion to sterol or nonsterol products, we studied the effect of treatment with TMD, a specific inhibitor of oxidosqualene cyclase (31), on the suppressive action of mevalonate. At the concentration of TMD used (20 μg/ml), the inhibition of incorporation of radiolabel from [14C]acetate into digitonin-n precipitable material is greater than 99% at all concentrations of mevalonate used in these experiments (data not shown).

Supplementation of CHO-K1 cell cultures with mevalonate (2-15 mM) produces a dose-dependent suppression of HMG-

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetoacetyl-CoA thiolase nmol/min/mg protein</th>
<th>HMG-CoA synthase nmol/min/mg protein</th>
<th>HMG-CoA reductase nmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>105.3 ± 6.0</td>
<td>1.57 ± 0.34</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>25-Hydroxycholesterol (1.2 μM)</td>
<td>57.6 ± 6.2</td>
<td>0.26 ± 0.10</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Mevinolin (2.5 μM)</td>
<td>175.0 ± 2.5</td>
<td>2.40 ± 0.36</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Mevinolin (2.5 μM) + mevalonate (0.4 mM)</td>
<td>143.3 ± 2.5</td>
<td>2.28 ± 0.57</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>Mevinolin (2.5 μM) + mevalonate (13.5 mM)</td>
<td>155.4 ± 18.3</td>
<td>0.85 ± 0.14</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>Mevinolin (2.5 μM) + 25-hydroxycholesterol (1.2 μM)</td>
<td>49.5 ± 2.4</td>
<td>0.33 ± 0.14</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Mevinolin (2.5 μM) + 25-hydroxycholesterol (1.2 μM) + mevalonate (0.4 μM)</td>
<td>48.3 ± 6.8</td>
<td>0.28 ± 0.12</td>
<td>0.01 ± 0.001</td>
</tr>
</tbody>
</table>
Regulation of HMG-CoA Reductase by Mevalonate

TABLE II

<table>
<thead>
<tr>
<th>Mevalonate</th>
<th>CHO-K1</th>
<th>Mevalonate</th>
<th>CHO-K1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>133.47 ± 6.5</td>
<td>112.6 ± 2.3</td>
<td>1.66 ± 0.11</td>
</tr>
<tr>
<td>25-Hydroxycholesterol (1.2 μM)</td>
<td>65.3 ± 11.5</td>
<td>58.0 ± 6.0</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>Mevalonate (0.4 mM)</td>
<td>116.1 ± 19.2</td>
<td>91.2 ± 8.4</td>
<td>1.73 ± 0.10</td>
</tr>
<tr>
<td>Mevalonate (13.5 mM)</td>
<td>88.5 ± 8.9</td>
<td>74.3 ± 3.5</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>25-Hydroxycholesterol (1.2 μM) +</td>
<td>64.9 ± 8.1</td>
<td>45.0 ± 7.0</td>
<td>0.02 ± 0.003</td>
</tr>
</tbody>
</table>

Fig. 1. Suppression of HMG-CoA synthase (○) and HMG-CoA reductase (●) activities of CHO cells by 25-hydroxycholesterol. For the study of synthase activity, cells were plated at 2.5 × 10^6/150-mm culture dish in 8 ml of F12FC5 on day 0. On day 1, the monolayers were rinsed twice with 4 ml of saline G and refed 5 ml of F12DPE5. Indicated amounts of 25-hydroxycholesterol were added in 10 μl of ethanol. After 48 h, cells were harvested by trypsinization on day 3, collected in 4 ml of F12FC5, and washed twice with phosphate-buffered saline prior to storage at -70°C. The thawed cell pellets were assayed for HMG-CoA synthase activity as described under "Experimental Procedures." The results are expressed as the mean ± S.E. of triplicate determinations from each of the two dishes at each data point. For the study of reductase activity, cells were plated at 2.5 × 10^6/60-mm culture dish in 3 ml of F12FC5 on day 0. On day 1, the monolayers were rinsed twice with 2 ml of saline G and refed 2 ml of F12DPE5. Indicated amounts of 25-hydroxycholesterol were added in 20 μl of ethanol. After 18 h, cells were harvested for the assay of HMG-CoA reductase as described under "Experimental Procedures." The results are expressed as the mean ± S.E. of assays from triplicate dishes at each data point. Control (100%) enzyme activities (mean ± S.E.) were 2.46 ± 0.12 and 0.99 ± 0.02 nmol/min/mg of protein for synthase and reductase, respectively.

Fig. 2. Suppression of HMG-CoA synthase (○) and HMG-CoA reductase (●) activities of CHO cells by mevalonate. Cells were grown as described in the legend to Fig. 1. On day 1, after the monolayers were refed F12DPE5, indicated amounts of mevalonate were added to triplicate dishes. Cells were harvested for the assay of reductase activity after 18 h of treatment and for the assay of synthase activity after 48 h of treatment. Control (100%) enzyme activities (mean ± S.E.) were 2.46 ± 0.12 and 0.78 ± 0.03 nmol/min/mg of protein for HMG-CoA synthase and HMG-CoA reductase, respectively.

Fig. 3. Suppression of HMG-CoA reductase activity of CHO cells by varying amounts of mevalonate in the absence (○) or the presence (●) of TMD. Cells were plated at 2.5 × 10^6/90-mm culture dish in 3 ml of F12FC5 on day 0. On day 1, the monolayers were refed 2 ml of F12DPE5 ± TMD (20 μg/ml). 3 h later, indicated amounts of mevalonate were added to triplicate dishes in each group. After 18 h, cells were harvested for the assay of reductase activity as described under "Experimental Procedures." Control enzyme activities (mean ± S.E.) were 0.62 ± 0.01 and 0.76 ± 0.02 nmol/min/mg of protein in the absence and the presence of TMD, respectively. The difference between the amounts of inhibition observed in the absence and the presence of TMD is plotted (△) to provide a measure of the loss of inhibition of reductase activity upon TMD treatment.

CoA reductase activity with near maximal (>95%) down-regulation at 15 mM mevalonate (Fig. 3). Pretreatment of cells with TMD (20 μg/ml) results in only a minor (~20%) prevention of this suppression even at the highest concentration of mevalonate. The difference between the inhibition curves in the absence and the presence of TMD (Fig. 3, open triangles) yields a measure of the protection afforded by TMD. If the suppression of HMG-CoA reductase activity by mevalonate treatment were entirely due to the formation of endogenous regulatory sterols, these data can be explained only by assuming a >80% leak through the TMD block (or <20% inhibition of sterol biosynthesis by TMD). However, based on the synthesis of digitonin-precipitable sterols from [14C] acetate, the actual inhibition produced by TMD is substantially greater than this figure. The results in Fig. 3 therefore demonstrate that the suppressive effect of mevalonate on
HMG-CoA reductase is for the most part independent of endogenous sterol formation. The extent of the suppression mediated by the sterol component is thus represented by the loss of inhibition in the presence of TMD (Fig. 3, open triangles).

In order to examine the significance of a possible leak of precursors through the TMD block in another fashion, we determined the effect of TMD on mevalonate regulation of HMG-CoA reductase in a somatic cell mutant of CHO cells (mutant 215) which is blocked in lanosterol demethylation (20). In several studies published previously, this mutant has been shown to be incapable of oxidative demethylation of lanosterol and by the detection methods used, is blocked 95% or better in this process. Treatment of these cells with mevalonate also results in a dose-dependent inhibition of HMG-CoA reductase activity (Fig. 4) similar to that seen in CHO-K1 cells (Fig. 3). Pretreatment of mutant 215 cells with TMD (20 µg/ml) alleviated <20% of this inhibition despite what should now be a cumulative 10,000-fold block in sterol synthesis in these cells.

Another system in which cells are blocked in lanosterol demethylation can be found in CHO-K1 cells treated with azole antymycotics such as ketoconazole (32). It has been reported that ketoconazole (30 µM) abolishes cholesterol synthesis in CHO-K1 cells through an inhibition of lanosterol 14α-demethylase (32). We therefore examined the capacity of mevalonate to act as a suppressor of HMG-CoA reductase activity in CHO-K1 cells treated with either ketoconazole or with a combination of TMD and ketoconazole. Surprisingly, pretreatment of cells with ketoconazole alone had no effect on the suppression of HMG-CoA reductase activity by mevalonate, whereas pretreatment with both ketoconazole and TMD prevented ≈20% of this inhibition (Fig. 5). A possible explanation for the similarity of results with both wild-type CHO-K1 cells and the mutant 215 cells (Figs. 3–5) is that the lanosterol that accumulates both in mutant 215 cells (20, 21) and in CHO-K1 cells treated with ketoconazole (32) is a weak regulatory sterol. However, as will be shown below, other sterols also accumulate in mutant 215 cells and in ketoconazole-treated CHO-K1 cells. It is therefore possible that one or more of these sterols is also regulatory or that the regulatory sterol is different in the two systems.

Analysis of Products Formed from Mevalonate under Conditions in Which Cholesterol Biosynthesis Is Inhibited—Although the literature documents that the conditions described above should produce massive inhibition of sterol synthesis from mevalonate, we wished to confirm that this was in fact the case under conditions in which mevalonate acts as a regulator of HMG-CoA reductase activity. We also sought to examine the identity of the products formed in the presence of high concentrations of mevalonate. We would expect that if there were any sterol synthesis under these conditions, lanosterol and 24(S),25-oxidolanosterol would accumulate since both the mutant 215 cells and the ketoconazole-treated CHO-K1 cells are blocked in lanosterol demethylation.

It has been reported previously that the major sterol product synthesized by 215 cells from mevalonate is lanosterol (20, 21). This result has previously been considered puzzling since this mutant is defective in 4α-methylsterol oxidase activity and therefore would be expected to accumulate 14-deamethyl lanosterol. Since previous analytical work on radioactive products formed from mevalonate in this mutant has been done by thin layer chromatography, we elected to analyze the products by HPLC with an on-line radiodetector. With one of the reverse phase systems (Fig. 6A), we were able to resolve a peak (peak 1) with a shorter retention time than lanosterol (peak 2). Overnight treatment of 215 cells with TMD results in a product (peak 4) that comigrates with squalene dioxide (Fig. 6B) at the expense of both of the major peaks normally found in 215 cells, suggesting that peak 1 is in fact a sterol.

Fig. 4. Effect of TMD on the suppression of HMG-CoA reductase activity of mutant 215 cells by mevalonate. Cells were plated at 2.5 × 10⁴/60-mm culture dish in 3 ml of F12HDF5. On day 1, the monolayers were refed 2 ml of F12HDF5 ± TMD (20 µg/ml). 3 h later, indicated amounts of mevalonate were added to triplicate dishes in each group. After 18 h, cells were harvested for the assay of reductase activity as described under “Experimental Procedures.” Control enzyme activities (mean ± S.E.) were 0.48 ± 0.02 and 0.57 ± 0.04 mmol/min/mg of protein in the absence (○) and the presence (●) of TMD, respectively. The degree of resistance of reductase activity in TMD-treated cells to mevalonate (△) is also plotted as described in Fig. 3.
for less than 5% of the total radioactivity incorporated into nonsaponifiable lipids, is also observed with a retention time of 12 min. This peak elutes neither with the cyclization product of squalene oxide, which is lanosterol, nor with the cyclization product of squalene dioxide, 24(S),25-oxidolanosterol, although its retention time is close to the latter. This material from peak 6 does, however, separate by more than 3 min from oxolanosterol when run on a normal phase HPLC column (data not shown). The retention time of the peak 6 compound is identical to that of lanosterol on nor- mal phase HPLC. It should be noted that both 32-oxolanosterol and 24(S),25-dihydrolanosterol; (peak 4) squalene 2,3,22,23-dioxide; (peak 5) squalene 2,3-oxide; (peak 6) unknown.

Two other findings are consistent with the conclusion that the peak 6 compound is not likely to be a regulatory sterol. Aliquots of this material being a sterol. Studies are currently in progress to identify the structure of this compound.

Product analyses were also performed in CHO-K1 cells treated with ketoconazole alone or ketoconazole + TMD and labeled with [3H]mevalonate (Fig. 7). In untreated CHO-K1 cells (Fig. 7A), the major radiolabeled product of mevalonate causes no suppression of HMG-CoA reductase activity (data not shown). The concentration of peak 6 material used in this experiment can be estimated to be 0.5–2.5 μg/ml assuming that this compound is an isoprenoid derived entirely from exogenous mevalonate. It should be noted that under the conditions used to generate the peak 6 compound (15 mM mevalonate and lipoprotein-containing medium), there is little endogenous synthesis of mevalonate. The lack of an inhibitory effect of the peak 6 compound on HMG-CoA reductase activity is in sharp contrast to that of several oxysterols that are potent suppressors of this enzyme at similar or lower concentrations in cultured fibroblasts (1). Also, when cells are labeled under conditions of Fig. 6D with [3H]squalene instead of with [3H]mevalonate, no radiolabel accumulates at the retention time of peak 6 (data not shown). Thus, peak 6 material appears to be neither regulatory nor a sterol. Studies are currently in progress to identify the structure of this compound.

![HPLC profiles of nonsaponifiable lipids in mutant 215 cells.](image1)

**FIG. 6.** HPLC profiles of nonsaponifiable lipids in mutant 215 cells. Cells were grown and treated as described in the legend to Fig. 4 except that triplicate dishes in each group received 20 μCi of [5-3H]mevalonate at the same time as unlabeled mevalonate. 18 h later, nonsaponifiable lipids were isolated from monolayers and resolved by reverse phase HPLC on a Versapak C18 column in acetonitrile/water (92/8, v/v) as described under “Experimental Procedures.” The chromatograms represent cells that were either untreated (panel A) or treated with 20 μg/ml TMD (panel B), 15 mM mevalonate (panel C), or 20 μg/ml TMD + 15 mM mevalonate (panel D). Approximately 100,000 dpm was applied to the column in each case. The minimum detection limit for the integration of a peak was 200 dpm. The numbered peaks were identified based on the coincidence of their retention times with those of authentic standards: (peak 1) possibly 14-desmethyl lanosterol (see “Results”); (peak 2) lanosterol; (peak 3) 24,25-dihydrolanosterol; (peak 4) squalene 2,3,22,23-dioxide; (peak 5) squalene 2,3-oxide; (peak 6) unknown.

![HPLC profiles of nonsaponifiable lipids in CHO cells treated with ketoconazole.](image2)

**FIG. 7.** HPLC profiles of nonsaponifiable lipids in CHO cells treated with ketoconazole. Cells were plated at 2.5 × 10⁶/60-mm culture dish in 3 ml of F12FC5 on day 0. On day 1, the monolayers were rinsed twice with 2 ml of saline G and refed 2 ml of either F12HDF5 medium (panel A) or medium containing 30 μM ketoconazole (panel B), 30 μM ketoconazole + 20 μg/ml TMD (panel C), or 30 μM ketoconazole + 20 μg/ml TMD + 15 mM mevalonate (panel D). To triplicate dishes in each group, 20 μCi of [5-3H]mevalonate was added. After 18 h, nonsaponifiable lipids were isolated from the monolayers and were resolved by reverse phase HPLC on a Versapak C18 column as described under “Experimental Procedures.” The chromatograms in panels A and B were obtained using methanol/water (95/5, v/v) whereas those in panel C and D were obtained using acetonitrile/water (92/8, v/v) as the mobile phase. Approximately 100,000 dpm was applied to the column in each case. The minimum detection limit for the integration of a peak was 200 dpm. The numbered peaks were identified based on the coincidence of their retention times with those of authentic standards: (peak 1) cholesterol; (peak 2) lanosterol; (peak 3) 24(S),25-oxidolanosterol; (peak 4) squalene 2,3,22,23-dioxide; (peak 5) squalene 2,3-oxide; (peak 6) unknown.
has the retention time of cholesterol (peak 1). Treatment of cells with 30 mM ketocanazole (Fig. 7B) results in a lack of detectable demethylation of lanosterol (peak 2) or oxidolanosterol (peak 3) and a partial block of cyclization of squalene oxide (peak 5) and dioxides (peak 4). When cells are treated with a combination of TMD and ketocanazole (Fig. 7C), the major product observed is squalene dioxide (peak 4) with some formation of squalene oxide (peak 5). Addition of increasing concentrations of mevalonate up to 15 mM to CHO-K1 pretreated with TMD and ketocanazole produces effects similar to those seen in TMD-treated 215 cells, including the formation of a compound (peak 6) with a retention time of 12 min (Fig. 7D). Again, no peaks that comigrate with either lanosterol or oxidolanosterol are observed as would be expected if there were any cyclization of the squalene oxide or squalene dioxide. These results thus support the contention that mevalonate-induced suppression of HMG-CoA reductase can occur in CHO cells in the absence of endogenous sterol synthesis.

**Effect of TMD on the Conversion of Exogenously Supplied Squalene Oxide to Regulatory Sterols—**Since some of the intermediates and end products of sterol synthesis can down-regulate HMG-CoA reductase, and since the production of these compounds is limited by the formation of mevalonate, we reasoned that squalene oxide would be a precursor to such inhibitors of HMG-CoA reductase in CHO-K1 cells. If TMD is effective in blocking the cyclization of squalene oxide even at high intracellular concentrations of that compound, we reasoned that any down-regulation of HMG-CoA reductase by exogenously supplied squalene oxide would be prevented by TMD treatment. The results of such an experiment (Fig. 8) verify our reasoning and confirm the effectiveness of TMD in blocking the production of squalene oxide. These results thus support the contention that mevalonate-induced suppression of HMG-CoA reductase in CHO cells is relatively small.

**Comparison of Effects of Fluoromevalonate and TMD on HMG-CoA Reductase Activity of CHO-K1 Cells—**In the above studies, we have examined the possible role of nonsterol mevalonate-derived products in the regulation of HMG-CoA reductase in response to exogenously supplied mevalonate. A complementary approach to this problem is to examine the effect of blockage of endogenously synthesized mevalonate conversion to products on the regulation of this enzyme. We chose to compare the effects of fluoromevalonate, a specific inhibitor of mevalonate pyrophosphate decarboxylase (35), an enzyme required for all isoprenoid synthesis in mammalian cells, and TMD (which is, of course, a specific inhibitor of sterol synthesis) on HMG-CoA reductase activity. At appropriate concentrations, fluoromevalonate produced a 98% inhibition of labeled mevalonate incorporation into total cellular isoprenylated proteins and is therefore an effective inhibitor of synthesis of at least one major nonsterol isoprenoid of CHO cells. Surprisingly, fluoromevalonate treatment was somewhat less effective in the inhibition of sterol synthesis producing a maximal inhibition of conversion of mevalonate to sterols of only 75% (data not shown). A comparison of the effects of fluoromevalonate and TMD on the regulation of HMG-CoA reductase activity in CHO-K1 cells (Fig. 9) indicates that fluoromevalonate produces a 4-fold stimulation of activity of this enzyme which is substantially greater than the approximately 50% stimulation produced by TMD. This finding is in agreement with the results shown in Figs. 3–5 that the contribution of biosynthetic sterols derived from mevalonate to the regulation of HMG-CoA reductase is relatively small.

**DISCUSSION**

When cultured cells are treated with mevalonic acid, HMG-CoA reductase activity is down-regulated. The magnitude of such suppression is at least as great as that produced by optimal doses of oxygenated steroids in CHO-K1 cells (see Figs. 1 and 3) as well as in other cell lines (1, 2). In contrast, mevalonate is ineffective relative to 25-hydroxycholesterol in down-regulation of acetoacetyl-CoA thiolase or HMG-CoA synthase. Data in this paper are also consistent with the conclusion that although mevalonate starvation results in a loss of regulation of HMG-CoA reductase by sterols, such multivalent control is not observed for HMG-CoA synthase or acetoacetyl-CoA thiolase (see Tables I and II). A large body of published data has been interpreted to argue for a common control mechanism of several enzymes of sterol biosynthesis by sterols. Of the enzymes of mevalonate biosynthesis, analysis of regulation of HMG-CoA synthase and HMG-CoA reductase, particularly the latter, has led to the conclusion that...
this common mechanism is transcriptional through sterol-responsive elements in the 5'-flanking regions of the respective genes (36). In contrast to the pleiotropic nature of sterol regulation of transcription of the genes of sterol synthesis in cultured fibroblasts, the regulation by mevalonate of HMG-CoA reductase appears to be unique among the enzymes studied to this point. We have proposed previously that this unique control mechanism for HMG-CoA reductase is at least in part translational (10). This concept has recently been confirmed by others (17). Since sterol regulation of the synthesis of several enzymes of cholesterol synthesis is pleiotropically transcriptional, whereas one component of the regulation of HMG-CoA reductase by mevalonate appears to be uniquely translational, it is likely that the translational control of HMG-CoA reductase is mediated at least in part through a nonsterol mevalonate-derived product. These ideas are consistent with the multivalent control hypothesis that was suggested some time ago (4) based solely on the behavior of HMG-CoA reductase activity in response to sterols under conditions of cellular mevalonate starvation.

It should be noted in this context that there is clear evidence that HMG-CoA reductase is also regulated through control of its rate of degradation. Some evidence has been presented (17) to suggest that a combination of sterol and nonsterol products of mevalonate is required to modulate the rate of degradation of reductase. Since the experiments reported in this paper deal only with the regulation of reductase activity, they do not address the possibility that in addition to the compartmentalization of the regulation of enzyme synthesis discussed above, there may be analogous compartmentalization of the regulation of reductase degradation.

If mevalonate can give rise to both sterol and nonsterol products, both of which apparently have regulatory significance for HMG-CoA reductase, it then becomes of interest to determine the relative contribution of each type of end product to the observed down-regulation of enzyme activity. The results in Figs. 3-5 indicate that upon treatment of proliferating cells with exogenous mevalonate, at least 80% of the observed inhibition is mediated by nonsterol products. This conclusion rests heavily upon the capacity of TMD to inhibit the conversion of mevalonate to sterols even at the high concentrations of mevalonate utilized in these experiments. To address this point, we have established that TMD is very effective (inhibition >99%) in blocking the conversion of acetate to digitonin-precipitable sterols at all concentrations of mevalonate utilized. Furthermore, after combining TMD treatment with a second block in cholesterol synthesis at lanosterol demethylation either by mutation (mutant 215) or by treatment with ketoconazole, the ability of mevalonate to cause a substantial down-regulation of HMG-CoA reductase activity remains unaffected. Since it has been reported that treatment of CHO cells with high concentrations of mevalonate can result in the formation of 32-oxylanosterols (33) that are regulatory but not digitonin-precipitable, we have examined the products formed from mevalonic acid in the presence of TMD and ketoconazole in CHO cells by HPLC and found no evidence of synthesis of any sterols, including 32-oxylanosterols. Ketoconazole alone, at the concentration used in our studies, has been reported to block 32-oxylanosterol formation in CHO cells (32). Further confirmation of the efficacy of TMD as an inhibitor of squalene oxide cyclization was obtained by demonstrating the capacity of TMD to block completely the suppression of HMG-CoA reductase activity by a broad range of exogenous squalene oxide concentrations.

Different results on the ability of TMD to act as an inhibitor of mevalonate conversion to cholesterol have been reported (16) in studies of a rat hepatoma line, H4. Although we have no experience with the utilization of TMD with rat hepatoma lines, we have found that TMD is quite ineffective as an inhibitor of the conversion of mevalonate to sterols in primary rat hepatocytes in culture (data not shown). Therefore, it is of significance that the cell line that we are utilizing in current studies is a TMD-sensitive cell line. These same authors (16) concluded that 2,3-iminosqualene was an effective inhibitor of the conversion of mevalonate to cholesterol in confluent H4 cells and that it did inhibit the ability of 4.6 mM mevalonate to act as a regulator of HMG-CoA reductase. However, they did not examine the regulatory capacity of mevalonate over a broad range of mevalonate supplements as we have presented in the current study.

Comparisons of the IC50 determined from dose-response curves of HMG-CoA reductase activity to mevalonate treatment are particularly informative. In Figs. 3-5, TMD treatment shifts the IC50 for mevalonate from a value of 3.5-4.0 to 5.5-6.0 mM, a factor of only 1.5. In contrast, the results of [14C]acetate incorporation into digitonin-precipitable material indicate a greater than 100-fold inhibition of sterol biosynthesis upon TMD treatment. If all of the suppression of HMG-CoA reductase caused by mevalonate could be accounted for by formation of a sterol regulator, it would be anticipated that a 100-fold inhibition of sterol synthesis would produce a 100-fold shift in the IC50. Clearly, this is not the case.

Another perspective on the quantitative significance of sterol versus nonsterol isoprenoid products of mevalonate metabolism in the regulation of HMG-CoA reductase can be gained by comparing the effects on reductase activity of 3- fluoromevalonate, an inhibitor of all isoprenoid synthesis, with those of TMD, a specific inhibitor of sterol synthesis. In such experiments, we are attempting to examine the capacity of these endogenously synthesized regulators to inhibit reductase activity in the absence of exogenous mevalonate. These data demonstrate that when HMG-CoA reductase activity is derepressed in cells by switching them to sterol-free medium, derepression is in fact far from complete. Blockage of endogenous isoprenoid synthesis with fluoromevalonate stimulates HMG-CoA reductase activity 4-fold under these conditions thereby indicating an 80% suppression of this enzyme by endogenously synthesized isoprenoid regulators. The contribution of endogenously synthesized sterol regulators to this inhibition based on the TMD data shown in Fig. 9 is quite small (about 10% of the total).

It is intriguing to speculate, as have others (4), that the regulation of HMG-CoA reductase by nonsterol prenyl derivatives is a phenomenon related to cell growth. In most cells, synthesis of cholesterol and other isoprenoids is really only required under conditions of proliferation since isoprenoids are long lived molecules. The existence of sterol and nonsterol mevalonate-derived regulators clearly reflects cellular growth requirements for cholesterol as well as nonsterol isoprenoid products, both of which can act on HMG-CoA reductase.

Independent regulation of the synthesis of these two classes of end products despite action on a common enzyme is likely achieved by utilizing different mechanisms of feedback regulation (transcriptional control for sterols, translational control for nonsterols) of HMG-CoA reductase synthesis. It is worth noting that in a nonproliferating cholesterogenic system such as mammalian liver, nonsterol regulation by mevalonate-derived products might not be as significant. Indeed, the bulk of the literature on mevalonate regulation of HMG-CoA reductase synthesis in animal liver indicates that it operates by a transcriptional control mechanism (37) as would
be the case if most of its regulatory effect were exerted through conversion to sterols.

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