

26-Hydroxycholesterol: regulation of hydroxymethylglutaryl-CoA reductase activity in Chinese hamster ovary cell culture

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Abstract The effect of 26-hydroxycholesterol and other intermediates in bile acid synthesis on HMG-CoA reductase activity was studied in Chinese hamster ovary (CHO) cell culture. Incubation of CHO cells for 5 hr in 0.25 μ M 26-hydroxycholesterol caused a 40% inhibition of HMG-CoA reductase activity. All other intermediates tested including 3 β -hydroxy-5-cholenoic acid and cholest-5-ene-3 β ,7 α ,26-triol, oxidation products of 26-hydroxycholesterol, had little or no inhibitory effect. It is proposed that 26-hydroxycholesterol has a selective biological role in the regulation of cholesterol synthesis.—Esterman, A. L., H. Baum, N. B. Javitt, and G. J. Darlington. 26-Hydroxycholesterol: regulation of hydroxymethylglutaryl-CoA reductase activity in Chinese hamster ovary cell culture. *J. Lipid Res.* 1983. **24**: 1304–1309.

Supplementary key words 26-hydroxycholesterol • 7 α -hydroxycholesterol • 3 β -hydroxy-5-cholenoic acid • bile acid intermediates • HMG-CoA reductase

Recently, we reported that 26-hydroxycholesterol (cholest-5-ene-3 β , 26-diol) normally occurs in adult human serum (1) and is deficient in amount in individuals with the genetically determined disease, cerebrotendinous xanthomatosis (CTX) (2), who lack the hepatic mitochondrial enzyme C27-steroid 26-hydroxylase (3). In view of the potent biological effects of 25-hydroxycholesterol (4), we undertook a study of the effect of 26-hydroxycholesterol and its derivatives on the activity of hydroxymethylglutaryl CoA (HMG-CoA) reductase (EC 1.1.1.34) in Chinese hamster ovary (CHO) cells, a well-established model for the study of sterol synthesis (5–7).

EXPERIMENTAL PROCEDURES

Materials

D,L-[¹⁴C]HMG-CoA and [³H]mevalonic acid were purchased from New England Nuclear. D,L,-HMG-CoA was obtained from P-L Biochemicals, Inc. Other biochemicals were from Sigma. Cholest-5-ene-3 β ,26-diol (26-hydroxycholesterol) was prepared from krypto-

genin (Syntex Research, Palo Alto, CA) by successive Clemmensen and Wolff-Kishner reduction described initially by Scheer, Thompson, and Mosettig (8) and as modified for the preparation of a radioactive compound (9). The purpose of the second step is to complete the reduction of the 16-keto group of kryptogenin which is the only other major product. Following crystallization of the 26-hydroxycholesterol as both the free sterol and the diacetate, it was analyzed after hydrolysis to the free sterol by both thin-layer and gas-liquid chromatography (9). No trace of the 16-keto derivative was found either by GLC (RRT compared to 26-hydroxycholesterol = 2.151) or TLC using silica gel G and a solvent system of chloroform-acetone 4:1 (v/v) (R_f 26-hydroxycholesterol = 0.67, 16-keto = 0.42). No other peaks were detected and therefore we believe the 26-hydroxycholesterol to be free of other sterols. Both cholest-5-ene-3 β ,7 α -diol (7 α -hydroxycholesterol) and cholest-5-ene-3 β ,7 α -26-triol (7 α ,26-hydroxycholesterol) were prepared using tertiary butyl perbenzoate (10) as described previously (11). 3 β -Hydroxy-5-cholenoic acid and 25-hydroxycholesterol were purchased from Steraloids. 5 β -Cholestane,3 α ,7 α -diol and 5 β -cholestane,3 α ,7 α ,12 α -triol were gifts from Dr. William Elliott, Department of Biochemistry, St. Louis University School of Medicine, St. Louis, MO. 3 β -Hydroxy-5-cholestenoic acid was a gift from Dr. Marcel Gut, Worcester Institute, Worcester, MA. All of the other sterols used were also found to give a single spot by TLC or a single peak by GLC.

Abbreviations: 26-hydroxycholesterol, cholest-5-ene-3 β ,26 diol; 25-hydroxycholesterol, cholest-5-ene-3 β ,25 diol; HMG-CoA reductase, hydroxymethylglutaryl coenzyme A reductase; Me₂SO, dimethylsulfoxide; CHO, Chinese hamster ovary cells; CTX, cerebrotendinous xanthomatosis; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; RRT, relative retention time.

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METHODS

Chinese hamster ovary cells (CHO-K1), a generous gift from Dr. L. Chasin, Columbia University, New York, were grown in F-12 media (Gibco, Grand Island, NY) supplemented with either 10% fetal bovine serum (FCS-M) or 10% delipidated fetal bovine serum (DEL-M). All incubations were in a humidified 5% CO₂ environment at 37°C.

Delipidated fetal bovine serum was prepared by extraction of lipids with n-butanol-diisopropyl ether 3:5 according to the method of Cham and Knowles (12), with the addition of a lyophilization step to remove the residual n-butanol. Prior to delipidation the fetal bovine serum contained 26-hydroxycholesterol, 0.7 µg/100 ml. Following delipidation the serum contained less than 0.1 µg/100 ml of 26-hydroxycholesterol as determined by isotope ratio mass spectrometry (1, 2). The fetal calf serum used did not contain 25-hydroxycholesterol which has a slightly earlier retention time by this technique (2).

HMG-CoA reductase activity

Cell culture. CHO-K1 cells were cultured following the protocol of Chang, Limanek, and Chang (6). The cells, 3.5×10^4 per 60-mm Falcon tissue culture dish, were plated in FCS-M and incubated for 48 hr. The cultures were washed with phosphate-buffered saline (PBS) and fed with DEL-M followed by a further 48-hr incubation. (Growth media was replenished every 24 hr). "Time 0 cultures" are defined as those cultures which were grown in DEL-M for 48 hr. Each compound was dissolved in dimethylsulfoxide and introduced to "time 0 cultures" at a final concentration of 0.14%. Control cultures also contained the same amount of solvent.

HMG-CoA reductase assay. Cell lysates were prepared by a hypotonic buffer-rapid cell scraping technique (6). HMG-CoA reductase activities were assayed by double isotope labeling (13) as modified by Chang et al. (6). Where appropriate, 10 units of *E. coli* alkaline phosphatase (Sigma Chemical Co.) was added to the pre-incubation mixture as described by Brown, Goldstein, and Dietschy (14). HMG-CoA reductase activity is expressed as pmoles of mevalonate formed \times mg protein⁻¹ \times minute⁻¹. Total cellular protein was determined by the method of Lowry et al. (15).

Uptake and metabolism of 26-hydroxycholesterol in cell culture. In one series of studies [^{16,22-³H}]26-hydroxycholesterol (sp. act. 3×10^6 dpm/µg) (9) was used in an identical manner as described above. Following incubation for 5 and 24 hr the media and cells were separated and collected. The cells were washed three times in PBS by gentle centrifugation.

Protein was precipitated using a ratio of 7:1 2,2-dimethoxypropane to aqueous phases (16) and the filtrate was brought to pH 8.3 with aqueous 5% NaHCO₃ and extracted with ethyl acetate. All the radioactivity was found in the organic phases and was either counted directly or analyzed further by separation on silica gel G in a solvent system of chloroform-acetone 4:1 (v/v).

RESULTS

Incubation of the cells for 5 hr with 26-hydroxycholesterol ranging in concentration from 0.025 to 2.5 µM gave a concentration-related reduction in HMG-CoA reductase activity (Fig. 1). All subsequent studies were done using a sterol concentration of 0.25 µM.

As shown in Fig. 2, the inhibition of HMG-CoA reductase activity increased to a maximum of 52% after 10 hr of incubation. Prolongation of the incubation to 24 hr did not have any greater inhibitory effect.

In these studies the increase in total cellular protein for control and 26-hydroxycholesterol-containing cultures at each time interval were not significantly different. DNA replication, determined by tritiated thymidine incorporation after 7 hr of incubation, also indicated no inhibition of cell growth. Plating efficiency studies indicated that no change had occurred in cultures containing 26-hydroxycholesterol.

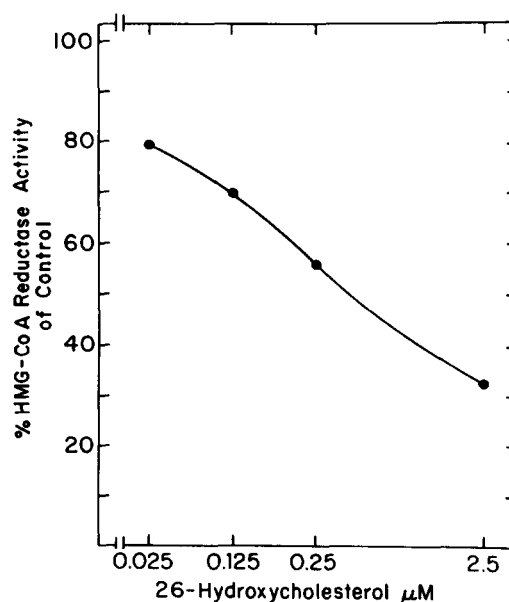


Fig. 1. Dose-response curve for 26-hydroxycholesterol. All cell cultures contained 0.14% Me₂SO with or without the indicated concentration of 26-hydroxycholesterol. Following a 5-hr incubation at 37°C the CHO K1 cells were harvested and assayed for HMG-CoA reductase activity and total protein. Data are presented as the mean of two independent studies consisting of two replicate cultures for each sterol concentration.

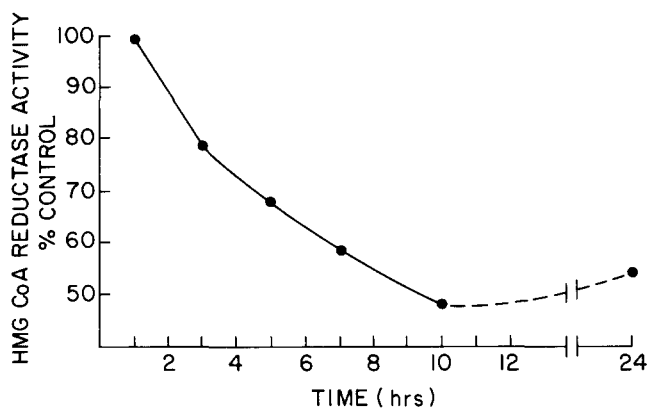


Fig. 2. Time-response curve for 26-hydroxycholesterol. All cell cultures contained 0.14% Me₂SO with or without 0.25 μM 26-hydroxycholesterol. At the indicated times following introduction of the sterol the cells were harvested and assayed for HMG-CoA reductase activity and total protein. Data are presented as the mean of three independent studies consisting of four replicate cultures for each time point.

The possibility of inhibition of HMG-CoA reductase activity by phosphorylation of the enzyme was determined by addition of *E. coli* alkaline phosphatase. No significant increase in enzyme activity was detected in either control or 26-hydroxycholesterol-containing cultures (Table 1).

Analysis of the cell culture for the distribution and metabolism of 26-hydroxycholesterol was done at 5 and 24 hr. At 5 hr, 4.3% of the radioactivity was recovered in the cellular fraction. Thin-layer chromatographic analysis (Fig. 3) of the radioactivity from the media and cell fractions indicated 95.0% and 85.5%, respectively, co-migrated with the 26-hydroxycholesterol (R_f 0.67). In the cellular fraction, 10.2% of the radioactivity was found in a zone less polar (R_f 0.94) than the parent compound. Saponification of the radioactivity obtained from the cellular fraction prior to chromatographic analysis indicated a loss of radioactivity at R_f 0.94 and an increase to 93.4% at R_f 0.67. Radioactivity at R_f 0.83 from the media fraction, representing 2.0% of the total

was unaffected by saponification and was not studied further.

Comparison of the inhibitory activity of equimolar amounts of other compounds after 5 hr of incubation is shown in Table 2. None of the compounds was found to be more inhibitory than 26-hydroxycholesterol. Although 26-hydroxycholesterol appears to have a greater inhibitory effect than 25-hydroxycholesterol at 5 hr, these differences become less striking at later time intervals (Table 3).

All compounds containing a 7α-hydroxyl group were less inhibitory than 26-hydroxycholesterol. No inhibition occurred with 7α-hydroxycholesterol.

DISCUSSION

The demonstration that oxygenated sterols are potent inhibitors of HMG-CoA reductase (4), the rate limiting enzyme in cholesterol synthesis, requires further evaluation of its physiological significance. Many of the compounds tested previously, such as cholest-5-ene,3β,25-diol (25-hydroxycholesterol), cholest-5-ene-3β-ol-7-one, and cholest-5-ene-3β,7β-diol, are known autoxidation products of cholesterol and probably do not occur in vivo as part of a metabolic pathway (6).

Under the conditions of our study, we found significant inhibition by both 25- and 26-hydroxycholesterol. Based on the findings after incubation with alkaline phosphatase, the mechanism of inhibition is probably the same as that established with studies of 25-hydroxycholesterol and relates to the amount of enzyme present rather than its activity (14, 17).

Evidence for the enzymatic synthesis of 25-hydroxycholesterol has not been consistent. Although Fredrickson and Ono (18) reported the formation of both 26-hydroxycholesterol and 25-hydroxycholesterol from incubates of liver mitochondria with cholesterol, Danielsson (19) was able to confirm the formation of 26-

TABLE 1. Reductase activity: effect of alkaline phosphatase

	HMG-CoA Reductase Activity pmol · mg ⁻¹ · min ⁻¹	% of Control
Control - AP ^a	466 ± 56 ^b	
Control + AP	433 ± 6	
+26-Hydroxycholesterol - AP	270 ± 6	57.9%
+26-Hydroxycholesterol + AP	234 ± 4	54.0%

^a AP, *E. coli* alkaline phosphatase. CHO cells were cultured as described in Methods and incubated with or without 26-hydroxycholesterol for 5 hr. Ten units of *E. coli* alkaline phosphatase was added to the cell lysates for 1 hr prior to the enzyme assay. HMG-CoA reductase activities are expressed as pmoles of mevalonic acid formed · mg protein⁻¹ · min⁻¹. The results indicate no change in the inhibitory effect of 26-hydroxycholesterol in the presence of alkaline phosphatase.

^b Mean ± SD.

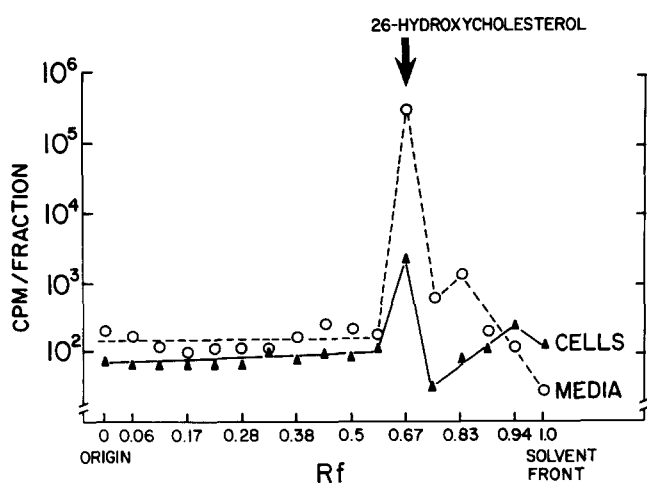


Fig. 3. Uptake and metabolism of 26-hydroxycholesterol in cell culture. Cell cultures and media were collected after a 24-hr incubation in the presence of $0.25 \mu\text{M}$ [$16,22\text{-}^3\text{H}$]26-hydroxycholesterol (sp act 3×10^6 dpm/ μg) and lipids were extracted as described in Methods. Following separation on silica gel G plates in a solvent system of chloroform-acetone 4:1 (v/v), 1-cm zones were scraped and counted. The less polar peaks in both the media and the cells disappeared following saponification.

hydroxycholesterol and considered 25-hydroxycholesterol to be an autoxidation product. Using human liver preparations, Björkhem (20) has found that mitochondrial C-26 hydroxylase activity is far greater than 25-hydroxylase activity. It is difficult to be certain in these *in vitro* preparations if the small amount of 25-hydroxycholesterol that is formed is dependent on an active enzyme since, in contrast to 26-hydroxycholesterol, it is readily found as the major autoxidation product of cholesterol (21). These findings *in vitro* are consistent with our analysis of normal human plasma (1, 2). In our method, both 25- and 26-hydroxycholesterol co-elute from the column and separate during gas-liquid chromatography so that they would appear as individual peaks. Recoveries of both compounds added to plasma

are the same and any analyses thus far regularly detect 26-hydroxycholesterol, but only occasionally is 25-hydroxycholesterol found, and in much lower concentration. Thus the findings to date are consistent with the view that mitochondrial C-26 hydroxylation of cholesterol is probably the major pathway and 25-hydroxycholesterol arises either by a minor enzymatic pathway or by intestinal absorption of the preformed sterol.

In contrast, both 7α -hydroxycholesterol and 26-hydroxycholesterol occur as a result of microsomal and mitochondrial enzyme activity, respectively, and are recognized intermediates in bile acid synthesis (11, 22). Recent studies indicate that the mitochondrial enzyme, C-27 sterol-26-hydroxylase not only gives rise to 26-hydroxycholesterol but also is essential for the normal oxidation of those bile acid intermediates formed in microsomes, such as 7α -hydroxycholesterol, 5β -cholestane- $3\alpha,7\alpha$ -diol, and 5β -cholestane- $3\alpha,7\alpha,12\alpha$ -triol (10, 23). In view of this information, we considered it important to evaluate the inhibitory potency of 26-hydroxycholesterol and its further metabolic products. Since significant metabolism of 26-hydroxycholesterol was not detected, we attribute the inhibition of HMG-CoA reductase activity to the effect of the free sterol. Under the conditions of our study, 7α -hydroxycholesterol did not cause inhibition. The data suggest that some stimulation of enzyme activity may occur, but this possibility cannot be evaluated further at this time. In other studies, at much higher concentrations, significant inhibition of HMG-CoA reductase activity by 7α -hydroxycholesterol has been reported (24). Of particular interest in our study is the finding that 7α -hydroxylation decreases the inhibition of 26-hydroxycholesterol. Such an event must occur *in vivo* to account for the metabolism of 26-hydroxycholesterol to both primary bile acids (11, 22). The finding that 26-hydroxycholesterol is the most potent of all the intermediates in a concentration that could occur *in vivo* implies a sensitivity and

TABLE 2. Effect of intermediates in bile acid synthesis on HMG-CoA reductase activity in CHO cell culture

Compound	Concentration μM^a	% HMG-CoA Reductase Activity % Control \pm SD
Cholest-5-ene- $3\beta,26$ -diol (26-hydroxycholesterol)	0.25	64.3 ± 6.6 (15) ^b
Cholest-5-ene- $3\beta,25$ -diol (25-hydroxycholesterol)	0.25	80.7 ± 8.2 (4)
3β -Hydroxy-5-cholestenoic acid	0.25	87.8 ± 20.4 (4)
3β -Hydroxy-5-cholenoic acid	0.25	89.0 ± 8.5 (4)
5β -Cholestane- $3\alpha,7\alpha$ -diol	0.25	90.3 ± 8.0 (4)
5β -Cholestane- $3\alpha,7\alpha,12\alpha$ -triol	0.25	95.75 ± 4.3 (4)
Cholest-5-ene- $3\beta,7\alpha,26$ -triol ($7\alpha,26$ -dihydroxycholesterol)	0.25	97.9 ± 10.9 (16)
7α -Hydroxycholesterol	0.25	103.7 ± 17 (6)

^a Cell cultures were incubated for 5 hr in the presence of the indicated sterol.

^b Number of studies in parentheses.

TABLE 3. Effect of 25-hydroxycholesterol vs. 26-hydroxycholesterol on HMG-CoA reductase activity

Incubation Period	HMG-CoA Reductase Activity ^a			% of Control	
	Control	+25-Hydroxy-cholesterol ^b	+26-Hydroxy-cholesterol ^b	+25-Hydroxy-cholesterol ^b	+26-Hydroxy-cholesterol ^b
hr		pmol · mg protein ⁻¹ · min ⁻¹			
5	551	455	379	80.7	68.8
10	536	294	272	54.8	48.3
24	126	79	72	63.0	57.7

^a Data are presented as the mean of two independent parallel studies consisting of four replicate cultures for each compound.

^b Concentration, 0.25 μ M.

specificity that fully supports the concept of a normal physiological role in the regulation of cholesterol synthesis.

Our studies do not address the mechanism of regulation. Furthermore, in approaching this aspect, it will be necessary to consider not only the free sterol but its fatty acid (1) and sulfate esters (25) which also occur in vivo. The present studies provide a rationale for further exploring the physiologic role of 26-hydroxycholesterol and its derivatives. ■■

A.L.E. and H.B. were post-doctoral fellows, Cornell University Medical College Training Grant # AM07142 N.B.J. was supported by grants # AM32995 and HL31160. G.J.D. was supported by grant # GM22372.

Manuscript received 24 November 1982 and in revised form 31 May 1983.

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