

Regulation of gene expression and synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by micellar cholesterol in CaCo-2 cells

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Abstract To investigate whether, and by what mechanisms, luminal (dietary) cholesterol regulates cholesterol synthesis in human intestinal cells, HMG-CoA reductase activity, gene expression, synthesis, and degradation were investigated in CaCo-2 cells exposed to taurocholate micelles containing cholesterol. In cells incubated with cholesterol solubilized in 5 mM taurocholate and 30 μ M monoolein, HMG-CoA reductase activity was decreased. 25-Hydroxycholesterol, delivered to the cells in the same manner as native cholesterol, was significantly more potent in inhibiting reductase activity and was used, therefore, to investigate mechanisms for sterol regulation. Cells incubated with taurocholate micelles without cholesterol lost cellular cholesterol into the medium causing an increase in HMG-CoA reductase activity and enzyme mass. Although steady-state levels of HMG-CoA reductase mRNA were increased under conditions of cholesterol efflux, synthesis rates of reductase protein were not increased. An increase in activity and enzyme mass in cells incubated with micelles alone, however, was accompanied by a significant decrease in the rate of degradation of reductase protein. In contrast, sterol influx from taurocholate micelles was associated with a marked decrease in HMG-CoA reductase activity and mass without altering mRNA levels except at high concentrations of the polar sterol which did decrease reductase mRNA levels by 50%. The absorption of apical sterol resulted in a significant decrease in the translational efficiency of reductase mRNA and a modest increase in the rate of degradation of the enzyme. Thus, although the primary function of the enterocyte is to transport luminal (dietary) cholesterol to other tissues of the body, apically derived cholesterol enters metabolic pools within the cell which regulates its own cholesterol synthesis. Dietary cholesterol, therefore, will regulate the contribution to the total body cholesterol pool of endogenously derived cholesterol from the intestine. The mechanism for this regulation of intestinal HMG-CoA reductase by luminal cholesterol occurs primarily at the post-transcriptional level. — Field, F. J., T. Shreves, D. Fujiwara, S. Murthy, E. Albright, and S. N. Mathur. Regulation of gene expression and synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* 1991. 32: 1811-1821.

Supplementary key words cholesterol • 25-hydroxycholesterol • micelles

Cholesterol requirements of most cells are met by two separate but interrelated processes. One process is the endogenous synthesis of cholesterol. This synthetic pathway, which involves over 20 reactions, is regulated primarily by the activity of HMG-CoA reductase which catalyzes the formation of mevalonate (1, 2). The other process involves the utilization of lipoprotein cholesterol following internalization of the lipoprotein bound to its surface receptor. Under most circumstances, the endogenous synthesis of cholesterol is required only if lipoprotein internalization is insufficient to meet the cholesterol requirements of the cell (3). To maintain cellular cholesterol homeostasis, there exists a rather potent negative feed-back system on the activity of HMG-CoA reductase which results in a decrease in the synthesis of cholesterol if excess sterol enters via the receptor. One mechanism for this feed-back regulation of HMG-CoA reductase activity by sterols is thought to occur at the level of gene expression leading to a decrease in gene transcription (4). Another factor that has been shown to regulate the expression of reductase is controlled degradation of the protein (5-7). The mechanism of this regulation is not well understood but it appears to depend upon the attachment of the enzyme to the endoplasmic reticulum (8, 9). Lastly, in experiments with Chinese hamster ovary cells, there is evidence for a decrease in translation efficiency of mRNA for HMG-CoA reductase resulting in decreased reductase protein and activity (10).

In regard to the regulation of cellular cholesterol metabolism, the small intestinal absorptive cell is unique. In addition to access of cholesterol via the LDL receptor and the biosynthetic pathway, the absorptive cell has ac-

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A. ¹To whom correspondence should be addressed.

cess to exogenous luminal cholesterol at its apical membrane. Although it is reasonable to assume that this influx of cholesterol at the apical membrane will contribute to the enterocyte cholesterol pool and result in a decrease in cholesterol synthesis, this remains controversial (11). Results of earlier studies have suggested that luminal bile acids rather than cholesterol regulate cholesterol biosynthesis in the intestine (12). In rat, most dietary studies have shown a lack of response of intestinal cholesterol synthesis to ingested cholesterol (13, 14). These observations have important ramifications as they relate to plasma cholesterol levels and the risk of atherosclerosis. It has been demonstrated that newly synthesized cholesterol from the intestine contributes directly to the plasma pool of cholesterol (15). Since the rate of intestinal cholesterol synthesis is second only to the liver (13), the apparent lack of regulation of endogenously synthesized cholesterol by exogenous cholesterol would result in continued high rates of cholesterol synthesis during times of significant cholesterol influx, a situation that may not be beneficial to the host. This lack of effect of luminal cholesterol on intestinal cholesterol metabolism, however, is species-dependent. Cholesterol synthesis in the intestines of rabbits, guinea pigs, and hamsters appears to be suppressed by dietary cholesterol (16–18). Mechanisms that explain the regulation of HMG-CoA reductase activity by luminal sterols in the intestine *in vivo* or *in vitro* (19–21) have not been previously addressed.

The present study was undertaken, therefore, to investigate whether luminal sterols regulate HMG-CoA reductase activity in the human intestinal cell line, CaCo-2. The results demonstrate that sterols absorbed from taurocholate micelles by the apical membrane of CaCo-2 cells decrease HMG-CoA reductase mass and activity. A mechanism for this regulation was demonstrated by pulse-chase experiments that showed a decrease in the translational efficiency of HMG-CoA reductase mRNA and an increase in the rate of degradation of the protein.

METHODS

Materials

[2-¹⁴C]acetate, [³H]cholesterol, [5-³H]mevalonic acid, 3-hydroxy-3-methyl[3-¹⁴C]glutaryl coenzyme A and [α -³²P]dATP were from New England Nuclear (Boston, MA). *Trans* [³⁵S]L-methionine was purchased from ICN Biomedicals Inc. (Irvine, CA). Cholesterol, sodium taurocholic acid, monoolein, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nucleotide adenine diphosphate, cycloheximide, dextran sulfate, actinomycin D, salmon sperm, 4-chloro-naphthol, protein A agarose, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). HMG-CoA was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). 25-Hydroxycholesterol was from

Steraloids (Wilton, NH). A cDNA probe of human HMG-CoA reductase (pH Red-102) was obtained from American Type Culture Collection (Rockville, MD). A cDNA for human α actin, PHM α A-1 (22), was graciously obtained from Dr. Peter Rubenstein, Department of Biochemistry, University of Iowa. A polyclonal antibody to human HMG-CoA reductase was generously provided by Dr. Paul Dawson, Department of Molecular Genetics and Internal Medicine, University of Texas Southwestern Medical Center (23). The murine monoclonal antibody, A9 (American Type Culture Collection, Rockville, MD), with specificity for human HMG-CoA reductase was purified from serum-free culture supernatants by saturated ammonium sulfate precipitation as described (24).

Cell culture

CaCo-2 cells were cultured as previously described (25). Experiments were performed in medium-199/Earle's (Gibco, Grand Island, NY) containing 1 mM HEPES, pH 7.4 (M199). The amount of cellular protein per dish did not vary, therefore, some of the data are expressed per dish. Viability of the cells was tested by trypan blue exclusion, LDH release, the amount of protein per dish, and light microscopic appearance. The experimental conditions had no effect on cell viability parameters.

Micelle preparation

Stock solutions of cholesterol and monoolein were prepared in chloroform. A stock solution of taurocholate was made in 95% ethanol. The lipids and bile salt were mixed and the solvents were evaporated under a stream of nitrogen. Medium-199 with HEPES was then added so that the final concentrations of the micelles when added to the medium covering the cells were: cholesterol (25–150 μ M), monoolein 0.03 mM, and taurocholate (0.5–5 mM). The micellar solution was warmed to 37°C and stirred vigorously before use. The amount and purity of the cholesterol in the clear micellar solutions were verified by gas-liquid chromatography.

HMG-CoA reductase mRNA quantitation

Total cellular RNA was extracted from CaCo-2 cells by the method described by Chomczynski and Sacchi (26) using guanidium thiocyanate. Northern blots were prepared using 1.1% agarose-formaldehyde gels as described by Maniatus, Fritsch, and Sambrook (27). RNA was transferred to membrane filters (Nytran, Midwest Scientific, Valley Park, MO) by capillary transfer and dried for 2 h at 80°C in a vacuum oven. The filters were pre-hybridized for 2 h at 42°C in 40 ml of buffer containing 20 ml of formamide, 2 ml of Denhardt's solution, 10 ml of 20 \times SSPE, 0.2 ml of 20% SDS, 0.4 ml of salmon sperm (10 mg/ml), and 7.4 ml of DEPC-water. cDNA probes were labeled with ³²P by random priming (28). Hybridi-

zation reactions were performed overnight with 0.1 μg ^{32}P -labeled cDNA (minimum of 5×10^7 cpm per filter) at 42°C in 6 ml of buffer containing 3 ml of formamide, 0.8 ml of Denhardt's solution, 1.5 ml of 20 \times SSPE, 0.03 ml of 20% SDS, 0.06 ml salmon sperm (10 mg/ml), and 1.2 ml of 50% dextran sulfate. After hybridization, the filters were washed twice for 15 min at room temperature in 6 \times SSPE and 0.1% SDS, once at 42°C for 30 min in 1 \times SSPE and 0.1% SDS, and once at 65°C for 30 min in 1 \times SSPE, 0.1% SDS. The filters were exposed to X-ray film with intensifying screens for 48–72 h. The probe was removed from the filters by washing the filter in 50% formamide/6 \times SSPE at 65°C for 30 min and rinsing it at 65°C in 2 \times SSPE for 15 min. The filter was then pre-hybridized and hybridized exactly as described above with the other cDNA probe. mRNA abundance of HMG-CoA reductase and actin were quantitated by laser-densitometry of the autoradiograms. The density of the band representing the mRNA of reductase was standardized to the density of the band for actin mRNA from the same filter.

Pulse-chase experiments

For these experiments, CaCo-2 cells were grown in 24-well plates and used five days after confluency. The amount of cellular protein per dish did not vary significantly and was approximately $500 \pm 25 \mu\text{g}$. Because of the smaller amount of protein per dish, toxicity was observed with 5 mM taurocholate. Therefore, in these experiments, sterols were solubilized in 1 mM of the bile salt. Cells were incubated for 1 h in 0.2 ml of methionine-free M199 to deplete the cells in methionine. To estimate the rate of synthesis of HMG-CoA reductase, cells were incubated for 30 min to 4 h in 0.2 ml of methionine-free medium containing 30 μCi of [^{35}S]methionine and the appropriate additions described for each experiment. At the end of each incubation, the cells were washed twice with 1 ml of M199 containing 500 μM of unlabeled methionine and 50 μM of unlabeled cysteine. [^{35}S]methionine-labeled HMG-CoA reductase was immunoprecipitated from the cells as described below.

To estimate the rate of degradation of HMG-CoA reductase, cells were grown in 24-well plates. At the start of the experiment, cells were incubated for 1 h in 0.2 ml of methionine-free M199. To steady-state label the intracellular proteins, cells were incubated for 4 h in methionine-free media containing 30 μCi of [^{35}S]methionine. The cells were then washed twice with M199 and incubated for 0, 1, 2, 4, 6, or 20 h in 1 ml of M199 containing 500 μM unlabeled methionine and 50 μM unlabeled cysteine and the appropriate additions that are described in the legends of each experiment. [^{35}S]methionine-labeled HMG-CoA reductase was immunoprecipitated from the cells as described below.

Immunoprecipitation of [^{35}S]methionine-labeled HMG-CoA reductase

After the incubations described above, the cells were solubilized in 0.2 ml of cell lysis buffer (10 mM Na phosphate, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin). The cells were sonicated for 15 sec at 4°C and centrifuged at 15,000 g for 10 min. Separate portions of the supernatant were removed for protein determination and estimation of incorporation of [^{35}S]methionine into total cellular proteins by precipitation with 10% trichloroacetic acid. A portion of the supernatant was taken and diluted to 0.5 ml with lysis buffer. The supernatant was precleared by incubation for 30 min with 50 μl of 10% (w/v) IgG sorb (The Enzyme Center, Maiden, MA). After recentrifugation, the supernatant was collected and 5 μg of anti-human HMG-CoA reductase polyclonal antibody was added and the solution was rocked for 1 h at 4°C. Fifty μl of 10% Protein-A agarose (sufficient to bind 0.2 mg of human IgG) was then added and the incubation continued for another hour. The antigen-antibody-Protein A agarose complex was sedimented by centrifugation for 1 min at 15,000 g . The pellet was washed five times with 1 ml of lysis buffer and the resulting pellet was resuspended in 50 μl of 1 \times Laemmli sample buffer containing 8 M urea (29). The proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) containing 7.5% polyacrylamide and 8 M urea. The stacking gel contained 4% acrylamide. After electrophoresis, the gels were treated with fixative solution (40% methanol, 7% acetic acid) for 30 min, washed three times with distilled water, and soaked for 30 min at room temperature in 1 M sodium salicylate. The gels were dried at 80°C for 2 h and the radioactive bands were detected by exposing the gels to Kodak X-Omat AR film for 48–72 h.

Immunoblot of HMG-CoA reductase

CaCo-2 cells were grown in 24-well plates. After the various treatments described in the text, cells were solubilized in 0.3 ml of lysis buffer and sonicated for 15 sec at 4°C. The homogenate was centrifuged at 15,000 g for 5 min to remove intact cells and cell debris. The proteins in the supernatant were separated by SDS-PAGE containing 7.5% polyacrylamide and 8 M urea in the separating gel and 4% in the stacking gel. The compositions of molecular mass standards were α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose 6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumerase (49 kDa), and lactic dehydrogenase (37 kDa). Proteins were transferred from the gels to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using a buffer containing

25 mM Tris-base, 192 mM glycine (pH 8.0), and 10% methanol (30). The membranes containing the transferred proteins were incubated for 30 min with blotting buffer (50 mM Tris-HCl, pH 8.0, 80 mM NaCl, and 2 mM CaCl₂) containing 5% non-fat dry milk and 0.2% Nonidet-40 (v/v). After blocking the reactive sites with 5% Blotto, the membrane was incubated for 2 h with 4 μg/ml of monoclonal antibody to HMG-CoA reductase (A9). The membranes were washed three times for 10–15 min each with 5% Blotto before adding biotin-SP-conjugated affinity pure F[ab']₂ fragment goat anti-mouse IgG and IgM (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 h. The membranes were again washed three times with 5% Blotto and incubated for 1 h with 1 μg/ml of peroxidase-conjugated streptavidin (Jackson Immunoresearch Laboratories, Inc.). Following this, the membranes were washed three times with 5% Blotto and once with phosphate-buffered saline. The antigen-monooclonal antibody to HMG-CoA reductase-biotinylated antibody-peroxidase-conjugated streptavidin complex was visualized by reacting the membrane with 60 mg 4-chloro-1-naphthol dissolved in 10 ml methanol, diluted in 100 ml of phosphate-buffered saline, pH 7.4, containing 0.02% hydrogen peroxide. The reaction product on the membrane was visualized under 254 nm UV light and photographed as described by Domingo and Marco (31).

Enzyme assays and other measurements

Activities were measured in total membrane preparations (32). 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity was measured as described (33). Specific activity of the substrate was 21,000 dpm/nmol. Cholesterol was determined by gas-liquid chromatography using cholestane as an internal standard (34). Protein was determined according to the method of Lowry et al. (35).

Statistical analysis

Student's unpaired *t* test was used to determine significance.

RESULTS

Effect of cholesterol flux on HMG-CoA reductase activity

To address the regulation of HMG-CoA reductase activity by cholesterol influx, CaCo-2 cells were incubated for 16 h in control medium (M199), medium containing increasing concentrations of cholesterol solubilized in 5 mM taurocholate and 30 μM monoolein, or medium containing micelles devoid of cholesterol. The results shown in Fig. 1A demonstrate that, in cells incubated with micelles containing cholesterol, HMG-CoA reductase activities were significantly decreased in a dose-dependent manner compared to activities observed in

cells incubated with micelles devoid of cholesterol. Above 150 μM of cholesterol, the micellar solution became cloudy; thus concentrations of cholesterol above 150 μM were not used.

To investigate the mechanisms for the regulation of HMG-CoA reductase activity by cholesterol influx, a more potent sterol regulator of cholesterol metabolism, 25-hydroxycholesterol, was solubilized in the micellar solution and incubated for 16 h with the cells (Fig. 1B). Compared to native cholesterol, the influx of the hydroxylated sterol resulted in a more profound inhibition of HMG-CoA reductase activity. Although the effects of

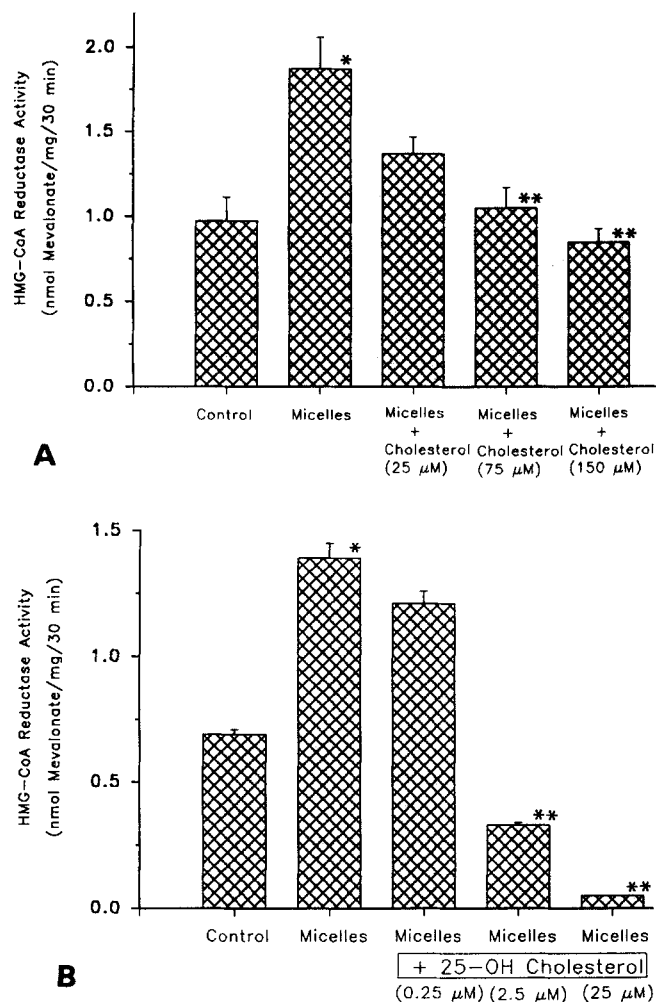


Fig. 1. Effect of micelles with or without cholesterol or 25-hydroxycholesterol on HMG-CoA reductase activity. CaCo-2 cells were incubated overnight (16 h) in medium alone (M199, control), medium containing 5 mM taurocholate and 30 μM monoolein (micelles), or micelles containing increasing concentrations of the appropriate sterol. After the incubations, total membranes were prepared and HMG-CoA reductase activities were determined; *n* = 3 dishes for each treatment group with the assays performed in triplicate. A Cholesterol, **P* < 0.05 vs. control; ***P* < 0.01 vs. micelles. B 25-Hydroxycholesterol, **P* < 0.05 vs. control; ***P* < 0.001 vs. control and micelles.

micellar sterols on reductase activity were observed by 6 h (36), enzyme analysis, RNA extractions, and immunoblotting for enzyme mass were more efficiently performed after an overnight incubation (16 h). As demonstrated in both Figs. 1A and 1B, the activity of HMG-CoA reductase was significantly increased in cells incubated with micelles devoid of cholesterol compared to the activity observed in cells incubated with control medium (M199). To explain this observation, it was postulated that reductase activity was increased to compensate for a loss of cellular cholesterol into the medium. To address this, CaCo-2 cells were incubated overnight with trace amounts of [³H]cholesterol to label the intracellular cholesterol pool. After washing the monolayer the next morning, cells were incubated for 6 h in control medium or medium containing 5 mM taurocholate and 30 μM monoolein. After the 6-h period, the amount of labeled cholesterol within cells and in the medium was determined. The results shown in the left panel of Fig. 2 demonstrate that significantly more labeled cholesterol was found in the medium containing the micelles compared to the amount of label found in control medium. The percentage of the intracellular labeled cholesterol that was found in the medium was 4.6% and 10.2%, for control and micellar incubations, respectively. Cholesterol mass was also measured in the medium after an identical experiment, and, as shown in the right panel of Fig. 2, the mass data paralleled the data obtained by labeled cholesterol. The combined results suggest that micelles cause an efflux of cholesterol from cells into the medium.

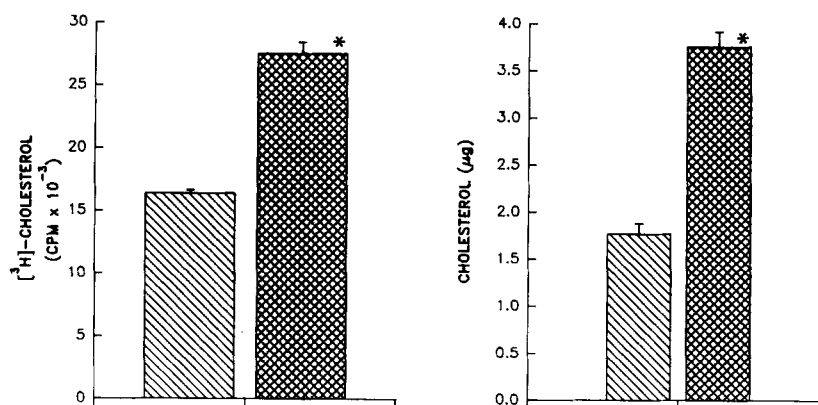


Fig. 2. Effect of micelles on cholesterol flux. CaCo-2 cells were incubated overnight in control medium (M199) containing trace amounts of [³H]cholesterol (1 mCi/ml). The following morning the cells were washed five times with 5 ml of M199. They were then incubated for 6 h in M199 or M199 containing 5 mM taurocholate and 30 μM monoolein. The medium was removed, centrifuged at 500 *g* to remove any debris or intact cells, and an aliquot was taken for counting. To measure the amount of cholesterol mass in the medium, the experiment was repeated but without the radiolabel. After centrifuging the medium, total lipids were extracted from the medium by chloroform-methanol-2:1 (v/v). Cholesterol was measured in the total lipid extract by gas-liquid chromatography (see Methods for reference). Left panel: radiolabel; right panel: mass; ▨: control; ▩: micelles; *n* = 6 dishes for each treatment; **P* < 0.01 vs. control.

Effect of cholesterol flux on HMG-CoA reductase protein

In data not shown, we found no evidence for a change in the phosphorylation state of reductase to explain the results observed in experiments described in Fig. 1. Therefore, to determine whether the regulation of HMG-CoA reductase activity by cholesterol flux was associated with changes in the amount of reductase protein, enzyme mass was estimated by immunoblotting. Fig. 3 shows these results. The band representing HMG-CoA reductase protein ran at a molecular weight of 94 kDa when compared to the separation of molecular weight standards run on the same gel. Compared to control cells incubated in M199 (A), HMG-CoA reductase protein was increased in cells incubated in medium containing micelles devoid of cholesterol (B). In cells incubated with micelles containing 150 μM of native cholesterol (C), reductase mass was not significantly altered and was similar to the amount of enzyme observed in cells incubated in medium containing micelles alone (B). In contrast, HMG-CoA reductase protein was significantly less in cells incubated in medium containing micelles and 25-hydroxycholesterol (D) compared to the amount observed in cells from the other treatment groups. With the exception of cells incubated with micelles containing native cholesterol, in which reductase activity was only modestly decreased, alterations in HMG-CoA reductase activity by cholesterol flux were reflected by parallel changes in reductase mass in CaCo-2 cells.

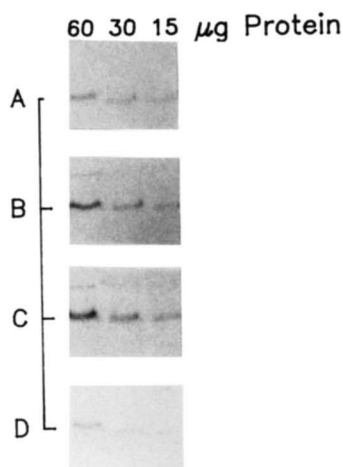


Fig. 3. Effect of micelles with or without cholesterol or 25-hydroxycholesterol on HMG-CoA reductase mass. CaCo-2 cells were incubated overnight in: A) control medium (M199); B) medium containing micelles; C) medium containing micelles and 150 μM cholesterol; or D) medium containing micelles and 2.5 μM 25-hydroxycholesterol. Cells were harvested, solubilized in lysis buffer, and sonicated. Cellular proteins (60, 30, and 15 μg) were separated by PAGE and transferred to Immobilon-P PVDF membranes for immunoblotting as described in Methods. The protein band representing HMG-CoA reductase migrated at a molecular weight of 94 kDa.

Effect of cholesterol flux on HMG-CoA reductase mRNA abundance

To address potential mechanisms for the regulation of reductase activity and mass by cholesterol flux, steady-state levels of mRNA of HMG-CoA reductase were estimated by Northern hybridization. α -Actin mRNA abundance was used to correct for any differences in transfer or loading of total RNA per sample. The results are shown in **Fig. 4**. HMG-CoA reductase mRNA approximated 4.3 kb (top blots) and α -actin mRNA 1.9 kb (bottom blots) in size. The amount of steady-state levels of HMG-CoA reductase mRNA was 2.5-fold greater in cells incubated in medium containing micelles devoid of sterol (lanes 3 and 4) compared to the amount of mRNA observed in control cells incubated in M199 alone (lanes 1 and 2). In cells incubated in medium containing micelles and 2.5 μM of 25-hydroxycholesterol (lanes 5 and 6), HMG-CoA reductase mRNA levels were not significantly altered when compared to levels found in cells incubated in medium containing micelles alone. mRNA abundance was decreased by 50%, however, in CaCo-2 cells incubated with micelles containing 25 μM of the polar sterol (lanes 7 and 8). **Table 1** summarizes the data comparing the regulation of HMG-CoA reductase activity, enzyme mass, and mRNA abundance by sterol flux. It is evident from these data that cholesterol efflux from CaCo-2 cells incubated in medium containing micelles alone was associated with increases in reductase activity, protein, and mRNA abundance. In contrast, with the lower

concentration of the hydroxylated sterol, which caused a significant decrease in reductase activity and enzyme mass, no changes were observed in mRNA abundance.

Effect of cholesterol flux on HMG-CoA reductase synthesis and degradation

The decrease in HMG-CoA reductase activity and enzyme mass resulting from an influx of sterol in CaCo-2 cells cannot be completely explained at the level of gene expression. Therefore, to address other potential mechanisms for this regulation, the effect of micelles with or without sterol on the rates of synthesis and degradation of HMG-CoA reductase was investigated with pulse-chase experiments. **Figs. 5 and 6** show these data. To estimate degradation rates of HMG-CoA reductase, cellular proteins were labeled to steady-state by 4 h of incubation with [^{35}S]methionine. The cells were then incubated with medium alone (A), medium containing micelles (B), or medium containing micelles and 25-hydroxycholesterol (C) in the presence of excess methionine. At the times indicated in **Fig. 5**, the cells were harvested, reductase was immunoprecipitated, and the immune complex was subjected to polyacrylamide gel electrophoresis. The band observed at 94 kDa on the autoradiogram represents radiolabeled HMG-CoA reductase. Under conditions of cholesterol efflux, i.e., in those cells incubated in medium containing micelles alone, the rate of degradation of reductase was markedly retarded (B and ∇). The $t_{1/2}$ for reductase in control cells was approximately 2.1 h (A and \bullet), whereas the $t_{1/2}$ for reductase in cells incubated with micelles was twice that, approximately 4.6 h. In contrast, the rate of degradation of HMG-CoA reductase was significantly increased in cells incubated in medium contain-

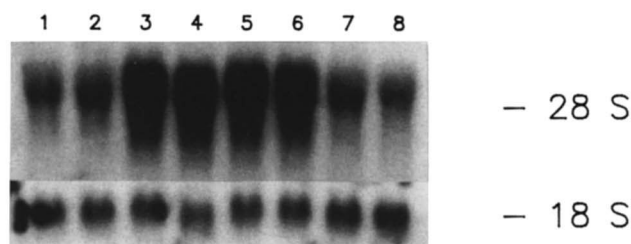


Fig. 4. Effect of micelles with or without 25-hydroxycholesterol on HMG-CoA reductase mRNA abundance. CaCo-2 cells were incubated overnight in control medium (lanes 1 and 2), medium containing micelles (lanes 3 and 4), or medium containing micelles and 2.5 (lanes 5 and 6) and 25 μM (lanes 7 and 8) of 25-hydroxycholesterol. Total RNA was then extracted from the cells and Northern blots were prepared by using 1.1% agarose-formaldehyde gels and applying 10 μg of total RNA per lane. After transfer to Nytran filters, the filters were probed first with labeled cDNA for reductase and then α -actin as described in Methods. mRNA abundance was quantitated by laser-densitometry of autoradiograms and standardized to the density of the actin band. Sizes of the mRNAs for reductase and actin were approximately 4.3 kb and 1.9 kb, respectively.

TABLE 1. Effect of cholesterol flux on HMG-CoA reductase activity, protein, and mRNA abundance

	HMG-CoA Reductase		
	Activity	Protein	mRNA
Control	1.0 ^a	1.0	1.0
Micelles ^b	2.0	3.0	2.5
Micelles + cholesterol (150 μ M)	0.85	3.4	nd ^c
Micelles			
+ 25-hydroxycholesterol (2.5 μ M)	0.49	0.50	2.7
+ 25-hydroxycholesterol (25 μ M)	0.12	nd ^c	1.3

^aArbitrary units.

^bOne or 5 mM taurocholate and 30 μ M monoolein.

^cNot determined.

ing micelles and the sterol ($t_{1/2} = 1.4$ h, C and \blacktriangledown) compared to the rate of degradation observed in cells incubated in medium containing micelles alone. The influx of sterol, however, had a modest effect on the degradation of HMG-CoA reductase when compared to the degradation rate observed in control cells (cells which were not losing cholesterol into the medium). The $t_{1/2}$ for reductase of control cells and cells incubated with micelles containing sterol were not that dissimilar.

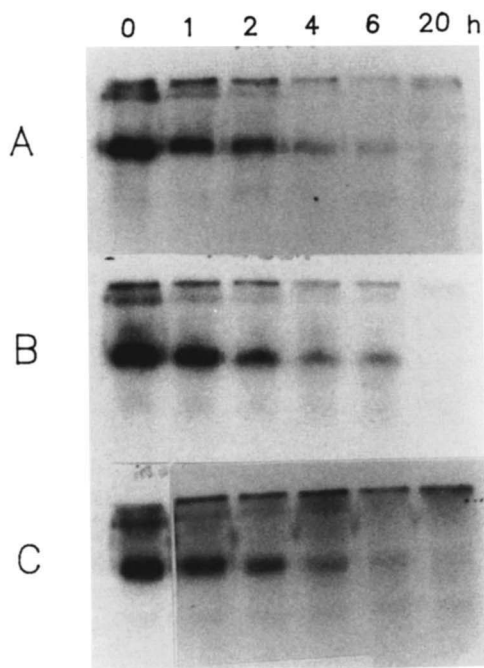
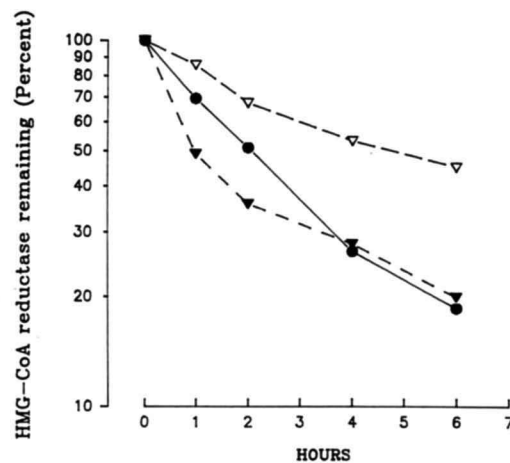


Fig. 5. Effect of micelles with or without 25-hydroxycholesterol on the turnover of ³⁵S-labeled HMG-CoA reductase. CaCo-2 cells were incubated for 1 h in methionine-free medium. They were then incubated for 4 h in methionine-free medium containing 30 μ Ci of [³⁵S]methionine. After washing the cells, medium containing 500 μ M of unlabeled methionine and 50 μ M of unlabeled cysteine and micelles or micelles and 2.5 μ M 25-hydroxycholesterol was added. At the appropriate time, the cells were solubilized in lysis buffer, sonicated, and HMG-CoA reductase was immunoprecipitated with 5 μ g of anti-human HMG-CoA reductase polyclonal antibody as described in Methods. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis. The gels were dried and radioactive bands were detected by exposing the gels to Kodak X-Omat AR film. The left panel shows the labeled reductase band at 0, 1, 2, 4, 6, and 20 h. A) control; B) micelles; and C) micelles and 2.5 μ M of 25-hydroxycholesterol. The right panel demonstrates the turnover of labeled reductase protein over 6 h; (●) control; (∇) micelles; (\blacktriangledown) micelles and 25-hydroxycholesterol.

Fig. 6 shows the results of the effect of sterol flux on the synthesis of HMG-CoA reductase. In this experiment, CaCo-2 cells were incubated for 4 h in control medium (A), medium containing micelles (B), or medium containing micelles and 25-hydroxycholesterol (C). The addition of [³⁵S]methionine was staggered so that all cells were harvested at the same time. The figure shows the resulting autoradiogram of the reductase band at 94 kDa after immunoprecipitation and polyacrylamide gel electrophoresis. In cells incubated with micelles containing the sterol, there was a profound decrease in the rate of incorporation of [³⁵S]methionine into immunoprecipitable HMG-CoA reductase compared to the rates observed in control cells and cells incubated in medium which contained micelles alone. Moreover, based on our observations in three separate experiments, the rate of incorporation of [³⁵S]methionine into immunoprecipitable reductase was 12% less in cells incubated with taurocholate micelles alone (B) compared to control cells (A).

DISCUSSION

The present study extends our information on the regulation of cholesterol metabolism by cholesterol flux in the



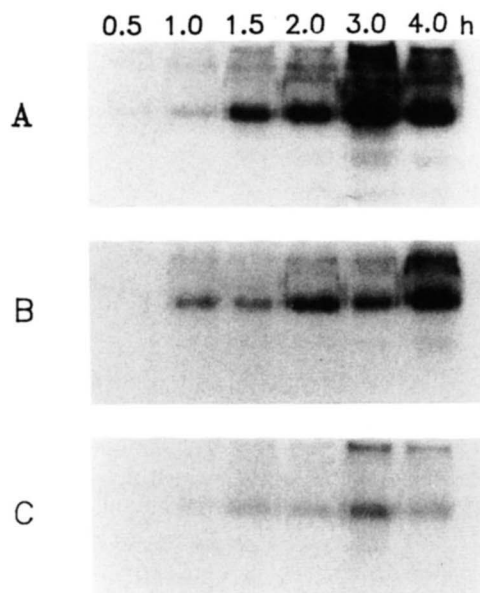


Fig. 6. Effect of micelles with or without 25-hydroxycholesterol on [^{35}S]methionine incorporation into HMG-CoA reductase. CaCo-2 cells were incubated for 1 h in methionine-free medium. After this the medium was changed to methionine-free medium containing micelles alone or micelles and $2.5\ \mu\text{M}$ 25-hydroxycholesterol. The incubation was continued for 4 h. The addition of [^{35}S]methionine was staggered so that all cells were harvested at 4 h. HMG-CoA reductase was immunoprecipitated and subjected to polyacrylamide gel electrophoresis as described in Fig. 5 and Methods. The figure depicts the rate of incorporation of label into HMG-CoA reductase. A) control; B) micelles; and C) micelles and $2.5\ \mu\text{M}$ 25-hydroxycholesterol.

intestine and provides new information on the mechanisms for the regulation of intestinal HMG-CoA reductase at the molecular level. We believe that under conditions of cellular cholesterol efflux, HMG-CoA reductase activity and protein are increased because of 1) an increase in mRNA abundance for reductase, and 2) a pronounced decrease in the rate of degradation of the enzyme. In contrast, after acute cholesterol influx into the intestinal cell, HMG-CoA reductase activity and protein were decreased secondary to 1) a marked decrease in the translational efficiency of its mRNA, and 2) a modest increase in the rate of degradation of reductase protein. At very high concentrations of sterol influx, which is not likely to occur *in vivo*, transcriptional regulation of HMG-CoA reductase must be included as a potential mechanism for regulating reductase activity.

In CaCo-2 cells incubated in medium containing micelles and native cholesterol, HMG-CoA reductase activity was modestly inhibited (approximately 60%) compared to reductase activity in cells incubated in medium with micelles alone. Compared to reductase activity in cells incubated in control medium (M199), however, influx of native cholesterol at its highest concentration decreased reductase activity by only 15%. This rather unimpressive effect of luminal cholesterol on human in-

testinal HMG-CoA reductase activity parallels results of earlier studies done under similar experimental conditions in which HMG-CoA reductase activities were decreased by 15% and 23% in human and canine intestinal explants incubated with cholesterol, respectively (19, 21). The effect of an influx of micellar cholesterol on HMG-CoA reductase activity in rabbit intestinal explants, however, was more pronounced. Stange et al. (20) observed an 85% decrease in reductase activity in rabbit explants incubated with 0.25 mM cholesterol solubilized in glycodeoxycholate micelles. This wide variability observed in the regulation of intestinal HMG-CoA reductase activity among species is not unexpected as the regulation of cholesterol metabolism in the gut is recognized to be species-dependent (11). We believe this is related to differences in intestinal cholesterol absorption and basal rates of cholesterol synthesis which occur among species. The present data, which are supported by earlier data of Gebhard, Stone, and Prigge (19), strongly suggest that in human intestine, the influx of luminal cholesterol does result in a decrease in cholesterol synthesis by the inhibition of HMG-CoA reductase activity. Since the influx of native cholesterol did not appear to alter the amount of reductase protein in CaCo-2 cells, the modest decrease in enzyme activity may be related to conformational changes of the enzyme secondary to lipid changes of the membrane.

It has been demonstrated in other cells that hydroxylated sterols are significantly more potent in regulating the activity of HMG-CoA reductase than is native cholesterol itself (37, 38). The human intestinal cell is no exception. 25-Hydroxycholesterol, in concentrations that were 60-fold less than native cholesterol, significantly inhibited reductase activity beyond that observed for the highest concentration of the native sterol. Because cholesterol and 25-hydroxycholesterol regulate HMG-CoA reductase activity through a common mechanism (39), the hydroxylated sterol was used as an investigative tool to exaggerate the regulation of HMG-CoA reductase activity so that specific mechanisms of regulation could be better addressed and defined. It is possible, however, that the true physiological regulator of intracellular cholesterol metabolism is an oxygenated sterol. In liver and untreated cell cultures, oxygenated sterols have been identified in concentrations that are sufficient to regulate HMG-CoA reductase activity (40, 41). The recent cloning and expression of an oxysterol-binding protein from rabbit liver suggest that this protein may play a role in the regulation of cholesterol metabolism by these modified sterols (42).

In previous reports, it has been shown that sterols can exert their regulation of HMG-CoA reductase activity by suppression of gene transcription (4, 43, 44). This mode of regulation is dependent upon a short regulatory sequence present in the 5'-flanking region of the gene which has been termed the sterol regulatory element (4). The results of the present study, however, do not support a sig-

nificant role of gene transcription for the short-term regulation of reductase activity by cholesterol influx in CaCo-2 cells. The influx of 2.5 μM 25-hydroxycholesterol, despite causing a 75% inhibition of HMG-CoA reductase activity and reducing the amount of reductase protein by 83%, did not significantly alter mRNA abundance of the enzyme. Other mechanisms must be implicated. One such mechanism is the regulation of degradation of reductase protein by sterol flux. It has been demonstrated previously that sterols will accelerate the degradation rate of reductase (6, 7). After the transfection of a full length cDNA for hamster HMG-CoA reductase into UT-2 cells, a mutant line of Chinese hamster ovary cells which lack HMG-CoA reductase, the rate of degradation of reductase protein was shown to be enhanced 2-fold by exposure of the cells to sterols (7). In other studies, 25-hydroxycholesterol has been shown to increase the rate of degradation of reductase in UT-1 and avian myeloblasts (6, 45). The sterol precursor, mevalonolactone, has also been shown to cause similar effects on reductase degradation in rat hepatocytes (5). The present data would also support a role for enzyme degradation as a mechanism for regulating the activity of HMG-CoA reductase by cholesterol influx in human intestinal cells. Pulse-chase experiments demonstrated a modest enhancement of the degradation rate of reductase protein by an influx of sterol.

In addition to an enhanced degradation rate of HMG-CoA reductase as was observed with sterol influx, sterol influx also markedly diminished the translational efficiency of reductase mRNA. Short-term pulse experiments with [^{35}S]methionine demonstrated a decrease in the incorporation of label into immunoprecipitable reductase by 25-hydroxycholesterol. Although it has been stated that sterols likely exert their effects on HMG-CoA reductase activity primarily through changes in transcription and degradation rates of the enzyme (46), Sinensky and Torget (10) and Faust et al. (6) have observed marked inhibition of HMG-CoA reductase synthesis by 25-hydroxycholesterol in CHO and UT-1 cells, respectively. Our data would also suggest that one major mechanism for regulating HMG-CoA reductase activity by sterol influx in CaCo-2 cells is the suppression of translation of reductase mRNA. The intestinal cell must respond rapidly to the ingestion of dietary sterols or the influx of biliary cholesterol into the lumen. Perhaps a direct effect of luminal sterols on translation of reductase message is the most rapid and efficient mechanism for inhibiting cholesterol synthesis in the gut.

In support of an earlier report by Stange et al. (20) in which an increase in HMG-CoA reductase activity was demonstrated in rabbit intestinal explants incubated with bile salt micelles, we also found an increase in reductase activity in CaCo-2 cells incubated with taurocholate micelles. After documenting cholesterol efflux from cells incubated in medium containing micelles, an explanation

as to why HMG-CoA reductase activity increased under these conditions became clear. In response to the loss of the sterol, cells were compensating by increasing their rate of cholesterol synthesis. It is likely that micelles were serving as cholesterol acceptors much like high density lipoproteins function as cholesterol acceptors in plasma or under similar cell culture conditions (47). In parallel with an increase in HMG-CoA reductase activity, reductase protein and mRNA abundance were also increased. In addition, the results of pulse-chase experiments demonstrated that in CaCo-2 cells incubated in medium containing micelles alone, the rate of turnover of HMG-CoA reductase was delayed without increasing the rate of enzyme synthesis. Although we cannot completely include or exclude a role for transcription of the reductase gene as a mode of regulation of reductase activity by cholesterol efflux, we believe it is the pronounced delay in enzyme degradation that most likely explains an increase in HMG-CoA reductase activity and protein occurring in CaCo-2 cells during cholesterol efflux.

Finally, this study was performed in the human intestinal cell line CaCo-2, a cell line that shares many similarities to the human intestinal absorptive cell (48). Results obtained from previous studies using this cell have provided new and important information on several aspects of intestinal lipid, lipoprotein, and apolipoprotein metabolism (33, 49–51). Although this cell line is considered by many investigators to be a valid model for the in vitro study of intestinal epithelial function (52–54), extrapolating results obtained from this cell to the mature human enterocyte should be done with caution, as differences do exist (50). ■

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