Effects of cyclosporin on cholesterol 27-hydroxylation and LDL receptor activity in HepG2 cells

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Abstract The hypothesis that mitochondrial sterol 27-hydroxylase plays a role in the sterol-mediated down-regulation of LDL receptor activity was evaluated in HepG2 cells. 27-Hydroxycholesterol was found to be more potent at suppressing LDL receptor activity than cholesterol (IC_{50} values of 8 μM and 142 μM for 27-hydroxycholesterol and cholesterol, respectively) when the sterols were delivered to cells from 2-hydroxypropyl-β-cyclodextrin (β-CD)-solubilized solutions. Cyclosporin, an immunosuppressant which has been shown to inhibit the 27-hydroxylation of sterols, was used to assess whether the formation of endogenous 27-hydroxycholesterol was required for the cholesterol-induced suppression of LDL receptor activity. Cyclosporin dose-dependently inhibited the 27- hydroxylation of cholesterol by HepG2 mitochondria (K_i = 0.25 μM) and HepG2 cell cultures (IC_{50} = 1 μM). At 1 μM, cyclosporin had no effect on LDL receptor activity, and did not prevent the suppression of LDL receptor activity caused by: *1)* the addition of β-CD-solubilized cholesterol, *2)* the receptor-mediated uptake of β-VLDL, or *3)* the inhibition of cholesterol esterification. In contrast, 10 μM cyclosporin was found to inhibit the esterification of cholesterol and to increase the cellular level of free cholesterol resulting in suppression of LDL receptor activity. These results suggest that if mitochondrial sterol 27-hydroxylase plays a role in the regulation of LDL receptor activity, it is not through the formation of potent regulatory oxysterols, but through its effects on the availability and/or size of the free cholesterol pool regulating LDL receptor activity.—**Winegar, D. A., J. A. Salisbury, S. S. Sundseth, and R. L. Hawke.** Effects of cyclosporin on cholesterol 27-hydroxylation and LDL receptor activity in HepG2 cells. *J. Lipid Res.* 1996. **37:** 179–191.

Supplementary key words 27-hydroxycholesterol • ACAT • 2-hydroxypropyl-β-cyclodextrin

Mammalian cells obtain the cholesterol they need for maintenance of membrane structure and regulation of cellular metabolic functions either by endogenous synthesis in which the enzyme HMG-CoA-reductase (HMGR) is rate-limiting, or by receptor-mediated uptake of low density lipoproteins (LDL) (1, 2). The proportion of cholesterol derived from each pathway may vary depending on the cell type. The liver, for example, expresses a much larger number of LDL receptors than do most other tissues in order to fulfill its additional cholesterol requirement for synthesis of bile acids and lipoproteins (3, 4). Studies in a variety of cultured cells have shown that both HMGR and the LDL receptor are subject to end-product feedback regulation such that increasing cellular sterol levels upon incubation in the presence of lipoproteins, nonlipoprotein cholesterol, or oxygenated sterols results in inhibition of HMGR activity and a decrease in the number of cell surface LDL receptors (1, 5, 6). The sterol-mediated decline in LDL receptor number is thought to be due entirely to a decrease in transcription of the LDL receptor gene (3, 6). The decline in HMGR activity, on the other hand, can be attributed to both a reduction in HMGR gene transcription and to an increase in the post-transcriptional degradation of the enzyme (1, 5, 6).

The exact nature of the physiologic regulator of HMGR and the LDL receptor is currently not known; however, a role for oxygenated derivatives of cholesterol has been strongly indicated. A number of oxysterols arising from enzymatic hydroxylation or spontaneous oxidation of cholesterol have been investigated for their effectiveness at inhibiting sterol synthesis, HMGR activity, and LDL metabolism in cultured cells (6–13). Most, if not all, exhibit greater potencies than purified nonlipoprotein cholesterol. It has been argued that this difference in potency is due to the more polar oxysterols gaining greater entry into cells so as to achieve higher

Abbreviations: β-CD, 2-hydroxypropyl-β-cyclodextrin; LPDS, lipoprotein-deficient serum; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ACAT, acyl-CoA:cholesterol acyltransferase; DDM, N,N-dibenzyl-N′-(2,6-diisopropylphenyl)malonamide.

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concentrations within the regulatory pool of sterols. There is evidence, however, to suggest that the mediator of LDL receptor expression may be a monooxygenated form of cholesterol generated in a cytochrome P450-catalyzed reaction (14–16). Furthermore, a cytosolic oxysterol binding protein has been identified that displays a relative affinity for oxysterols that correlates with relative potency in suppressing HMGR (17, 18).

Several recent reports have investigated the possible role of 27-hydroxycholesterol in the regulation of cellular cholesterol homeostasis (8, 9, 12, 13, 19–21). 27-Hydroxycholesterol is a normal component of human serum that is formed from cholesterol by a mitochondrial cytochrome P450 enzyme (sterol 27-hydroxylase) that is expressed in most tissues, including the vascular endothelium (19, 22–25). In vitro studies have shown that the addition of 27-hydroxycholesterol to the media of hepatic and nonhepatic cells in culture results in potent suppression of sterol synthesis, HMGR activity, and LDL metabolism (9–13, 18–20). In the liver, 27-hydroxycholesterol may be metabolized to bile acids through a proposed alternative "acid pathway" that leads predominately to the formation of chenodeoxycholic acid (19, 26, 27). Individuals lacking the ability to synthesize 27-hydroxylated sterols due to single point mutations in the gene encoding sterol 27-hydroxylase suffer from the rare sterol storage disorder known as cerebrotendinous xanthomatosis (CTX) (28, 29). In CTX subjects, normal regulation of sterol homeostasis is generally absent as there is decreased bile acid synthesis accompanied by increased cholestanol synthesis and LDL receptor activity (30, 31). This condition is thought to contribute to the abnormal tissue deposition of cholesterol and accelerated atherosclerosis that are characteristic of the disease.

While these findings are consistent with an important biological role for 27-hydroxycholesterol, many questions remain unanswered as to its relative importance in maintaining normal cell function. The present work further explores the possibility that 27-hydroxycholesterol may be responsible for the sterol-mediated down-regulation of LDL uptake. This study focused on the regulation in hepatic tissues as the liver is a major site of receptor-dependent LDL uptake and degradation as well as the site where cholesterol secretion, esterification, and metabolism to bile acids occurs. The hepatoma cell line HepG2 was used as a model for human hepatocytes. HepG2 cells express most liver-specific functions relating to cholesterol and triglyceride metabolism, including high affinity, regulatable LDL receptor activity (reviewed in ref. 32). Here, we have prepared aqueous solutions of cholesterol and 27-hydroxycholesterol solubilized in 2-hydroxypropyl-β-cyclodextrin (β-CD) for the purpose of examining their effects on LDL receptor regulation. We have also made use of a recently identified inhibitor of the sterol 27-hydroxylase pathway, cyclosporin (33–35), to assess whether sterol 27-hydroxylation is critical to the suppression of LDL receptor activity caused by lipoprotein and non-lipoprotein (i.e., β-CD-solubilized) cholesterol.

MATERIALS AND METHODS

Materials

The HepG2 cell line was obtained from the American Type Culture Collection (ATCC #HB8065) (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM; high glucose with L-glutamine and 25 mM HEPES buffer), trypsin/EDTA, penicillin G, and streptomycin were from GIBCO-BRL (Gaithersburg, MD). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium was from JRH Biosciences (Lenexa, KS). Human low density lipoproteins (LDL) and ^{125}I-labeled LDL (217–643 cpm/ng) were prepared by Organon Teknika Biotechnology Research Institute (Rockville, MD). Rabbit very low density lipoprotein (β-VLDL) was prepared by Biomedical Technologies Inc. (Stoughton, MA). 2-Hydroxypropyl-β-cyclodextrin (β-CD) was purchased as a sterile 45% (w/v) (286 mM) solution from Pharmatec, Inc. (Alachua, FL). 27-Hydroxycholesterol was obtained from Research Plus Inc. (Bayonne, NJ). The cyclosporin stock was a 50 mg/ml solution in Cremophor EL (Sandimmune, Sandoz) diluted to the indicated concentrations with DMSO. The acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor N,N-dibenzyl-N′-(2,6-diisopropylphenyl)-malonamide (DDM) was generously provided by Dr. James Chapman, University of South Carolina. The bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co (Rockford, IL). Organic solvents for high performance liquid chromatographic (HPLC) analysis of cholesterol, cholesteryl ester, and 27-hydroxycholesterol were purchased from EM Science (Gibbstown, NJ). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of β-CD-solubilized [^{14}C]cholesterol and [^{14}C]27-hydroxycholesterol

Oxidation products were removed from [4-^{14}C]cholesterol (52 mCi/mmol, Amersham) by dissolving 250 μCi in 8 ml hexane, loading onto a silica Sep-Pak cartridge (Millipore), and eluting the cholesterol with 8 ml 2% 2-propanol in hexane. After drying under N_2, the cholesterol was dissolved in the 286 mM β-CD stock solution to ~0.7 μCi/μl by stirring overnight in an amber vial at room temperature. The final radiolabeled choles-

terol/β-CD solution was stored at 4°C until use. [4-^{14}C]27-hydroxycholesterol was synthesized enzymatically from radiolabeled cholesterol. Briefly, [^{14}C]cholesterol/β-CD (8 μCi) was incubated with 5 mg female rat liver mitochondria in a total volume of 2 ml buffer containing 100 mM Tris-HCl (pH 7.7), 1 unit isocitrate dehydrogenase, and 6.425 mg/ml isocitrate. Incubations were carried out at 37°C for 8 h. The reaction was stopped by the addition of 2 ml ethanol, and the resulting products were extracted with 10 ml hexane–ethyl acetate 1:1 containing 0.005% butylated hydroxytoluene. Extracts were resuspended in hexane and applied to a silica Sep-Pak cartridge as described for cholesterol purification, except that following elution of the cholesterol fraction with 2% 2-propanol in hexane, 27-hydroxycholesterol was (~0.8 μCi) eluted from the cartridge with an additional 8 ml of 20% 2-propanol in hexane. The final [^{14}C]27-hydroxycholesterol preparation, judged to be 94% pure by HPLC, was resuspended in the 286 mM β-CD stock solution to 0.052 μCi/μl (1 mM).

Culturing of HepG2 cells

HepG2 cell stocks were maintained in 75-cm^2 flasks containing DMEM high glucose supplemented with 25 mM HEPES buffer, 10% (w/v) fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded at 5 × 10^4 cells/well into 22.6-mm multiwell dishes and grown for 3–4 days to ~90% confluency, at which time the media were changed to DMEM containing 10% lipoprotein-deficient serum (LPDS). After 24 h, the media were replaced with fresh DMEM/LPDS containing the appropriate compounds dissolved in DMSO and/or sterols dissolved in β-CD.

Lipoprotein binding studies

The uptake (binding and internalization) of ^{125}I-labeled LDL was evaluated as described by Goldstein et al. (36). Briefly, after a 24-h incubation in DMEM/LPDS with the indicated concentrations of compounds and/or sterols, the media were removed and the cells were washed two times, 5 min each, with PBS. The experiment was started when fresh DMEM/LPDS containing ^{125}I-labeled LDL (10 μg/ml) was added to each well in the absence or presence of 300 μg/ml of unlabeled LDL. Unless otherwise indicated, compounds and/or sterols were not present during this incubation. After 3 h at 37°C, the multiwell dishes were cooled on ice, the media were removed, and the cells were washed three times rapidly with cold wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 2 mg/ml BSA. This was followed by two 10-min washes with the same buffer and one rapid wash with cold wash buffer without BSA. The cells were dissolved in 0.1 N NaOH and cell-associated radioactivity was determined. An aliquot was removed to determine cellular protein content. Specific LDL uptake was calculated by subtracting the cell-associated radioactivity measured in the presence of unlabeled LDL from that measured in its absence.

Cholesterol 27-hydroxylase assay

HepG2 mitochondria were isolated from T-75 flasks of cells grown in DMEM in the confluent state for 2 weeks. Each of the flasks was scraped into 3.5 ml cold saline and cells were pelleted at 800 *g*. Cell pellets were resuspended in 1.5 ml homogenization buffer (200 mM sucrose/100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA and DTT) and sonicated for 40 sec (Ultrasonic Processor with microtip attachment, Heat Systems Inc., power setting = 2.5, 50% duty cycle). Sonicated cells were centrifuged for 15 min at 500 *g*, the supernatant was respun for 20 min at 10,000 *g*, and the isolated mitochondrial pellets from two flasks were combined, resuspended in 1.5 ml of homogenization buffer, and respun at 10,000 *g* as before. The final washed mitochondrial pellets were resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.7) and assayed immediately for cholesterol 27-hydroxylase activity. All mitochondrial isolation steps were performed at 4°C with an average yield of 1 mg mitochondrial protein/T-75 flask of HepG2 cells.

Mitochondrial sterol 27-hydroxylase was measured in a total volume of 530 μl containing ~100 μg mitochondrial protein, 1 unit isocitrate dehydrogenase, 5 mM isocitrate, 10–160 μM [^{14}C]cholesterol solubilized in 5 μl of a 45% aqueous solution of β-CD, and 50 mM Tris-Cl buffer at pH 7.7. Reactions were initiated with isocitrate, incubated with shaking at 37°C for 20 min, and stopped by the addition of 1 ml ethanol. Reaction volumes were brought to 2 ml with saline and radiolabeled products were extracted with 5 ml hexane–ethyl acetate 1:1 containing 0.005% BHT. Dried extracts were resuspended in 250 μl 2% 2-propanol in hexane and reaction products were separated by normal phase HPLC as described below. In cyclosporin inhibition experiments, cyclosporin (final concentration = 0.250–1 μM) was added to mitochondrial incubations in 5 μl DMSO.

Analysis of cholesterol, cholesteryl ester and 27-hydroxycholesterol

After incubation of cells with [^{14}C]cholesterol or [^{14}C]27-hydroxycholesterol and removal of the media for analysis, cells were washed twice with PBS and then scraped into 1 ml 0.9% saline. Cells were sonicated for 30 sec and an aliquot was removed for protein determination. After the addition of 1 ml 95% ethanol to cell lysates or media samples, sterols were extracted with 5 ml hexane–ethyl acetate 1:1 containing 0.005% BHT. [^{3}H]cholesteryl oleate (~10,000 dpm) was added to all

samples as an internal standard. The recovery of internal standard was routinely 90–98%. Dried extracts of the sterol fraction were resuspended in 1% methyl *t*-butyl ether (MTBE) in hexane and reaction products were separated according to the procedure of Pedersen, Björkhem, and Gustafsson (37) using a normal phase HPLC system (Waters mPorasil silica column, mobile phase: 1% acetic acid–2.5% 2-propanol–96.5 hexane; flow rate = 1.25 ml/min) equipped with a Radiomatic FLO-ONE\Beta radiochromatographic detector (FLO-SCINT V at 1 ml/min) to quantitate cholesterol (retention time (RT) = 15 min), cholesteryl ester (RT = 4 min), and 27-hydroxycholesterol (RT = 23 min). When desired, half of each sterol extract was saponified as follows. Dried extracts were resuspended in 1 ml 95% ethanol and 100 μl 33% KOH and heated overnight at 60°C. After the addition of 1 ml 0.9% saline, hydrolyzed products were extracted with 5 ml hexane and separated by HPLC as described above.

Other methods

Lipoprotein-deficient serum (LPDS) was prepared according to the method of Fabricant and Broitman (38). The incorporation of [^{14}C]acetate (1 μCi/well, 1 mM final concentration) into cellular cholesterol was measured over a 6-h period as previously described (39). [^{14}C]cholesterol was separated from other radiolabeled products by normal phase HPLC under the same conditions described above. Protein concentrations were determined by the method of Smith et al. (40) using the Pierce BCA Protein Assay Reagents with bovine serum albumin as a standard. Enzyme kinetic data was analyzed with an in-house computer program (Enzyme Kinetic Analysis Version 2.3).

RESULTS

Effects of β-CD-solubilized cholesterol and 27-hydroxycholesterol on LDL uptake

A generally recognized problem encountered when working with sterols in cell culture systems is that sterols added by means of organic solvents to cells grown in culture may precipitate upon dilution in the aqueous media. Recently, several groups have demonstrated that β-CD can be used as a vehicle to deliver sterols into cells by the formation of aqueous-soluble inclusion complexes (35, 41, 42). Most of these reports involved the addition of β-CD-solubilized sterols to in vitro enzyme assay systems. In the current work, β-CD was used to introduce cholesterol or 27-hydroxycholesterol into intact HepG2 cells. The receptor-mediated uptake of ^{125}I-labeled LDL was then measured in these sterol-loaded cells.

In initial experiments, it was learned that β-CD alone (i.e., uncomplexed with sterols) stimulated LDL uptake under a variety of culture conditions (**Table 1**). When cells were cultured for 24 h in the presence of 5 μl/well of the 286 mM β-CD stock solution (2.86 mM final concentration) without a media change prior to a 3-h labeling period with [^{125}I]-labeled LDL, uptake was increased 2.2-fold above control (Table 1: condition B). This increase was found to be due in part to a direct stimulation of LDL uptake by β-CD, as the addition of this vehicle to the media only during the 3-h labeling period with ^{125}I-labeled LDL produced a small, but significant rise in LDL receptor activity (Table 1: condition C). When cells cultured for 24 h in the presence of β-CD were washed prior to the addition of ^{125}I-labeled LDL, the level of LDL uptake remained significantly elevated above control (Table 1: condition D). In order to minimize potential β-CD-dependent effects on LDL uptake, cells treated with β-CD-solubilized sterols were washed free of the vehicle prior to the addition of ^{125}I-labeled LDL (Table 1: condition D).

To determine the effects of increasing concentrations of cholesterol and 27-hydroxycholesterol on LDL uptake by HepG2 cells, the sterols were diluted into the culture medium from either 15 mM or 25 mM stock solutions of β-CD-solubilized cholesterol or 27-hydroxycholesterol, respectively. These concentrations represent the maximum solubility of the sterols in the 286 mM aqueous stock solution of β-CD (fully loaded) (41). Both sterols decreased the cellular uptake of LDL in a dose-dependent manner (**Fig. 1**); however, the effect was much more pronounced with 27-hydroxycholesterol. Half-maximal inhibition was attained at concentrations

TABLE 1. Effect of β-CD on LDL receptor activity

Treatment	^{125}I-Labeled LDL Uptake	% Control
	ng/mg protein	
A. Control	200.7 ± 10.9	100
B. β-CD, 24 h + 3 h labeling	433.4 ± 33.8[a]	215.9
C. β-CD, 3 h labeling	273.8 ± 33.8[b]	136.4
D. β-CD, washed	254.0 ± 15.9[c]	126.6

Cells were incubated for 24 h in DMEM containing 10% LPDS (0.5 ml/well) followed by these treatments. A) Control: fresh LPDS media for 24 h, media were removed, cells were washed twice with PBS prior to the addition of fresh LPDS media containing 10 mg/ml ^{125}I-labeled LDL; B) β-CD, 24 h + 3 h labeling: fresh LPDS media containing 2.86 mM (final concentration) β-CD for 24 h with no media change prior to the addition of ^{125}I-labeled LDL; C) β-CD, 3 h labeling: same as control except β-CD was added at the same time as the ^{125}I-labeled LDL; and D) β-CD, washed: fresh LPDS media containing β-CD (2.86 mM final concentration) for 24 h, media were removed, cells were washed twice with PBS prior to the addition of fresh LPDS media containing ^{125}I-labeled LDL. The uptake of ^{125}I-labeled LDL over a 3-h period at 37°C was measured as described in Materials and Methods. The values shown are means (± SD) of three determinations and are representative of two similar experiments.

Compared to controls, all β-CD treatments had significant effects on LDL receptor activity: [a]$P < 0.005$, [b]$P < 0.02$, [c]$P < 0.001$.

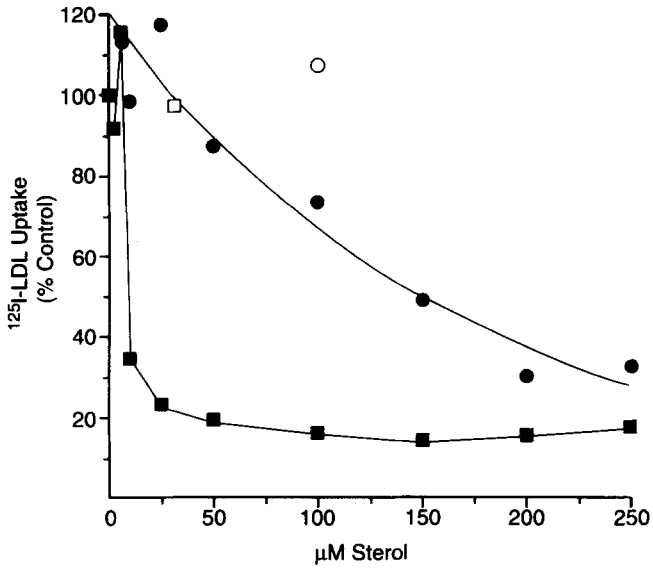

Fig. 1. Effect of increasing concentrations of cholesterol or 27-hydroxycholesterol on ^{125}I-labeled LDL uptake by HepG2 cells. Cells were incubated for 24 h in DMEM containing 10% LPDS. The media were replaced with fresh media containing the indicated concentrations of β-CD-solubilized cholesterol (circles) or 27-hydroxycholesterol (squares). After 24 h, the cells were washed, fresh media were added, and the uptake of ^{125}I-labeled LDL was measured as described in Materials and Methods. The closed symbols represent the level of LDL uptake measured in cells exposed to the indicated concentrations of sterols added from β-CD stock solutions that were fully loaded with sterols (25 mM 27-hydroxycholesterol or 15 mM cholesterol stock solutions). The open symbols represent LDL uptake measured in cells exposed to 30 μM 27-hydroxycholesterol or 100 μM cholesterol added from a β-CD stock solution only partially filled with sterols (3 mM 27-hydroxycholesterol or 10 mM cholesterol stock solutions). Values are the means of three determinations and are expressed as percentages of ^{125}I-labeled LDL uptake in control incubations. Control values averaged 165 ± 11 ng/mg.

of 142 μM for cholesterol and 8 μM for 27-hydroxycholesterol. These IC_{50} values were similar to those previously reported in HepG2 cells by Bellosta et al. (9) in which the sterols were supplied to the medium in ethanol.

The potencies of these β-CD-solubilized sterols in inhibiting LDL uptake were greatly reduced when they were delivered to cells from submaximally concentrated stock solutions in which the hydrophobic cavities of the β-CD molecules were only partially filled with sterols. Figure 1 shows that the addition of 100 μM cholesterol or 30 μM 27-hydroxycholesterol from β-CD stock solutions fully loaded with sterol resulted in reductions in LDL uptake of 30 and 80%, respectively (Fig. 1, closed symbols); however, there was no effect of the sterols at these concentrations when added from partially filled, 10 mM cholesterol and 3 mM 27-hydroxycholesterol β-CD stock solutions (open symbols). These results suggest that fully loaded β-CD more effectively delivers sterols to cells than partially filled β-CD. Unless otherwise noted, the β-CD used in all experiments described here was fully loaded with sterol.

To estimate the cellular levels of cholesterol and 27-hydroxycholesterol that were associated with a 50% reduction in LDL receptor activity, cells were incubated with either [^{14}C]cholesterol or [^{14}C]27-hydroxycholesterol solubilized in β-CD under the same conditions used to measure LDL uptake. The amounts of radiolabeled cholesterol, cholesteryl ester, and 27-hydroxycholesterol present in either the cells or media were then determined by normal phase HPLC. When

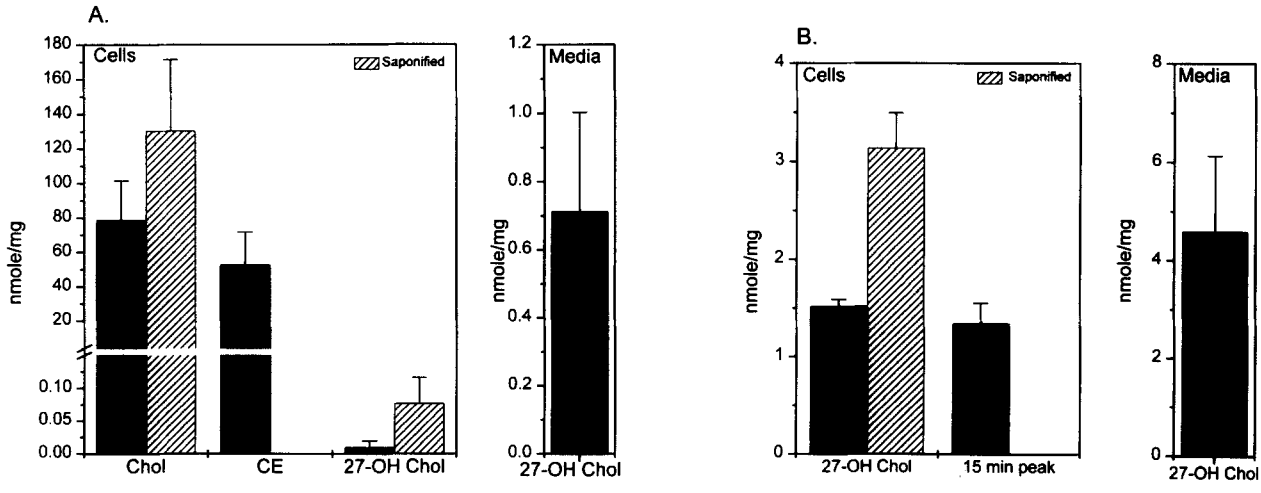

Fig. 2. Distribution of ^{14}C-labeled sterols in cells and media. A: HepG2 cells were incubated for 24 h in DMEM containing 10% LPDS. Fresh LPDS media containing 150 μM [^{14}C]cholesterol (25.6 nCi/nmol) solubilized in β-CD (fully loaded) was added for a further 24 h. Cells and media were analyzed separately for radioactive sterols by normal phase HPLC as described in Materials and Methods. A portion of the cell lysate was saponified prior to sterol analysis. The solid bars correspond to the amounts (nmol/mg cell protein) of cholesterol (Chol), cholesteryl ester (CE), 27-hydroxycholesterol (27-OH Chol), or an unknown metabolite eluting with a retention time of 15 min (15 min peak) recovered in the cell and media fractions prior to saponification. The hatched bars correspond to the amounts of these sterols measured in the cellular fraction after saponification. The values represented are means (± SD) of triplicate incubations with cells and are indicative of at least one other experiment. B: Experimental conditions and the analysis of media and cell fractions were exactly as indicated in A, except 10 μM [^{14}C]27-hydroxycholesterol (6.6 nCi/nmol) solubilized in β-CD (fully loaded) was added for the 24 h incubation.

HepG2 cells were exposed to 150 μM [^{14}C]cholesterol (25.6 nCi/nmol) for 24 h (**Fig. 2A,** solid bars), 78% of the radiolabel was incorporated in the cells as either free cholesterol (78.8 nmol/mg protein) or cholesterol ester (52.6 nmol/mg protein). The amount of [^{14}C]27-hydroxycholesterol found associated with the cells was very low (0.01 nmol/mg protein) relative to that detected in the media (0.71 nmol/mg protein). This finding is in agreement with Reiss et al. (24) who recently reported the accumulation of 27-hydroxycholesterol in the media of HepG2 cells cultured in the presence of β-CD-solubilized cholesterol. Saponification of the cells (Fig. 2A, hatched bars) resulted in increased amounts of both 27-hydroxycholesterol and free cholesterol. These results suggest that while the majority of the 27-hydroxycholesterol synthesized from cholesterol by HepG2 cells is secreted into the media, nearly all of that remaining in the cells exists there in an esterified form.

When cells were incubated with 10 μM [^{14}C]27-hydroxycholesterol (6.6 nCi/nmol) for 24 h (Fig. 2B, solid bars), approximately 30% of the added radiolabel was found associated with the cells, distributed nearly evenly between 27-hydroxycholesterol (1.5 nmol/mg protein) and an unidentified metabolite (1.3 nmol/mg protein) eluting with a retention time of 15 min on normal phase HPLC. The unknown peak is likely to represent a 27-hydroxycholesterol ester as saponification of the cell samples resulted in the corresponding loss of this peak and an increase in the 27-hydroxycholesterol peak. Considering the low level of cellular [^{14}C]27-hydroxycholesterol (0.01 nmol/mg protein) observed after the incubation of cells with [^{14}C]cholesterol at its IC_{50} concentration relative to the cellular level (1.5 nmol/mg protein), associated with a 50% suppression of LDL uptake by [^{14}C]27-hydroxycholesterol, it can be argued that 27-hydroxycholesterol itself does not mediate the suppression of LDL receptor activity by β-CD-solubilized cholesterol.

Inhibition of sterol 27-hydroxylase by cyclosporin

To examine the possibility that endogenously formed 27-hydroxycholesterol or some cellular product of 27-hydroxycholesterol metabolism may mediate the cholesterol-induced suppression of LDL receptor activity, we studied the effects of cyclosporin on LDL uptake at concentrations that inhibit the 27-hydroxylation of cholesterol in HepG2 cells.

First, we determined the relative affinities of HepG2 sterol 27-hydroxylase for substrate (cholesterol) and inhibitor (cyclosporin) by examining the mechanism of cholesterol 27-hydroxylation inhibition using freshly isolated HepG2 mitochondria. The Lineweaver-Burk plot shown in **Fig. 3** was used to determine an apparent K_m for cholesterol of 93 μM and a V_{max} of 44 pmol/min per mg, with the K_i for cyclosporin estimated at 0.259 μM.

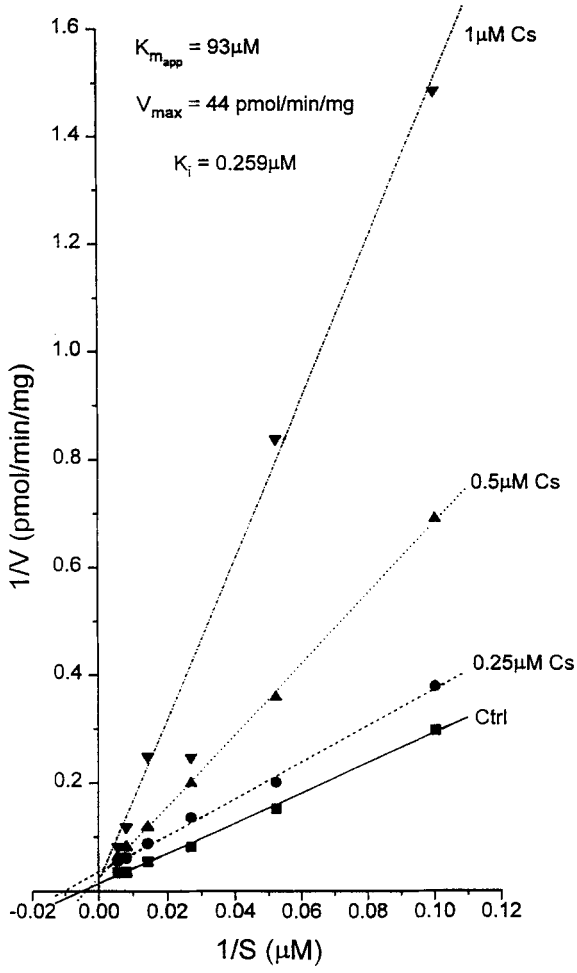

Fig. 3. Inhbition of sterol 27-hydroxylase activity in HepG2 mitochondria by cyclosporin. Mitochondria isolated from HepG2 cells grown in the confluent state for 2 weeks were assayed for cholesterol 27-hydroxylase activities as described in Materials and Methods. Final cholesterol concentrations varied between 10 and 160 μM. To examine the mechanism of cholesterol 27-hydroxylation inhibition by cyclosporin, cyclosporin was added to mitochondrial incubations at final concentrations ranging from 0.250 mM to 1 mM. Shown are the Lineweaver-Burk plots for determination of enzyme kinetics in the presence and absence of cyclosporin.

The mechanism of cholesterol 27-hydroxylation inhibition appeared to be competitive. This study suggested that a cyclosporin concentration range between 0.25 and 2.5 μM could be used to inhibit endogenous 27-hydroxylase activity in HepG2 cultures.

Next, we determined the optimal concentration of cyclosporin required to inhibit the synthesis of [^{14}C]27-hydroxycholesterol from [^{14}C]-β-CD-solubilized cholesterol in HepG2 cultures. **Table 2** shows that cyclosporin dose-dependently decreased the amount of [^{14}C]27-hydroxycholesterol detected in the cells and secreted into the media during a 24-h incubation period. At 1 μM cyclosporin, the levels of cellular and secreted 27-hydroxycholesterol were reduced by 90% and 49%, respec-

TABLE 2. Effect of cyclosporin on the distribution of [^{14}C]sterols in cells and media

Treatment	Media	Cells		
	27-OH Cholesterol	27-OH Cholesterol	Cholesteryl Ester	Cholesterol
Control	0.91 ± 0.18	0.026 ± 0.005	60.4 ± 7.1	61.6 ± 4.1
Cyclosporin, 250 nM	0.96 ± 0.38	0.018 ± 0.002	60.5 ± 5.0	63.8 ± 4.6
Cyclosporin, 1 μM	0.43 ± 0.09	0.003 ± 0.005	55.7 ± 3.3	65.0 ± 3.5
Cyclosporin, 2.5 μM	0.24 ± 0.05	ND	49.1 ± 4.7[a]	77.3 ± 3.4[a]
Cyclosporin, 10 μM	0.17 ± 0.02	ND	41.1 ± 4.2[b]	108.0 ± 11.6[c]

HepG2 cells were incubated for 24 h in DMEM containing 10% LPDS. Fresh LPDS media containing 150 μM [^{14}C]cholesterol solubilized in β-CD (25.6 nCi/nmol) with the indicated concentrations of cyclopsorin was added for a further 24 h. Cells and media were analyzed separately for radioactive sterols by HPLC as described in Materials and Methods. Data are expressed as ^{14}C (nmol/mg cell protein) recovered in the media or cells as 27-hydroxycholesterol, cholesterol, or cholesteryl ester. ND, not determined. The values shown are means (±SD) of three determinations.
[a]$P < 0.005$; [b]$P < 0.01$; [c]$P < 0.001$.

tively. Cyclosporin also caused a dose-dependent decrease in the esterification of [^{14}C]cholesterol at concentrations at or above 1 μM (Table 2). **Figure 4** shows the effects of 10 μM cyclosporin on the metabolism of [^{14}C]cholesterol to total radiolabeled sterols detected in the cells and media. Note that cyclosporin inhibited the 27-hydroxylation and esterification of cholesterol as well as the metabolism of cholesterol to polar sterol intermediates eluting with retention times greater than 25 min. At 10 μM cyclosporin, a 32% decrease in cholesterol esterification, combined with potent inhibition of cholesterol 27-hydroxylation, gave rise to a 76% increase in cell-associated free cholesterol. This latter result differs considerably from what was observed with the ACAT inhibitor DDM (**Fig. 5**). When DDM was used at 40 nM to produce a similar 46% decrease in the synthesis of cholesteryl esters from [^{14}C]β-CD-solubilized cholesterol, there was less of an accumulation of cell-associated free cholesterol (only an 16% increase), while the formation of both cellular and secreted 27-hydroxycholesterol increased 3-fold.

Control experiments conducted to determine the effects of cyclosporin alone on LDL receptor activity in HepG2 cells showed no effects of the drug at 1 μM; however, 10 μM cyclosporin reduced LDL uptake by 50% (**Fig. 6**). Data from the previous experiments (Table 2) suggest that this suppression of LDL receptor by 10 μM cyclosporin may be caused by the increase in cellular free cholesterol levels resulting from cyclosporin's inhibition of both cholesterol esterification and cholesterol 27-hydroxylation. In a separate set of control experiments, it was also noted that at concentrations above 1 μM, cyclosporin can produce a direct, concentration-dependent decrease in the uptake of LDL, even if only present in the culture media during the 3-h incubation period with ^{125}I-labeled LDL (D. Winegar, unpublished data). To minimize potential nonspecific interferences with LDL uptake, 1 μM cyclosporin was used in all experiments described here. In addition, after incubations with the drug, the cells were washed twice and fresh media were added prior to measurement of LDL receptor activity.

To investigate whether the inhibition of cholesterol 27-hydroxylase activity by cyclosporin could block the cholesterol-induced suppression of LDL receptor activity, three approaches were taken to increase the free cholesterol content of the cell's putative regulatory sterol pool. In the first series of experiments, cells were loaded with cholesterol by incubation with 150 μM of the sterol solubilized in β-CD. As shown previously in Fig. 1, 150 μM β-CD-solubilized cholesterol caused a 50%

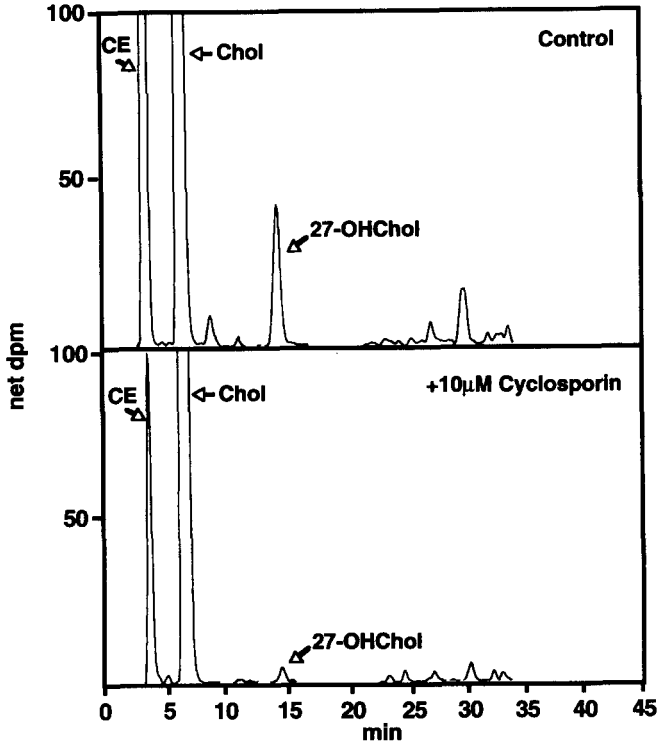

Fig. 4. Effect of cyclosporin on the metabolism of [^{14}C]cholesterol to ^{14}C-labeled sterols. HepG2 cells were incubated for 24 h with DMEM containing 10% LPDS. Fresh LPDS media containing 150 μM [^{14}C]cholesterol (25.6 nCi/nmol) solubilized in β-CD (fully loaded) was added in the absence or presence of 10 μM cyclosporin for a further 24 h. Cells and media were combined and analyzed for radioactive sterols by normal phase HPLC as described in Materials and Methods. Shown are radioactive traces representative of combined cell and media samples obtained from control (top) and treated (bottom) cells.

reduction in LDL uptake compared to the control level (Fig. 6). The presence of 1 μM cyclosporin during these incubations did not prevent the cholesterol-induced suppression of LDL uptake (Fig. 6). This concentration of cyclosporin inhibited total cholesterol 27-hydroxylation (media and cells combined) by 50%, but had no effect on cholesterol esterification (Table 2). This suggests that the synthesis of 27-hydroxycholesterol may not be required for the suppression of LDL receptor activity by β-CD-solubilized cholesterol in HepG2 cells.

To rule out possible artifacts resulting from the delivery of large amounts of cholesterol to cells by a nonphysiological route (i.e., β-CD), cells were loaded with extracellular cholesterol by the receptor-mediated uptake of lipoproteins through the LDL receptor. Kamps and Van Berkel (43) previously showed that in HepG2 cells, cholesterol can be delivered more efficiently to the intracellular pool regulating LDL receptors by cholesteryl ester-rich β-migrating VLDL (β-VLDL) than by LDL. For this reason, we studied the effects of cyclosporin on the suppression of LDL receptor activity induced by β-VLDL. When HepG2 cells were cultured for 24 h in media containing 5 μg/ml β-VLDL (~140 μM free cholesterol), LDL uptake was suppressed by 45% (Fig. 6). The presence of 1 μM cyclosporin during these incubations with β-VLDL did not block this sterol-induced decline in LDL uptake.

A third approach taken to expand the cell's regulatory cholesterol pool was to inhibit the esterification of intracellular free cholesterol with the ACAT inhibitor DDM.

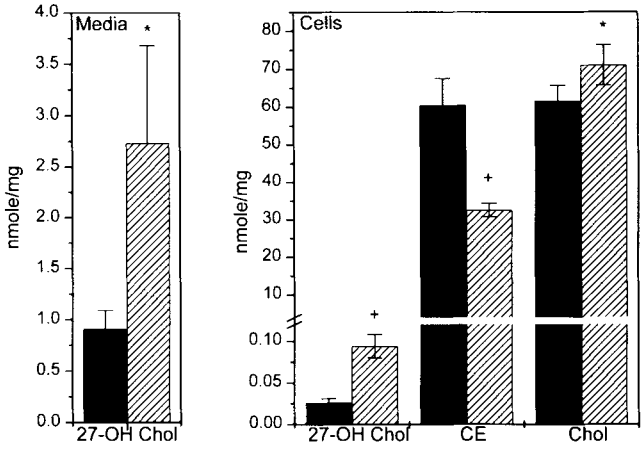

Fig. 5. Effect of DDM on the synthesis of 27-hydroxycholesterol from [^{14}C]β-CD-solubilized cholesterol. HepG2 cells were incubated for 24 h in DMEM containing 10% LPDS. Fresh LPDS media containing 150 μM [^{14}C]cholesterol (25.6 nCi/nmol) solubilized in β-CD (fully loaded) in the absence (solid bars) or presence (hatched bars) of 40 nM DDM was added for a further 24 h. Cells and media were analyzed separately for radioactive sterols by HPLC as described in Materials and Methods. Data are expressed as ^{14}C (nmol/mg cell protein) recovered in the media or cells as 27-hydroxycholesterol, cholesterol, or cholesteryl ester; ND, none detected. The values shown are means (± SD) of three determinations; $^{*}P < 0.05$; $^{+}P < 0.001$.

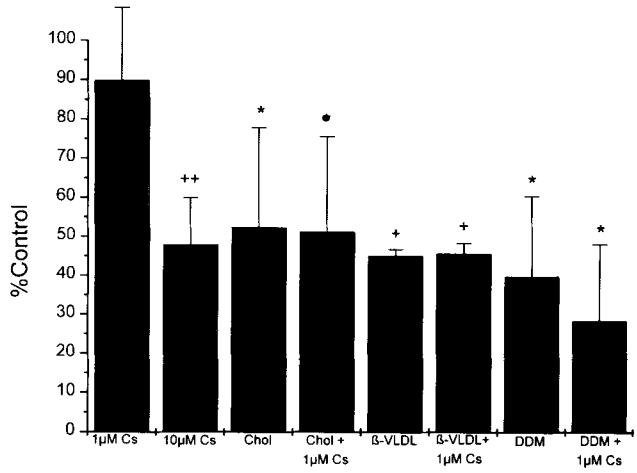

Fig. 6. Effect of cyclosporin on the suppression of LDL receptor activity induced by cholesterol, β-VLDL, or DDM. HepG2 cells were incubated for 24 h with DMEM containing 10% LPDS. Fresh LPDS media containing the following additions was added for a further 24 h: (l–r) 1 μM cyclosporin (Cs); 10 μM Cs; 150 μM β-CD-solubilized cholesterol; 150 μM β-CD-solubilized cholesterol + 1 μM Cs; 1 μg/ml β-VLDL; 1 μg/ml β-VLDL + 1 μM Cs; 1 μM DDM; 1 μM DDM + 1 μM Cs. All drugs were prepared in DMSO. The uptake of ^{125}I-labeled LDL was measured as described in Materials and Methods. Values are the means of two or three experiments and are expressed as percentages (means ±SD) of ^{125}I-labeled LDL uptake in control incubations. Control values averaged 219 + 98 ng/mg. Compared to controls: $^{*}P < 0.05$, $^{+}P < 0.01$, $^{++}P < 0.001$. Compared to treated cells, 1 mM cyclosporin had no statistically significant effect on LDL receptor activity.

In preliminary experiments, it was found that the esterification of [^{14}C]β-CD-solubilized cholesterol by HepG2 cells was maximally inhibited (98%) in the presence of 1 μM DDM (D. Winegar, unpublished data). When cells were cultured for 24 h in media containing 1 μM DDM, LDL uptake was reduced 60% below the control level (Fig. 6). This effect was not altered by the addition of 1 μM cyclosporin during these incubations. Taken together, these results suggest that LDL receptor activity in HepG2 cells is primarily regulated by the size of the intracellular pool of free cholesterol and not by the endogenous synthesis of 27-hydroxycholesterol.

DISCUSSION

We have explored the potential role of 27-hydroxycholesterol in the regulation of cellular cholesterol homeostasis by studying the effects of both exogenously added and endogenously generated cholesterol and 27-hydroxycholesterol on LDL receptor activity in HepG2 cells. To compare the effects of the exogenous sterols on the uptake of ^{125}I-labeled LDL, aqueous solutions of cholesterol and 27-hydroxycholesterol solubilized in β-CD were prepared. Whereas β-CD has been used routinely to efficiently deliver sterols to broken cell enzyme assay systems, its use as a vehicle for introducing sterols

into intact cells in culture has been limited (35). In control experiments, we found that uncomplexed β-CD stimulated LDL uptake in HepG2 cells by both direct and indirect means (Table 1). Considering β-CD's propensity for forming aqueous-soluble inclusion complexes with lipophilic substances (41, 42), and its demonstrated ability to induce the release of cellular cholesterol (44), it is possible that when added to cell culture media, uncomplexed β-CD may encapsulate and solubilize both the cholesterol contained on the surface of LDL particles (direct effect) and cellular plasma membrane cholesterol (indirect effect) in its vacant hydrophobic cavity. Changes in plasma membrane cholesterol content are known to affect the fluidity of the lipid bilayer, often resulting in altered protein-protein interactions and changes in enzymatic activity (45). β-CD has been shown to modify plasma membrane function in rat adipocytes and soleus muscles such that glucose transport is activated in these cells (46). β-CD itself does not appear to be cytotoxic to a variety of cell types, even at concentrations 15-fold greater than that used in the current study (47). One would expect the removal of β-CD from the cell mileau prior to the labeling period with ^{125}I-labeled LDL (Table 1, condition D) to eliminate potential direct effects of β-CD on LDL receptor activity that may result from the complexation of β-CD with lipoprotein cholesterol. Alternately, this washing procedure would not be expected to influence changes in plasma membrane cholesterol composition that may occur when cells are cultured with β-CD for long time periods (indirect effects). As it was beyond the scope of this study to explore the mechanism whereby β-CD stimulates LDL receptor activity, potential β-CD-dependent effects were minimized by using only fully complexed β-CD and by washing cells free of the vehicle prior to the addition of ^{125}I-labeled LDL.

In agreement with earlier reports (9, 12, 13), we found exogenous 27-hydroxycholesterol to be much more potent at suppressing LDL receptor activity than exogenous cholesterol (IC_{50}s = 8 μM and 142 μM for 27-hydroxycholesterol and cholesterol, respectively). One explanation for this increased potency of 27-hydroxycholesterol may be the oxysterol's more rapid entry into the intracellular sterol pool regulating cholesterol biosynthesis and metabolism. Lange, Ye, and Strebel (48) have shown that 25-hydroxycholesterol and cholesterol move between the plasma membrane and endoplasmic reticulum by a common transport mechanism; however, 25-hydroxycholesterol enters this pathway at an accelerated rate. 27-Hydroxycholesterol may be taken up by cells in a similar manner. It is possible that LPDS media may contain small amounts of 27-hydroxycholesterol that are removed more efficiently from the media than β-CD-solubilized cholesterol and thus account for the observed cholesterol-mediated suppression of LDL receptor activity. Our finding that the potency of both sterols was greatest when delivered to cells from stock solutions that were fully saturated with sterols suggested that β-CD in the "fully loaded" state may release less cholesterol from plasma membrane pools (44) than "partially filled" β-CD having an empty hydrophobic cavity.

[^{14}C]cholesterol and [^{14}C]27-hydroxycholesterol solubilized in β-CD were used to determine whether, at a given level of suppression of LDL receptor activity, the amounts of cellular 27-hydroxycholesterol synthesized endogenously from cholesterol were equivalent to the amounts of 27-hydroxycholesterol taken up by the cells from exogenous sources. The cellular levels of [^{14}C]27-hydroxycholesterol associated with a 50% reduction in LDL receptor activity were estimated at 1.5 nmol/mg protein after the incubation of cells with [^{14}C]27-hydroxycholesterol, and 0.1 nmol/mg protein after the incubation of cells with [^{14}C]cholesterol. Although these data argue against the involvement of 27-hydroxycholesterol in the suppression of LDL receptor activity mediated by exogenous cholesterol, it should be considered that there may be differences between the subcellular distribution of exogenously added 27-hydroxycholesterol and the endogenously generated oxysterol that are critical for biological activity.

Consistent with reports of 27-hydroxycholesterol esters in human plasma and aortic tissues (29, 49), we found that a significant portion of the [^{14}C]27-hydroxycholesterol detected in the cells and media appeared to be esterified (Figs. 2A and 2B). Only about 20% of this esterification could be inhibited by the ACAT inhibitor DDM (D. Winegar, unpublished data). ACAT-independent esterification of 27-hydroxycholesterol was recently demonstrated using discoidal bilayer particles and purified lecithin:cholesterol acyltransferase (LCAT) (50). In this study, LCAT catalyzed the esterification of 27-hydroxycholesterol at both the 3-β-OH and 27-OH positions. The significance of this esterification remains to be determined.

To investigate whether the inhibition of cholesterol 27-hydroxylation could prevent the cholesterol-induced suppression of LDL receptor activity, cyclosporin was used as a tool to inhibit the sterol 27-hydroxylase pathway. A recent study of the effect of cyclosporin on bile acid synthesis in HepG2 cells showed that the drug inhibits the synthesis of chenodeoxycholic acid by selectively interfering with both cholesterol 27-hydroxylation and the 27-hydroxylation of 7α-hydroxylated bile acid intermediates (35). In initial experiments, we confirmed that cyclosporin inhibits the 27-hydroxylation of β-CD-solubilized cholesterol in a dose-dependent manner in both HepG2 mitochondria and whole cell cultures (Fig.

3 and Table 3). Unexpectedly, we found that at concentrations > 1 μM, where the drug inhibited the 27-hydroxylation of [^{14}C]cholesterol by more than 50%, cholesterol esterification was also reduced. At 10 μM cyclosporin, the combined inhibition of these two pathways resulted in a large increase in the cellular free cholesterol level (Table 3). These findings suggest that cyclosporin inhibits multiple pathways of cholesterol metabolism which can result in the accumulation of intracellular free cholesterol. In contrast, at a similar degree of inhibition of cholesterol esterification by the ACAT inhibitor DDM, we observed an increase in the synthesis of 27-hydroxycholesterol without significant accumulation of intracellular free cholesterol (Fig. 5). From these results, it appears that sterol 27-hydroxylase and ACAT may have access to the same substrate pool of free cholesterol such that the inhibition of ACAT-dependent cholesterol esterification directs substrate toward sterol-27-hydroxylase. The finding that 1 μM cyclosporin had no effect on LDL uptake while 10 μM cyclosporin reduced uptake by 50% (Fig. 6) suggests a correlation between the level of inhibition of cholesterol metabolic pathways by cyclosporin and the drug's effects on LDL receptor activity. The decrease in LDL uptake at 10 μM cyclosporin may have been due to the large increase in intracellular free cholesterol resulting from cyclosporin's inhibition of both cholesterol esterification and cholesterol 27-hydroxylation as dose–response studies with DDM suggested that greater than 50% inhibition of cholesterol esterification was necessary for suppression of LDL receptor activity (D. Winegar, unpublished data). Cyclosporin had no significant effect on the synthesis of cholesterol from [^{14}C]acetate at concentrations up to 10 μM (D. Winegar, unpublished data). The drug did, however, lower LDL uptake when added to cells only during the 3-h incubation period with ^{125}I-labeled LDL (D. Winegar, unpublished data). This effect may be related to cyclosporin's reported association with lipoproteins (51). A further investigation into the effects of cyclosporin on cellular LDL receptor activity may help to explain the increases in LDL cholesterol observed in patients receiving cyclosporin treatment (52).

To examine whether 27-hydroxycholesterol was responsible for the cholesterol-induced suppression of LDL receptor activity, we compared the effects of cholesterol loading on LDL uptake in the absence and presence of 1 μM cyclosporin. Cholesterol was introduced into the intracellular sterol pool regulating LDL receptor activity in HepG2 cells by incubating cells with one of the following at a concentration sufficient to produce a 50% suppression of LDL uptake: *1)* nonlipoprotein cholesterol solubilized in β-CD which was taken up by cells through the plasma membrane; *2)* lipoprotein cholesterol in the form of β-VLDL which entered cells through the LDL receptor; or *3)* the ACAT inhibitor DDM which expanded intracellular regulatory cholesterol pools by inhibiting the esterification of endogenous free cholesterol. We found that 1 μM cyclosporin, which inhibited cholesterol 27-hydroxylation by 50%, did not block the suppression of LDL uptake induced by cholesterol entering the cell's regulatory pool by the three major routes examined. It is possible that a level of inhibition of 27-hydroxycholesterol synthesis greater than that obtained with 1 μM cyclosporin may be required for a significant effect on LDL receptor activity. These experiments clearly demonstrate the limitations of using cyclosporin as a tool for exploring the role of 27-hydroxycholesterol in the control of cholesterol homeostasis. The development of more specific inhibitors of sterol 27-hydroxylase would be helpful for future studies in this area. Our results suggest that if mitochondrial sterol 27-hydroxylase plays a role in the regulation of LDL receptor activity, it is not through the formation of potent regulatory oxysterols, but through its effects on the availability and/or size of the free cholesterol pool regulating LDL receptor activity. It is currently thought that sterol regulation of LDL receptor gene transcription occurs by a novel mechanism that involves transcriptional factors known as sterol regulatory binding proteins (SREBPs) (53–55). In the absence of sterols, membrane-bound SREBP-1 (125 kDa) undergoes proteolytic cleavage to a soluble 68 kDa fragment that is transported to the nucleus where it binds to sterol regulatory element 1 (SRE-1) of the LDL receptor gene and activates gene transcription. Sterols inhibit the cleavage of membrane-bound SREBP-1 to the 68 kDa fragment, blocking transcription of the LDL receptor gene. While oxysterols have been found to be much more potent than cholesterol at inhibiting LDL receptor gene transcription, our present findings suggest that it is the intracellular free cholesterol pool that is most likely responsible for the regulation of SREBP-1-mediated transcription.

Axelson and Larsson (56) recently reported that LDL-derived 27-hydroxycholesterol mediates the suppression of HMGR in human fibroblasts. They showed a temporal correlation between the production of 27-hydroxycholesterol and inhibition of HMGR activity in normal fibroblasts grown in the presence of LDL. In fibroblasts from CTX patients and in normal fibroblasts treated with cyclosporin, the suppressive effect of LDL on HMGR was reduced 10- to 20-fold. It was noted that the weakened effect of LDL on HMGR in cyclosporin-treated cells may be due to the drug's inhibition of LDL uptake. We observed a similar 50% reduction in LDL uptake by 10 μM cyclosporin in HepG2 cells (Fig. 6). It was not determined whether LDL uptake was also impaired in the CTX fibroblasts used in these experiments.

Although LDL receptor activity is expected to be up-regulated in CTX disease (57), elevated plasma LDL cholesterol concentrations in some subjects (58) suggest that there may be variations in LDL receptor activity within the CTX population that should be considered.

27-Hydroxycholesterol has been detected in human plasma and in normal and atherosclerotic human aorta (59, 60) and mRNA for the sterol 27-hydroxylase are present in a wide range of tissues (19, 23). Recent studies in cultured endothelial cells and macrophages have led to the exciting proposal that the 27-hydroxylation of cholesterol, which is locally formed by peripheral cells, may serve as a mechanism for the removal of excess cholesterol from extrahepatic tissues (24, 25). It is possible that the secretion of 27-hydroxycholesterol by peripheral cells may reflect their lack of the hepatic enzymes that metabolize cholesterol or 27-hydroxycholesterol to bile acids. In this respect, HepG2 cells share some common features with peripheral cells. These cells express normal LDL receptors and carry out most liver-specific functions relating to cholesterol and triglyceride metabolism (32); however, their ability to metabolize cholesterol to bile acids is deficient because of low 7α-hydroxylase activity (S. Sundseth, unpublished data). It is probable that the synthesis and secretion of 27-hydroxycholesterol by HepG2 cells reflects the inability of this cell line to adequately convert cholesterol to bile acids. The current data implying a potential relationship between sterol 27-hydroxylase and ACAT warrant further investigation, particularly considering the interest in ACAT inhibitors as direct-acting anti-atherosclerotic agents. Further evaluation of the role of 27-hydroxycholesterol in vascular tissue should include studies looking at the effects of ACAT inhibition on 27-hydroxycholesterol levels in plasma and developing atherosclerotic lesions.

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