The Hypcholesterolemic Agent LY295427 Reverses Suppression of Sterol Regulatory Element-binding Protein Processing Mediated by Oxysterols*

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The sterol LY295427 reduces plasma cholesterol levels in animals by increasing the expression of hepatic low density lipoprotein (LDL) receptors. Here we trace the hypocholesterolemic activity of LY295427 to an ability to reverse oxysterol-mediated suppression of sterol regulatory element-binding protein (SREBP) processing. Micromolar concentrations of LY295427 induced the metabolism of LDL in oxysterol-treated cultured cells and inhibited the stimulation of cholesteryl ester synthesis mediated by oxysterols. cDNA microarray and RNA blotting experiments revealed that LY295427 increased levels of the LDL receptor mRNA and those of other SREBP target genes. The compound stimulated the accumulation of SREBPs in the nuclei of cells grown in the presence of oxysterols within 4–6 h of addition to the medium. Induction required components of the normal SREBP-processing pathway, including the SREBP cleavage-activating protein and the Site 1 protease. LY295427 overcame the suppression of SREBP processing mediated by several oxysterols but not by LDL-derived cholesterol. We conclude that LY295427 achieves a therapeutically desirable end point by an unique mechanism of action.

The accumulation of cholesterol1 in cells activates a feedback mechanism that delimits further buildup of this essential but toxic lipid (1). Suppression is mediated by decreasing the production of active sterol regulatory element-binding proteins (SREBPs).2,2 transcription factors that are required for the expression of genes in the cholesterol supply pathways (2, 3). In the presence of excess sterols, SREBP is maintained as an inactive precursor in the membranes of the endoplasmic reticulum and transcription from supply pathway genes is low. Sterol loss activates a processing pathway in which the SREBP precursor is transported from the endoplasmic reticulum to the Golgi apparatus and therein cleaved by proteases to release the amino-terminal half of the protein from the membrane-bound precursor. The cleaved protein migrates to the nucleus and activates the transcription of the low density lipoprotein (LDL) receptor gene and genes encoding enzymes in the cholesterol biosynthetic pathway, leading to increased cellular cholesterol.

cDNAs and genes encoding proteins that participate in this complex mechanism of feedback regulation have been identified recently. These include DNAs encoding two closely related SREBPs (1 and 2) (4, 5), an SREBP cleavage activating protein (SCAP) (6), which functions to retain SREBPs in the endoplasmic reticulum in the presence of sterols and to escort them to the Golgi in the absence of sterols (7, 8), and the Site 1 and Site 2 proteases of this subcellular compartment (9, 10). All of these proteins are tightly associated with the membranes of the cell, presumably to allow close monitoring of and response to fluctuations in the levels of bilayer sterols.

It has been known for many years that oxysterols are far more potent than cholesterol in exerting negative feedback regulation on the sterol biosynthetic and uptake pathways (11, 12). When dissolved in ethanol and added to the medium of cultured cells at concentrations of less than 1 μM, the oxysterols 25-hydroxycholesterol or 27-hydroxycholesterol inhibit the proteolysis of SREBP precursors, which in turn suppresses the synthesis of cholesterol and the uptake of LDL cholesterol via the LDL receptor. In contrast, the addition of cholesterol in ethanol causes at best only modest suppression. The mechanisms by which oxysterols mediate their potency have not been defined, nor has it proven straightforward to determine whether oxysterols play a regulatory role in the whole animal. Although several enzymes that synthesize (13) and degrade (14, 15) oxysterols have been characterized, their physiological significance in vivo remains unproven.

In a pioneering series of studies, Lin et al. (16, 17) exploited the potency of oxysterols and knowledge of the feedback regulation of cholesterol metabolism to identify small molecules that modulate the system. To this end, libraries of compounds were screened using an assay that detected the reversal of oxysterol-mediated suppression of a human LDL receptor promoter reporter gene. Among the active compounds identified was the sterol LY295427 (Fig. 1), which had an EC50 value in the low micromolar range. The closely related compound LY306309, differing only in the stereochemistry at carbon 3 (Fig. 1), was inactive. When administered to cholesterol-fed hamsters, LY295427 reduced plasma LDL levels by as much as 69% (18). Experiments with cultured cells and liver extracts...
prepared from treated hamsters indicated that LY295427 increased the levels of unidentified oxysterol-binding proteins (19); however, the relationship between these findings and the decrease in serum cholesterol levels was not elucidated.

In the current studies we show that LY295427 reverses the oxysterol-mediated suppression of SREBP processing in cultured cells. The consequences of this reversal are the activation of multiple SREBP-responsive genes, including the LDL receptor gene, and a concomitant increase in the metabolism of LDL, thus explaining the hypocholesterolemic effects of the compound. When considered with the in vivo activity of LY295427, these findings support a role for oxysterols in the regulation of cholesterol metabolism in the whole animal.

**EXPERIMENTAL PROCEDURES**

**Materials**—LY295427 and LY306039 were kind gifts of Julio Medina (Tularik Inc., South San Francisco, CA), and Robert A. Gadski (Eli Lilly and Co., Indianapolis, IN). The compounds were dissolved in ethanol to produce stock solutions of 10 mM. 24(S,25-Epoxycholesterol was obtained from E. J. Corey (Harvard University, Cambridge, MA). Cholesterol, 25-hydroxycholesterol, 22(R)-hydroxycholesterol, 27-hydroxycholesterol, desmosterol, 25-acetylolesterol, and 25-fluorocolesterol were obtained from Steraloids Inc. (Wilton, NH). The protease inhibitors N-acetyl-leucinal-norleucinal, pepstatin A, leupeptin, Pefabloc, and dithiothreitol were purchased from Sigma. Sodium mevalonate and sodium compactin were prepared as described (20). Human lipoprotein-deficient serum (HLPPS) and newborn calf lipoprotein-deficient serum were prepared as described (21). Cell culture medium was purchased from Life Technologies, Inc.

**Chinese Hamster Ovary Cell Lines**—CHO-7 cells, which were derived from CHO-K1 cells by adaptation to continuous growth in lipoprotein-deficient serum (22), were maintained and seeded in medium A (1:1 mixture of Ham’s F(12)-medium and Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) newborn calf lipoprotein-deficient serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) at 37 °C and 8–9% CO2. On day 0 of an experiment, CHO-7 cells were seeded at a density of 7 × 10^5 cells/10-cm plate and thereafter treated as described below. SRD-12B and SRD-13A are CHO-7-derived lines that are deficient in SCAP (23) and the Site 1 protease (24), respectively. Both SRD cell types were seeded at a density of 5 × 10^5 cells/10-cm plate on day 0 of an experiment and maintained in medium B (1:1 mixture of Ham’s F-12 medium and DMEM containing 5% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, 5 μg/ml cholesterol, 20 μM oleate, and 1 mM sodium malonate) at 37 °C and 8–9% CO2. On day 2, CHO-7 and SRD cells were incubated in medium C (medium A supplemented with 10 μg/ml cholesterol, 50 μM sodium mevalonate, and 50 μM sodium compactin) in the absence or presence of 1 μM 25-hydroxycholesterol and 20 μM LY295427 for 24 h. One h prior to the termination of the experiment, N-acetyl-leucinal-norleucinal was added to the culture medium at a final concentration of 25 μM. CHO-7 cells were then harvested and separated into membrane and nuclear fractions as described (25). SRD cells were harvested, and pellets were resuspended in SDS lysis buffer (25) and then stored at −80 °C until assayed for protein concentration and use in immunoblotting experiments.

In experiments with CHO-7 cells as well as the cell lines listed below, oxysterols were added in combination with cholesterol to suppress the expression of cholesterogenic genes. The oxysterol is the active suppressive agent in this mixture; the inclusion of cholesterol prevents cell death that can occur following acute inhibition of cholesterol biosynthesis and lipoprotein uptake (12).

**Human Fibroblast Cell Lines**—Diploid human fibroblasts and SV589 cells, an immortalized line of fibroblasts expressing the SV7-SV7 large T antigen, were maintained in medium D (DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) at 37 °C and 5% CO2. SV589 cells were seeded on day 0 at a density of 1.7 × 10^5 cells/15-cm plate. On day 1, the cells were switched to medium E (DMEM supplemented with 10% HLPPS, 10 μg/ml cholesterol, 50 μM sodium mevalonate, and 50 μM sodium compactin) in the absence or presence of 1 μM 25-hydroxycholesterol and 20 μM LY295427. Following a 24-h incubation, the cells were harvested and separated into membrane and nuclear fractions (25).

**Immunoblotting**—Protein concentrations were determined using a micro BCA assay kit (Pierce) according to the manufacturer’s instructions in Costar poly styrene 96-well plates (Corning, Inc., Corning, NY). Proteins were separated on 8% (w/v) polyacrylamide/0.1% (v/v) SDS gels (16 × 15 cm) together with prestained molecular mass markers (Bio-Rad). Proteins were transfected to Hybond C Extra nitrocellulose membranes (Amersham Pharmacia Biotech) at 200 volts for 30 min using a transblot apparatus (Hoeffer Scientific Instruments, San Francisco, CA). Immunoblot analyses of hamster SREBP-1 and SREBP-2 were accomplished with a rabbit polyclonal antibody (IgG-179; StressGen Biotechnologies Inc., Victoria, BC) directed against the NH2-terminal regions of these proteins (26). Human SREBP-2 was detected using a mouse monoclonal antibody (IgG-112D) that recognizes the NH2 terminus of the protein.3 Rabbit anti-rat immunoglobulin heavy chain-binding protein antibody was purchased from StressGen Biotechnologies Inc. (Collevelle, PA), and peroxidase-conjugated donkey anti-rabbit IgG and donkey anti-mouse IgG were acquired from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Antibodies were diluted in phosphate-buffered saline (PBS), pH 7.4, containing 5% (v/v) newborn calf serum, 5% (v/v) nonfat dry milk, and 0.05% (v/v) Tween 20 (Sigma). Antibody complexes were visualized using SuperSignal CL-horseradish peroxidase (Pierce) according to the manufacturer’s instructions. Immunobots were exposed to X-Omat film and developed. The chemiluminescent signal was removed by treating filters with 2% (w/v) SDS, 62.5 mM Tris-Cl, pH 6.8, 100 mM 2-mercaptoethanol for 30 min at 68 °C, prior to a second round of immunoblotting.

**Cholesterol and Triglyceride Esterification Assay**—Human fibroblasts were plated at 2 × 10^5 cells/6-cm dish on day 0 in medium D and refed on day 3. On day 5, cells were washed with PBS, and fresh DMEM supplemented with 10% (v/v) HLPPS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate was added. On day 7, the medium was replaced with fresh DMEM lacking glutamate and supplemented with 2 mg/ml bovine serum albumin. LY295427 was added to dishes at a final concentration of 20 μM. After 20 min at 37 °C, either human LDL or 25-hydroxycholesterol was added followed by a 5-h incubation at 37 °C. Lastly, 2.2 mM [3H]oleate was added to each dish, and the incubation continued at the same temperature for 2 h. The cells were harvested and extracted with organic solvent (hexane/isopropanol, 3:2, v/v) to isolate the lipid fraction. [3H]Cholesterol oleate (~34,000 dpm) was added to each sample as a recovery standard, and [14C]oleate incorporation into steryl esters was assayed using thin layer chromatography (21).

**LDL Metabolism**—The measurement of LDL binding, uptake, and degradation in cultured human fibroblast cells was performed as described (21). Briefly, fibroblasts were plated at 2 × 10^5 cells/6-cm dish on day 0 in medium D and refed on day 2. On day 5, cells were washed with PBS and overlaid with fresh DMEM supplemented with 10% (v/v) FCS or HLPPS. LY295427 was added to a final concentration of 20 μM. After 20 min at 37 °C, either human LDL or 25-hydroxycholesterol was added followed by a 5-h incubation at 37 °C. Lastly, 2.2 mM [14C]oleate was added to each dish, and the incubation continued at the same temperature for 2 h. The cells were harvested and extracted with organic solvent (hexane/isopropanol, 3:2, v/v) to isolate the lipid fraction. [3H]Cholesterol oleate (~34,000 dpm) was added to each sample as a recovery standard, and [14C]oleate incorporation into steryl esters was assayed using thin layer chromatography (21).

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**FIG. 1. Chemical structures of LY295427, an active hypocholesterolemic agent, and LY306039, an inactive stereoisomer. The two compounds differ only in the stereochemistry of the hydroxyl group at carbon 3.**
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**TABLE I**
*Effects of LY295427 on LDL metabolism in human fibroblasts*

| Treatment                           | Binding | Internalization | Degradation |
|-------------------------------------|---------|----------------|-------------|
| Experiment A                        |         |                |             |
| None                                | 108     | 572            | 3433        |
| LY295427                            | 121     | 743            | 4054        |
| Sterols                             | 56      | 269            | 1320        |
| Sterols + LY295427                  | 103     | 649            | 3257        |
| LDL                                 | 22      | 81             | 375         |
| LDL + LY295427                      | 17      | 137            | 522         |
| Experiment B                        |         |                |             |
| None                                | 149     | 1021           | 5233        |
| LY295427                            | 134     | 1235           | 4941        |
| Sterols                             | 43      | 241            | 788         |
| Sterols + LY295427                  | 151     | 897            | 4429        |
| FCS                                 | 27      | 212            | 650         |
| LDL + LY295427                      | 47      | 336            | 908         |

**Sphingomyelinase Treatment—CHO-7 cells** were grown as described above and treated with different concentrations of sphingomyelinase (Sigma catalog number S-8633) exactly as described (27). After a 90-min incubation with enzyme, the cells were harvested and resuspended in SDS lysis buffer (25), and SREBP peptides were detected by immunoblotting.

**RNA Isolation and Blotting**—Total RNA was isolated from SV589 cells using Trizol Reagent (Life Technologies, Inc.), and poly(A)⁺ mRNA was isolated using Oligotex spin column kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA was quantified by spectral analysis. Aliquots of poly(A)⁺ mRNA were separated by agarose gel electrophoresis (28), transferred to nylon filters, and hybridized for 16 h in solutions containing 50% (v/v) formamide and 1 × 10⁶ cpm/ml ³²P-labeled probe (>10⁶ cpm/μg DNA) produced by random hexanucleotide priming. Blots were washed with 0.1 × SSC, 1% (w/v) SDS prior to exposure to Kodak X-Omat AR film at −80 °C using an intensifying screen.

**cDNA Microarray Analyses**—Poly(A)⁺ mRNA isolated from SV589 cells grown in medium supplemented with 1 μM LY295427 was subjected to hybridization analyses using a microarray representing ~10,000 human cDNAs (Incyte Genomics, St. Louis, MO). A second independent analysis of the same SV589 poly(A)⁺ mRNA was performed on a custom human cDNA chip arrayed at Tularik, Inc.

**RESULTS**

The consequences of LY295427 treatment for cellular cholesterol metabolism were determined by treating diploid human fibroblasts with 30 μM compound for 48 h and then measuring the binding, uptake, and degradation of [¹²⁵I]LDL (Table I). In two separate experiments, cells grown in lipoprotein-deficient medium, conditions that result in the induction of LDL receptor expression, metabolized microgram amounts of LDL. The quantity of LDL metabolized in this medium was not altered by treatment with LY295427. Cells grown in the presence of a mixture of sterols (5 μg/ml cholesterol and 0.4 μg/ml 25-hydroxycholesterol) metabolized far less LDL because of oxygen-stress-mediated suppression of LDL receptor expression. Incubation of the sterol-treated cells with LY295427, however, comple-
Fig. 3. Time course of gene expression in LY295427-treated human SV589 cells. Dishes of SV589 cells were set up on day 0 as described under “Experimental Procedures.” On day 1, the cells were refed medium E supplemented with sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) and 20 μM LY295427. The cells were harvested at time 0 (lane 1) and following 6 h (lane 2), 12 h (lane 3), or 24 h (lane 4) of incubation. Poly(A)+ mRNA was isolated, and 5-μg aliquots were analyzed by blot hybridization with 32P-labeled cDNA probes encoding the LDL receptor (GenBank™ accession number NM000527), SREBP-2 (GenBank™ accession number NM004599), mouse stearyl-CoA desaturase (GenBank™ accession number BC007474), and rat cyclophilin (GenBank™ accession number M19533). After washing, filters were exposed to x-ray film for 15–24 h.

The presence of LY295427 but to a lesser extent (1.8 to 2.1-fold; Table II).

These results were confirmed and extended in a series of RNA blotting experiments carried out in SV589 cells grown in the presence of sterols and LY295427 for different lengths of time. As shown by the data in Fig. 3, mRNA levels for the LDL receptor, SREBP-2, and stearyl-CoA desaturase, three genes identified in the cDNA array experiments, were low in cells grown in the presence of sterols. The amounts of these mRNAs began to increase 6 h after the addition of LY295427 to the sterol-containing medium, and they reached maximal levels at 24 h. In contrast, the levels of an mRNA transcribed from the cyclophilin gene, which is not sterol-responsive, remained unchanged throughout the time course of the experiment (Fig. 3).

A majority of the genes whose expression was altered by LY295427 were targets of the SREBP-1 and SREBP-2 transcription factors (30), suggesting that the compound in some manner affects the activity of these DNA-binding proteins. To test this hypothesis, we monitored the processing of SREBP's in CHO-7 cells treated with LY295427. When grown in the absence of sterols, CHO-7 cells contained ample amounts of both SREBP-1 and -2 precursor and mature forms (Fig. 4, lanes 1 and 5). Conversely, when grown in the presence of sterols, CHO-7 cells contained the membrane-bound precursor but little or no nuclear SREBP (lanes 2 and 6). The addition of LY295427 did not affect levels of the SREBP precursors or their processed nuclear forms in cells grown in the absence of sterols (lanes 3 and 7), but it completely prevented the decrease in the nuclear forms normally observed in the presence of sterols (lanes 4 and 8).

LY295427 was similarly able to reverse the suppression of SREBP processing caused by 25-hydroxycholesterol in human SV589 cells. The data of Fig. 5 showed that as the concentration of this oxysterol was increased from 0 to 5 μg/ml there was a progressive decrease in the amount of SREBP-2 in the nuclear fraction. This decrease was prevented by inclusion of 20 μM LY295427 in the culture medium. The addition of LDL to the medium at concentrations ranging from 5 to 100 μg/ml also suppressed the processing of SREBP-2; however, LY295427 did not reverse this suppression (Fig. 5). The levels of the LDL receptor and HMG-CoA synthase mRNAs were monitored in this same experiment, and these correlated directly with the amounts of SREBP-2 present in each condition (data not shown). These results agreed with those presented in Table I and indicated that LY295427 reversed the inhibition of SREBP processing mediated by oxysterols but not by LDL.

The hydrolysis of cellular sphingomyelin by the enzyme sphingomyelinase triggers the movement of cholesterol from the plasma membrane to the endoplasmic reticulum, which in turn causes suppression of SREBP processing (27). To determine whether LY295427 could reverse this suppression, CHO-7 cells were treated with different concentrations of sphingomyelinase in the presence or absence of 20 μM compound and SREBP-2 processing was followed in cell lysates (Fig. 6). Suppression was observed when the level of sphingomyelinase exceeded 100 milliunits/ml (lanes 1–4), and the inclusion of LY295427 in the culture medium had no effect on this regulation (lanes 5–8).

Sterol-regulated proteolysis of SREBP involves the cooperative actions of the sterol-sensing protein SCAP and two cleavage enzymes, the Site 1 and Site 2 proteases (31). To determine whether the ability of LY295427 to facilitate SREBP processing in the presence of sterols also required these proteins, the effect of the compound in CHO-7 cell lines lacking SCAP or the Site 1 protease was assessed (Fig. 7). In control experiments, wild type CHO-7 cells grown in the presence of sterols contained only the SREBP-2 precursor (lane 1). The processing of this form to the active nuclear protein was induced when LY295427 was added to the sterol-containing medium (lane 2). In a line of CHO-7 cells deficient in SCAP (SRD13A cells), no SREBP-2 proteins or peptides were detected, regardless of the presence or absence of sterols or LY295427 (lanes 3–6). The absence of SCAP chaperone activity renders SREBP unstable in SRD13A cells (23), and thus these results suggested that LY295427 was not acting by increasing the stability of the transcription factor. CHO-7 cells deficient in the Site 1 protease (SRD12B cells) contained the precursor form of SREBP-2 when cultured in the presence of sterols (lane 8), and, as expected, these cells were unable to cleave the precursor into the active form when sterols were removed from the medium (lane 7). LY295427 was unable to induce SREBP cleavage when these cells were grown in the presence or absence of sterols (lanes 9 and 10). In no cell line was the level of a control protein (immunoglobulin heavy chain-binding protein) changed (Fig. 7, lower panel). Similar results were obtained when the processing of SREBP-1 was followed (data not shown). Together these data indicated that the cleavage of SREBP in the presence of sterols and LY295427 involved two of the same components that process the protein in the absence of sterols.

Dose-response experiments indicated that micromolar concentrations of LY295427 were needed to reverse the suppressive effects of oxysterols on SREBP processing in SV589 cells (Fig. 8). An increase in the nuclear form of SREBP-2 was first detected at 5 μM LY295427 (lane 4). Enhanced production of processed SREBP-2 correlated with the increase of LY295427 up to 60 μM (lanes 5–8), which represented the solubility limit of the compound. In marked contrast, 60 μM LY306039, the 3β-stereoisomer of LY295427, did not affect SREBP-2 processing (Fig. 8, lane 9).

The time course of LY295427 action in SV589 cells was assessed in two ways. First, we determined the ability of LY295427 to reverse the suppressive effects of oxysterols when the two were added simultaneously to the culture medium. As shown by the data of Fig. 9A, in the absence of the compound, sterols decreased the amounts of nuclear SREBP-2 by 50% at 4 h (lane 2) and by greater than 95% at 8 and 12 h (lanes 3 and...
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Identification of mRNAs with increased expression after LY295427 treatment of human SV589 cells

In Experiment 1, poly(A)+ mRNA isolated from SV589 cells grown in medium E supplemented with 1 μg/ml 25-hydroxycholesterol ± 20 μM LY295427 was subjected to hybridization analyses using a human microarray (Incyte Genomics, St. Louis, MO) as described under "Experimental Procedures." In Experiment 2, the same SV589 poly(A)+ mRNA was subjected to hybridization analysis except that a custom cDNA chip arrayed at Tularik, Inc. was used. ND, not detected, either because the cDNA for this gene product was not present on the microarray or because the hybridization signal for the cDNA was not above background.

| Gene                                      | Function                                      | Fold increase | GenBank™ accession number |
|-------------------------------------------|-----------------------------------------------|---------------|---------------------------|
| **Experiment 1**                          |                                               |               |                           |
| Stearyl-CoA desaturase                    | Fatty acid biosynthesis                       | 7.3           | AB032261                  |
| Methyl sterol oxidase                     | Cholesterol biosynthesis                      | 6.9           | AA157955                  |
| Isopentenyl diphosphate isomerase         | Cholesterol biosynthesis                      | 6.6           | H08899                    |
| Farnesyl-diphosphate farnesyltransferase  | Cholesterol biosynthesis                      | 5.4           | X69141                    |
| Mevalonate kinase                         | Cholesterol biosynthesis                      | 4.9           | X75311                    |
| 3-Hydroxy-3-methylglutaryl-CoA reductase   | Cholesterol biosynthesis                      | 4.5           | H16650                    |
| Squalene epoxidase                        | Cholesterol biosynthesis                      | 3.7           | AP098865                  |
| LDL receptor                              | Cholesterol biosynthesis                      | 3.6           | NM_000527                 |
| 7-Dehydro-cholesterol Δ5-reductase        | Cholesterol biosynthesis                      | ND            | BE378962                  |
| Mevalonate diphosphate decarboxylase      | Cholesterol biosynthesis                      | 2.8           | U49260                    |
| ATP citrate lyase                         | Lipid biosynthesis                            | 2.3           | AW967351                  |
| SREBP-2                                   | Transcription of lipid metabolism genes       | ND            | AA608556                  |
| Isopentenyl-diphosphate δ-isomerase       | Cholesterol biosynthesis                      | ND            | X17025                    |
| Farnesyl diphosphate synthase             | Cholesterol biosynthesis                      | 2.1           | D14697                    |
| Sterol C4-methyl oxidase                  | Cholesterol biosynthesis                      | ND            | NM_006745                 |

| **Experiment 2**                          |                                               |               |                           |
| Stearyl-CoA desaturase                    | Fatty acid biosynthesis                       | 6.5           |                           |
| Methyl sterol oxidase                     | Cholesterol biosynthesis                      | ND            |                           |
| Isopentenyl diphosphate isomerase         | Cholesterol biosynthesis                      | ND            |                           |
| Farnesyl-diphosphate farnesyltransferase  | Cholesterol biosynthesis                      | ND            |                           |
| Mevalonate kinase                         | Cholesterol biosynthesis                      | ND            |                           |
| 3-Hydroxy-3-methylglutaryl-CoA reductase   | Cholesterol biosynthesis                      | ND            |                           |
| Squalene epoxidase                        | Cholesterol biosynthesis                      | ND            |                           |
| LDL receptor                              | Cholesterol biosynthesis                      | ND            |                           |
| 7-Dehydro-cholesterol Δ5-reductase        | Cholesterol biosynthesis                      | ND            |                           |
| Mevalonate diphosphate decarboxylase      | Cholesterol biosynthesis                      | ND            |                           |
| ATP citrate lyase                         | Lipid biosynthesis                            | ND            |                           |
| SREBP-2                                   | Transcription of lipid metabolism genes       | ND            |                           |
| Isopentenyl-diphosphate δ-isomerase       | Cholesterol biosynthesis                      | ND            |                           |
| Farnesyl diphosphate synthase             | Cholesterol biosynthesis                      | ND            |                           |
| Sterol C4-methyl oxidase                  | Cholesterol biosynthesis                      | ND            |                           |

LY295427 reverses sterol-mediated suppression of SREBP processing in CHO-7 cells. On day 0, the cells were set up as described under "Experimental Procedures." On day 2, cells were refed medium C supplemented with vehicle (lanes 1 and 5), sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol, lanes 2 and 6), 20 μM LY295427 (lanes 3 and 7), or LY295427 and sterols (lanes 4 and 8). The cells were harvested after 24 h, and fractions enriched in membrane and nuclear proteins were prepared by centrifugation. Aliquots of protein (40–65 μg) from each fraction were separated by SDS-PAGE, transferred to a nylon membrane, and incubated with 5 μg/ml rabbit anti-hamster SREBP-1 IgG-1D2 (lanes 1–4) or 5 μg/ml mouse anti-hamster SREBP-2 IgG-7D4 (lanes 5–8). In the left lower panel, the cleaved form of SREBP-1 in the nuclear fraction is indicated by an arrow, and an intense cross-reacting protein is indicated by an asterisk. The positions to which prestained protein markers migrated to in the polyacrylamide gel are indicated on the right of the lumigrams. The blots were developed with a chemiluminescence reagent and exposed to film for 5–15 s.

25-HC, μg/ml

LY295427, 20 μM

SREBP-2, nuclear

LDL, μg/ml

LY295427, 20 μM

SREBP-2, nuclear

LY295427 required a minimum of 4–6 h to begin reversing the suppressive effect of oxysterols on SREBP processing.

LY295427 reversed the suppression of SREBP processing mediated by several different oxysterols and cholesterol derivatives. The data of Fig. 10 show that 20 μM compound reversed the suppressive effects of 25-hydroxycholesterol, 27-hydroxycholesterol, 24(S),25-epoxycholesterol, desmosterol, 25-acetylcholesterol, and 25-fluorocholesterol. The oxysterol 22(R)-hydroxycholesterol did not alter SREBP-2 processing in the absence or presence of LY295427. Similarly, in no experiment did the inactive isomer LY306039 alleviate sterol suppression of SREBP-2 processing (Fig. 10).

Cells accumulate sterols in the form of fatty acid esters, which are synthesized in the endoplasmic reticulum by acyl-Coenzyme A cholesterol acyltransferase. To determine the effect of LY295427 on cholesterol esterification, the incorporation of [14C]oleate into cholesteryl esters was measured in diploid human fibroblasts supplemented with LDL or 25-hydroxycholesterol. When grown in the absence of sterols, the cells esterified small amounts of cholesterol, and LY295427 addition did
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FIG. 6. LY295427 does not reverse suppression of SREBP processing caused by sphingomyelinase. CHO-7 cells were plated on day 0 as described under "Experimental Procedures." The medium was removed on day 2, the cells were washed twice with PBS, and fresh medium supplemented with 10 μg/ml of an acylcholesterol acyl transferase inhibitor (Sandoz 58–035) and 20 μM LY295427 (as indicated) was added. After 30 min, sphingomyelinase was added to the medium at the indicated concentrations, and the incubation continued for 90 min. The cells were harvested, lysed with a buffer containing SDS, and aliquots (50 μg) of the resulting lysates were subjected to SDS-PAGE followed by immunoblotting. Precursor (P) and mature (M) forms of SREBP-2 were detected with a mouse anti-hamster SREBP-2 monoclonal antibody (IgG-7D4) and chemiluminescent reagents. mu, milliunits.

FIG. 7. LY295427 action requires an intact SREBP processing system. On day 0, CHO-7, SRD-12B, or SRD-13A cells were set up as described under "Experimental Procedures." On day 2, the cells were refed medium C supplemented with vehicle (ethanol), sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol), or sterols plus 20 μM LY295427, as indicated, for 24 h. The cells were harvested and lysed with a buffer containing SDS, and aliquots (80 μg) of the resulting lysates were subjected to SDS-PAGE followed by immunoblotting. Precursor (P) and mature (M) forms of SREBP-2 were detected with a mouse anti-hamster SREBP-2 monoclonal antibody (IgG-7D4) and a chemiluminescent system. Signals were removed from the blot, and the filter was subjected to a second round of immunoblotting using a rabbit antirat immunoglobulin heavy chain-binding protein (BIP) antibody to confirm that equal amounts of protein were analyzed in each lane (lower panel). Lumigens were generated by exposing the treated filters to x-ray film for 5–15 s.

not alter this activity (Fig. 11). As expected (32, 33), the addition of increasing amounts of 25-hydroxycholesterol to the medium progressively increased the rate of cholesteryl ester formation (Fig. 11, left panel). This stimulation was abrogated when both 25-hydroxycholesterol and LY295427 were included in the medium. The addition of LDL also increased the amount of cholesteryl esters formed in the cells; however, this stimulation was not affected by LY295427 (Fig. 11, right panel). The compound also did not affect the incorporation of [14C]oleate into triacylglycerols under any of the tested culture conditions (data not shown). These data indicated that LY295427 reversed the effects of oxysterols but not LDL-associated sterols on cholesteryl esterification.

DISCUSSION

The current experiments reveal the mechanism of action of LY295427, a hypocholesterolemic agent originally isolated by the Eli Lilly Corporation. The compound has the unique ability to override the suppressive effects of oxysterols on the production of active SREBP transcription factors. When added to culture medium containing an oxysterol, LY295427 induces expression of the LDL receptor and the metabolism of LDL and enhances the transcription of many genes involved in cholesterol metabolism. Gene induction is a consequence of derepression of SREBP processing and occurs within 4–6 h of compound addition. A stereoisomer of LY295427, differing only in the orientation of a hydroxyl group at carbon 3 of the molecule (LY306039; Fig. 1), does not induce SREBP processing. LY295427 action requires the presence of SCAP and the Site 1 protease and thus appears to activate the normal processing pathway that generates SREBP when levels of sterols decrease in the cell. LY295427 reverses the suppression of SREBP processing mediated by numerous oxysterols and cholesterol derivatives but does not affect suppression effected by LDL or by...
treatment of cells with sphingomyelinase. The compound decreases the stimulation of cholesteryl ester formation in cells incubated with oxysterols but not that mediated by LDL. We conclude that LY295427 selectively interferes with the ability of oxysterols to regulate SREBP processing and cholesterol metabolism in cultured cells.

There are several possible mechanisms by which LY295427 could disrupt the regulation of SREBP processing. First, the compound could activate a surrogate proteolysis pathway. Treatment of cells with the protein kinase inhibitor staurosporine leads to activation of one or more caspase enzymes that cleave SREBP precursors to release transcriptionally active fragments (34–36). In a similar fashion, LY295427 could induce these or other proteases of the apoptotic pathways in cultured cells causing inappropriate activation of SREBPs. This mechanism appears unlikely because LY295427 activity in staurosporine-sensitive CHO-7 cells requires SCAP and the Site 1 protease (Fig. 7) and because SREBP-responsive genes are activated by the compound, whereas stress-responsive genes such as those encoding heat shock proteins, p53, or NFκB are not (Table II).

Second, LY295427 could cause selective precipitation of oxysterols in a manner similar to the ability of digitonin to render cholesterol insoluble (37). We could detect, however, no such precipitation activity when [3H]25-hydroxycholesterol (1 μg/ml) was incubated with seven different concentrations of LY295427 (1–60 μM, 0.4–24 μg/ml) under conditions identical to those used to reverse the suppression of SREBP processing by oxysterols in cultured cells and under which digitonin quantitatively precipitated cholesterol (data not shown). Furthermore, LY295427 did not prevent the ability of oxysterols to decrease the incorporation of acetate into sterols in HepG2 cells (18), again suggesting that oxysterols remain soluble and active in the presence of the compound and able to mediate posttranscriptional regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (38–40).

A third mechanism by which LY295427 may act is through altering the transport of cholesterol from the plasma membrane to the regulatory pool that is sensed by the SCAP-SREBP complex in the endoplasmic reticulum. Several lines of evidence from the current experiments support this hypothesis, beginning with the observation that LY295427 specifically overrides suppression mediated only by oxysterols. 25-Hydroxycholesterol stimulates the movement of cholesterol from the plasma membrane, where a majority of the sterol resides, to the endoplasmic reticulum, in which it exerts regulatory effects and serves as a substrate for the synthesis of cholesteryl esters (33, 41). The mechanism by which oxysterols induce movement of cholesterol within the cell has not been defined; however, if LY295427 were to interfere with this transport, SREBP cleavage would be activated in the presence of oxysterols and the stimulation of cholesteryl ester formation would be attenuated. These are exactly the outcomes observed in treated cells (Figs. 5 and 11).

The failure of LY295427 to mediate these effects when cells are grown in the presence of LDL (Table I and Figs. 5 and 11) may reflect the delivery of LDL cholesterol to the endoplasmic reticulum by a different pathway (42, 43). The stimulation of cholesteryl ester formation by LDL can be of greater magnitude

![Fig. 10. LY295427 reverses inhibition of SREBP processing mediated by different sterols in immortalized human fibroblasts.](http://www.jbc.org/)

![Fig. 11. Effect of LY295427 on cholesteryl ester formation in human diploid fibroblasts.](http://www.jbc.org/)

**LY295427 Mechanism of Action**
SREBP proteolysis, the current results further suggest that if, as appears to be the case in cultured cells, the compound diminish SREBP processing by different mechanisms, only one of which is sensitive to the compound.

It seems unlikely that LY295427 is acting by directly inhibiting a sterol transport protein given the 4–6-h period required for the compound to act (Fig. 9). These kinetics are more suggestive of an effect on gene transcription or protein turnover. SREBP precursor-SCAP complexes are usually present in the endoplasmic reticulum regardless of the presence or absence of sterols (Fig. 4), and the movement of the complex from the endoplasmic reticulum to the Golgi can be detected within 15 min of a reduction in cellular cholesterol (8). Furthermore, pulse-chase experiments indicate that the regulatory pool of cholesterol in the endoplasmic reticulum turns over very rapidly ($T_{1/2}$ = 40 min) (48). These observations indicate that an acute inhibition of transport should have a rapid effect on SREBP processing rather than the slow time course observed for LY295427 action.

cDNA array experiments have so far revealed only SREBP-1c and for retention of the SREBP-SCAP complex within the endoplasmic reticulum via different pathways.

In conclusion, the in vitro experiments presented here suggest that the hypcholesterolemic effects of LY295427 observed in vivo (16, 18) are due to a derepression of SREBP processing. If, as appears to be the case in cultured cells, the compound specifically antagonizes the ability of oxysterols to decrease SREBP proteolysis, the current results further suggest that oxysterols may play an important regulatory role in the whole animal. Our future experiments will be directed toward identifying the target protein through which LY295427 acts.
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The Hypocholesterolemic Agent LY295427 Reverses Suppression of Sterol Regulatory Element-binding Protein Processing Mediated by Oxysterols
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