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Down-regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase mRNA Levels and Synthesis in Syrian Hamster C100 Cells by the Oxidosqualene Cyclase Inhibitor [4′-(6-allyl-ethyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone (Ro 48–8071): Comparison to Simvastatin

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ABSTRACT. *In vivo* inhibition of 2,3-oxidosqualene:lanosterol cyclase (OSC, E.C. 5.4.99.7)—the enzyme which catalyzes the cyclization of monooxidosqualene to lanosterol—does not result in elevated 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) activity. This trait is attributed to increased levels of oxysterols, produced upon partial inhibition of OSC, that suppress HMGR and other sterol-responsive genes. The OSC inhibitor [4′-(6-allyl-ethyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone (Ro 48–8071) was shown earlier to lower low-density lipoprotein (LDL) cholesterol in hamsters with no increase in hepatic HMGR, in contrast to simvastatin. To delineate the regulatory mechanism(s) by which Ro 48–8071 reduces cholesterol synthesis without raising HMGR levels, Syrian hamster C100 cells were incubated with either Ro 48–8071 or simvastatin, and their effects on cholesterol synthesis and LDL uptake, as well as on HMGR mRNA levels and rates of synthesis, were determined. Using RNase protection and radioimmunoprecipitation assays, we found that, in the absence of LDL in the culture medium, both HMGR mRNA levels and synthesis were reduced with concentrations of Ro 48–8071 inhibiting cholesterol synthesis by 50–75%, whereas LDL uptake was either reduced or unchanged. In contrast, simvastatin, at concentrations inhibiting cholesterol synthesis by the same 50–75%, increased both HMGR mRNA levels and synthesis, as well as LDL uptake. In the presence of LDL, HMGR mRNA levels and synthesis along with LDL uptake were little affected after incubation with Ro 48–8071. Still, simvastatin markedly increased both HMGR mRNA levels and synthesis in cells incubated in the presence of LDL, leaving LDL uptake unaffected. These data suggest that inhibition of OSC by Ro 48–8071 results in an indirect down-regulation of HMGR mRNA levels and synthesis. BIOCHEM PHARMACOL **56**;4: 439–449, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Cholesterol; 3-hydroxy-3-methylglutaryl CoA reductase; 2,3-oxidosqualene:lanosterol cyclase; Ro 48–8071; post-transcriptional; transcriptional

Ro 48–8071 is a specific and potent inhibitor of OSC§ (E.C. 5.4.99.7), whose pharmacological properties were recently described [1]. It lowered LDL cholesterol in hamsters with no hepatotoxicity and did not reduce liver and heart coenzyme Q10 levels. As anticipated from the self-limited regulatory loop triggered by inhibition of OSC [2–7], hamsters treated with Ro 48–8071 showed no increase in *ex vivo* activity of hepatic HMGR, squalene synthase and OSC, in contrast to simvastatin [1].

OSC, a microsomal enzyme which catalyzes the cyclization of 2,3-oxidosqualene to lanosterol [8], is a peculiar target in the cholesterol synthetic pathway. Firstly, because OSC is located downstream of farnesylpyrophosphate, inhibitors of this enzyme are unlikely to block the production of nonsterol isoprenoids, coenzymes Q and dolichol, and to affect protein prenylation, in contrast to statins [1, 9–12]. Secondly, partial inhibition of OSC should trigger a negative feedback regulatory loop resulting from: 1) epoxidation of squalene to MOS followed by 2) epoxidation of MOS to

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§ *Abbreviations*: C100 cells, Syrian hamster kidney C100 cells; DMEM, Dulbecco's modified Eagle's medium; DOS, dioxidosqualene; HMGR, HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low-density lipoprotein; LPDS, lipoprotein deficient serum; MOS, monooxidosqualene; OSC, 2,3-oxidosqualene:lanosterol cyclase; Ro 48–8071, [4′-(6-allyl-ethyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone; RP, ribosomal protein; and SRE, sterol regulatory element.

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DOS; 3) preferential cyclization of DOS versus MOS to 24(S),25-epoxylanosterol [2, 7, 13]; and 4) conversion to 24(S),25-epoxycholesterol [1, 2, 5, 6, 14, 15], which is a potent down-regulator of HMGR expression [4–7]. Although OSC inhibitors effectively block cholesterol synthesis [14] and reduce feedback regulation of sterol-responsive genes [16, 17], they do not necessarily mediate an increase in HMGR as reported for HMGR inhibitors [1, 2]. The reason for this difference between these two classes of inhibitors is not fully understood, although it has been proposed that increased production of MOS, DOS, or 24,25-epoxycholesterol in cells treated with OSC inhibitors could play a role in this regulatory process [4, 6].

The aim of this paper was to further delineate regulatory mechanism(s) through which the novel OSC inhibitor Ro 48–8071 mediates its effects on HMGR expression under conditions that minimize any effect of HMGR degradation on the apparent rate of synthesis [18, 19]. The baby hamster cell line C100 was selected for this study because it exhibits a four-fold amplification of the HMGR gene and expresses high levels of HMGR compared to parental cell line SV28, which facilitates measurements of HMGR mRNA and synthesis [20, 21]. The C100 cells are not considered to be mutant, and there is no evidence that the HMGR gene sequence has been altered in these cells. As in other cell lines with one gene copy, HMGR is regulated in C100 cells, transcriptionally by oxysterols [22] and post-transcriptionally by mevalonate-derived isoprenoids or nonsterols [17, 18, 23].

We present evidence that inhibition of OSC by Ro 48–8071 results in down-regulation of HMGR synthesis and mRNA, and that this effect is mediated in part at the posttranscriptional level. Our study also shows that Ro 48–8071 and simvastatin, an HMGR inhibitor, exert distinctly different effects on the feedback control of HMGR mRNA levels and synthesis.

MATERIALS AND METHODS

Chemicals

[4′-(6-Allyl-ethyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl)-methanone (Ro 48–8071) was synthesized as described [1], and dissolved in DMSO as a 10 mM stock solution. Simvastatin was a generous gift from Merck & Co.; it was prepared as the active opened lactone form [24], neutralized with HCl and brought to a final concentration of 1 mM in distilled water. The following were from commercial sources: [2-^{14}C]sodium acetate, 2.15 GBq/mmol (Amersham); [Na^{125}I] from Medipro AG; [^{35}S]methionine, 40.7 TBq/mmol (ICN Radiochemicals). All solvents were from Fluka and all other chemicals were from Sigma.

Isolation and Iodination of Human LDL

Plasma from blood of healthy volunteers collected on EDTA was obtained at the Blutspendezentrum in Basel and stored at −20°. LDLs were isolated by sequential ultracentrifugation at a density of $1.019 < d < 1.063$ g/mL [25], stored at 4° at a concentration of 0.5–2 mg/mL in PBS containing 0.2 mM EDTA and 40 μM 2,6-di-*tert*-butyl-4-methylphenol (BHT), and used within the next two weeks. LDLs were iodinated with ^{125}I at a specific activity of 500–800 dpm/ng of apolipoprotein [26, 27], and stored at 4° in PBS containing 0.2 mM EDTA and 40 μM 2,6-di-*tert*-butyl-4-methylphenol. Purity of each preparation was checked by agarose gel electrophoresis (Paragon Lipogel, Beckman).

Cells and Culture Conditions

The baby hamster kidney cell line C100 [21] was obtained from Dr. Robert Simoni, Department of Biologic Sciences, Stanford Medical School. Cells were cultured routinely at 37° in a humidified 5% CO_2, 95% air atmosphere in Ham's F12 medium containing penicillin (100 IU/mL) and streptomycin (100 IU/mL), supplemented with 10% (v/v) fetal calf serum (Life Technologies), and passaged once a week with Trypsin + EDTA (Life Technologies). LPDS was prepared from fetal calf serum by KBr density flotation, followed by repeated dialysis and sterile filtration.

Incorporation of ^{14}C-Acetate into Nonsaponifiable Lipids of Cultured C100 Cells

C100 cells were harvested using Trypsin + EDTA (Life Technologies), and washed once by centrifugation in DMEM-5% LPDS. In this set-up F12 medium was replaced by DMEM to obtain optimal attachment of cells to culture glass tubes. Cells were counted, diluted and seeded in sterile collagen-coated screw-cap glass tubes at a density of 3.5×10^4 cells in 700 μL of DMEM-5% LPDS per tube. Twenty-four hours after seeding, each tube received: 1) 100 μL of DMEM-5% LPDS with or without 10-fold concentrated human LDL; 2) 100 μL of DMEM-5% LPDS containing ^{14}C-acetate; and 3) 100 μL of intermediate dilutions of Ro 48–8071 or simvastatin in DMEM-5% LPDS, to reach the appropriate concentrations of drug in a final volume of 1 mL. Final concentration of LDL was 0 or 100 μg/mL, and ^{14}C-acetate was 74 kBq/mL. After an additional incubation of 18 hr at 37°, cells were washed once with 2 mL of PBS. Lipids were saponified directly in the culture glass tube, extracted and separated by TLC together with lipid standards as described [1]. For further identification, nonsaponifiable lipids were also analyzed by reverse phase HPLC as described [1]. Data for synthesis of cholesterol and other nonsaponifiable lipids were expressed as dpm of ^{14}C-acetate incorporated per tube in cholesterol or total nonsaponifiable lipids excluding free fatty acids.

Uptake of ^{125}I-LDL by Cultured C100 Cells

C100 cells were harvested using Tryspin + EDTA (Life Technologies) and washed once by centrifugation in F12–2% LPDS. Cells were counted, diluted and seeded in wells of 48-well culture plates at a density of 3.5×10^4 cells

in 400 μL of F12–2% LPDS per well. Twenty-four hours after seeding, each well received: 1) 50 μL of F12–2% LPDS with or without 10-fold concentrated human LDL; and 2) 50 μL of intermediate dilutions of Ro 48–8071 or simvastatin, to reach the appropriate concentrations of drug in a final volume of 500 μL. The final concentration of LDL was 0 or 100 μg/mL. Cells were incubated a further 18 hr at 37° prior to testing ^{125}I-LDL uptake, as described previously [28] with few modifications, as follows. Cells were washed once with 500 μL of F12–2% LPDS and preincubated for 30 min in F12–2% LPDS in order to eliminate receptor-bound nonradioactive LDL. Then, medium was removed by aspiration, and each well received 300 μL F12–2% LPDS supplemented with 1.5 mM $CaCl_2$ containing 5 μg/mL of ^{125}I-LDL. Samples were also tested in the presence of an excess of 100 μg/mL of nonlabeled LDL to measure nonspecific uptake. After 2 hr of incubation at 37°, the cells were washed with 3 × 1 mL of PBS at room temperature, and directly solubilized by adding 250 μL of BCA protein reagent (pH > 11, Pierce) and microwaving at 150 W for 1 min. After 30 min at 37°, 200-μL aliquots of solubilized cells in the BCA reagent were transferred into the wells of a 96-well microplate, and absorbance was measured at 562 nm in a microplate reader (Molecular Devices) to determine protein content. Standard solutions of BSA were run in parallel for calibration. Then, 180-μL aliquots of each radioactive sample in the BCA reagent were transferred into plastic tubes and counted. Data were calculated as ng of apolipoprotein taken up per mg of cellular protein.

RNA Isolation

Cells were lysed in 5.0 M guanidinium isothiocyanate, 0.5% sodium lauryl sarcosine, and 0.025 M sodium citrate, pH 7.0. RNA was purified from this lysate by the single-step acid guanidinium isothio-cyanate:phenol:chloroform extraction procedure [22, 29].

RNase Protection Assays

Both HMGR and RP S17 mRNAs were determined by RNase protection assays as described previously [23]. SP6 RNA polymerase was used to synthesize antisense RNA transcripts complementary to HMGR mRNA from *Eco*RI cut pRedD2, which contains a 400-bp *Hin*dIII fragment of the coding region for hamster HMGR. To detect RP S17 mRNA, pRPS17, which contains a 369-bp *Pst*I fragment representing a full-length cDNA for hamster RP S17 mRNA [30], was linearized with *Ava*II. Antisense RNA transcripts 200 bp in size were subsequently transcribed from *Ava*II-restricted plasmid DNA using T7 RNA polymerase. RNA was transcribed from each of the linearized templates according to the conditions of Melton *et al.* [31] using [α-^{32}P]CTP to label transcripts. Approximately 150,000 dpm of each ^{32}P-labeled RNA transcript were added to RNA samples and hybridized at 50° for 16 hr as described [23]. All subsequent protocols were performed using an RPA II Ribonuclease Protection Assay Kit (Ambion). Hybridized RNAs were digested with RNase A and T1 according to the manufacturer's instructions, and then subjected to electrophoresis on 6–8% polyacrylamide:7 M urea gels. Protected RNA species representing HMGR and RP S17 mRNAs were visualized by autoradiography. Relative changes in mRNA were estimated by laser densitometric scanning of autoradiographs.

Labeling Cells with [^{35}S]-Methionine

One million C100 cells were inoculated into 60-mm tissue culture plates and incubated for 24 hr in DMEM-5% FCS. The medium was then changed to DMEM-2% LPDS supplemented with Ro 48–8071 or simvastatin, either in the absence or presence of LDL (100 μg/mL), and cells were cultured for 16 hr. Subsequently, cells were incubated for 1 hr in methionine-free medium containing 2.8 MBq/mL of ^{35}S-methionine.

Radioimmunoprecipitation of HMGR

The procedure used was that of Hardeman *et al.* [20] modified by Peffley et al. [32]. Cells labeled with ^{35}S-methionine were harvested by scraping into ice-cold 50 mM HEPES, 0.15 M NaCl, and 5 mM EDTA, pH 7.4. The cells were lysed and HMGR solubilized in 1% Triton X-100 in 0.5 M KCl, 20 mM EDTA, 100 mM sucrose, 3 mM phenylmethane sulfonyl fluoride, 300 μM leupeptin, and 10 mM dithiothreitol in 5 mM K_3PO_4, pH 7.0. The lysate was cleared of insoluble material by centrifugation at 8,000 *g* at 4° for 15 min. HMGR was immunoprecipitated with a rat-specific HMGR antibody as described [32]. The anti-HMGR antibody was a kind gift from Dr. Peter Edwards, Department of Medicine, University of California at Los Angeles. Electrophoresis, fluorography and quantitation of HMGR were done as described [18].

RESULTS

Incorporation of ^{14}C-Acetate into Nonsaponifiable Lipids of Cultured C100 Cells

In the absence of LDL, incorporation of ^{14}C-acetate into cellular cholesterol was inhibited dose dependently by Ro 48–8071 with an IC_{50} of 1.3 nM (Fig. 1A, B). This inhibition was associated with the production of radioactive MOS and DOS at concentrations >3 nM. Radioactivity of a polar lipid with an Rf of 0.16 on TLC increased with increasing concentrations of Ro 48–8071 up to 10 nM, and decreased further at higher concentrations. This polar lipid was characterized as 24,25-epoxycholesterol by TLC and HPLC [1], in agreement with Panini *et al.* [14]. At 3–30 nM Ro 48–8071, radioactivity of 24,25-epoxycholesterol was approximately one-third of that in cholesterol of control cells. Nevertheless, the mass of 24,25-epoxycholesterol relative to cholesterol in treated cells might have been

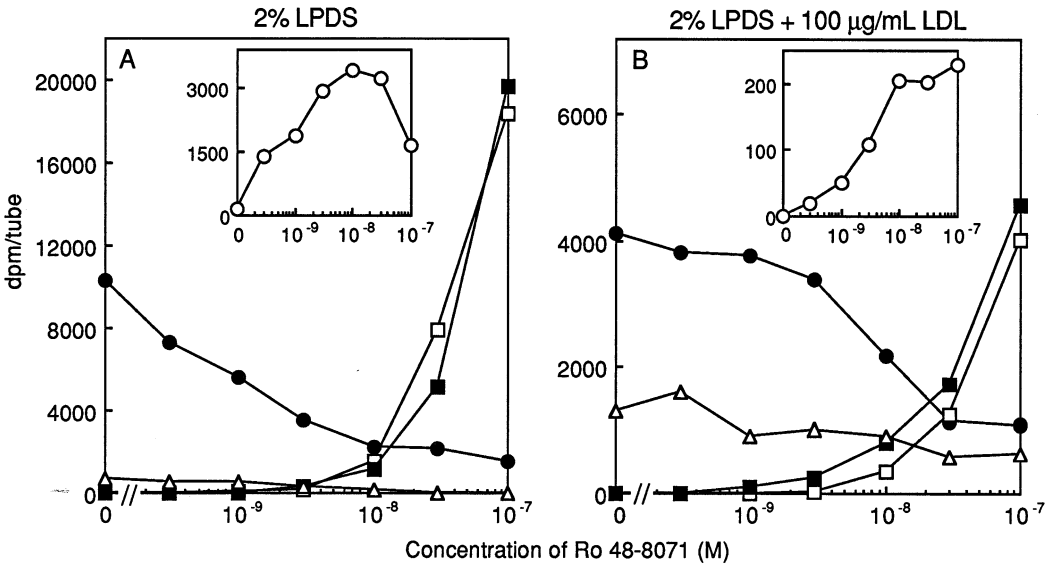

FIG. 1. Effects of Ro 48–8071 on the incorporation of ^{14}C-acetate into nonsaponifiable lipids of cultured C100 cells. C100 cells cultured in sterile collagen-coated glass tubes were preincubated for 24 hr in medium-LPDS, and then incubated for 18 hr in medium-LPDS containing 2 μCi/mL of ^{14}C-acetate with increasing concentrations of Ro 48–8071, in the absence (A) or presence (B) of 100 μg/mL of LDL. After washing the cells with PBS, lipids were extracted, saponified, extracted and separated by TLC, prior to identification and quantification by PhosphorImager scanning. The symbols are: cholesterol (●), DOS (□) and MOS (■), squalene (△) and in insert 24,25-epoxycholesterol (○). Data are expressed as dpm of ^{14}C-acetate incorporated per lipid into each culture tube, and each point is the mean value of two determinations.

less because of preexisting cholesterol. The relative increase in radioactive 24,25-epoxycholesterol was similar to that found in Hep-G2 cells under the same conditions, but higher than that reported for BIBX79 [2]; in the latter work, cells were exposed to the OSC inhibitor for 16 hr prior to a coincubation of 2 hr with ^{14}C-acetate, which might have limited the amount of 24,25-epoxycholesterol produced. Low amounts of radioactive squalene relative to cholesterol were detected, which were reduced with increasing concentrations of Ro 48–8071. Ro 48–8071 caused no accumulation of desmosterol, lanosterol or other nonsaponifiable lipids. Overall, the carbon flow from acetate measured as the net incorporation in total nonsaponifiable lipids was reduced at concentrations up to 30 nM of Ro 48–8071 and increased afterward (Fig. 2A) mostly because of massive incorporation into DOS and MOS.

In order to match *in vivo* conditions where exogenous, LDL-derived cholesterol can modify the balance of cellular regulatory metabolites modulating HMGR and LDL receptor, the effects of Ro 48–8071 and simvastatin were measured in the presence of 100 μg/mL of LDL. Ro 48–8071 inhibited ^{14}C-acetate incorporation into choles-

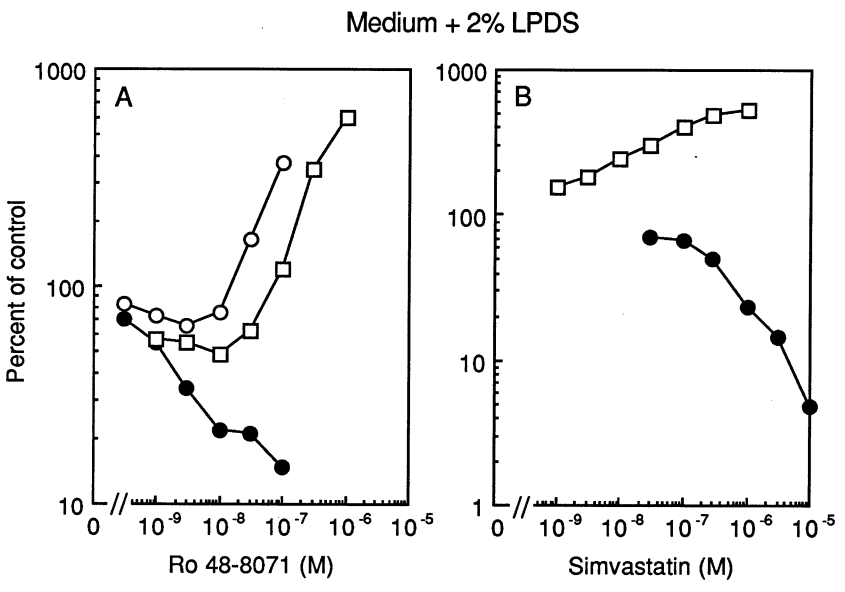

FIG. 2. Effects of Ro 48–8071 and simvastatin on ^{14}C-acetate incorporation into total nonsaponifiable lipids and cholesterol, and ^{125}I-LDL uptake in the absence of LDL. C100 cells were preincubated for 24 hr in 48-well culture plates in medium-LPDS, and then incubated for 18 hr in medium-LPDS with increasing concentrations of either Ro 48–8071 (A) or simvastatin (B). Then, uptake of ^{125}I-LDL was measured after 2 hr of incubation as described in Materials and Methods. LDL uptake (□) is expressed in percent of control (no drug added). Control value for uptake of ^{125}I-LDL was 27–40 ng of apolipoprotein per mg of cellular protein, and each point is the mean value of at least three determinations. Incorporation of ^{14}C-acetate into total nonsaponifiable lipids excluding free fatty acids (○) and cholesterol (●) was measured as in Fig. 1 and expressed in percent of control; each point is the mean value of two determinations.

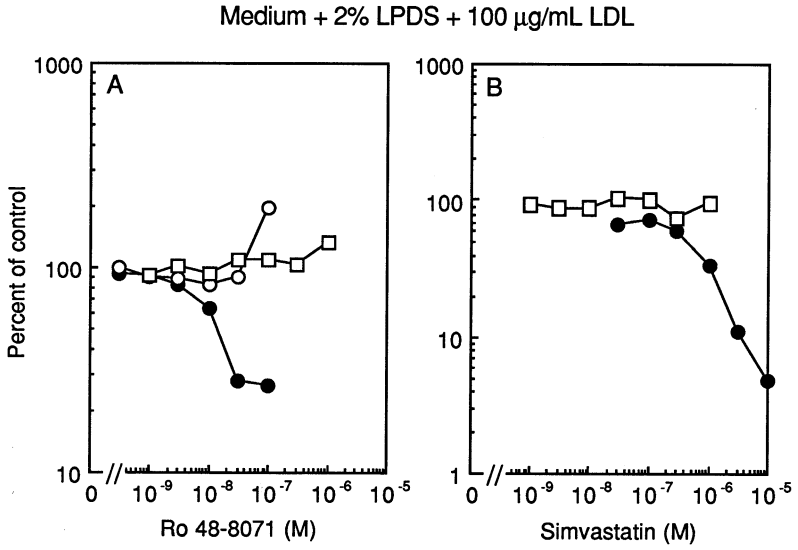

FIG. 3. Effects of Ro 48–8071 and simvastatin on ^{14}C-acetate incorporation into total nonsaponifiable lipids and cholesterol, and ^{125}I-LDL uptake in the presence of LDL. C100 cells were preincubated for 24 hr in 48-well culture plates in medium-LPDS, and then incubated for 18 hr in medium-LPDS with increasing concentrations of either Ro 48–8071 (A) or simvastatin (B), in the presence of 100 μg/mL of LDL. Then, uptake of ^{125}I-LDL (□) and incorporation of ^{14}C-acetate into total nonsaponifiable lipids excluding free fatty acids (○) and cholesterol (●) were measured, calculated and plotted in percent of control (no drug added) as in Fig. 2. Control value for uptake of ^{125}I-LDL was 14–20 ng of apolipoprotein per mg of cellular protein.

terol dose dependently in cells incubated in the presence of LDL, although it was ~10 times less potent than in the absence of LDL with an IC_{50} of 15 nM (Fig. 1B and Fig. 3A). Radioactive 24–25-epoxycholesterol did not exhibit the biphasic dose-response curve found in the absence of LDL, and increased up to 6% of cholesterol radioactivity in control cells at 100 nM Ro 48–8071. At 1.3 nM of Ro 48–8071, i.e. the IC_{50} for ^{14}C-acetate incorporation into cholesterol in the absence of LDL, radioactive 24,25-epoxycholesterol was 18% of radioactive cholesterol in control cells. In contrast, at 15 nM Ro 48–8071, i.e., the IC_{50} for ^{14}C-acetate incorporation in cholesterol in the presence of LDL, radioactive 24,25-epoxycholesterol was only 5% of radioactive cholesterol in control cells, perhaps because of alterations in carbon partitioning between the sterol and epoxysterol branches of the pathway, and of isotopic dilution by exogenous, nonradioactive cholesterol. OSC inhibition in the presence of LDL was also associated with the production of MOS and DOS, though in a lesser amount relative to radioactive cholesterol as compared to cells incubated in the absence of LDL. In the presence of LDL, the carbon flow from acetate measured as the net incorporation into total nonsaponifiable lipids was unchanged at concentrations up to 10 nM of Ro 48–8071, and increased three-fold thereafter. No radioactivity associated with nonsaponifiable lipids was found in the medium.

In contrast to Ro 48–8071, the HMGR inhibitor simvastatin should affect the production of mevalonate carbon for all isoprenoids and sterols, and incorporation of ^{14}C-acetate into cholesterol is therefore a reasonable approximation of the carbon flow in the pathway. Simvastatin reduced incorporation of ^{14}C-acetate into cholesterol with an IC_{50} of 300 nM and 450 nM in the absence or presence of LDL, respectively (Figs. 2B and 3B) with no accumulation of nonsterol and sterol metabolites.

Uptake of ^{125}I-LDL

The uptake of ^{125}I-LDL by C100 cells was measured after a treatment of 18 hr with increasing concentrations of either Ro 48–8071 or simvastatin, in the absence or presence of 100 μg/mL of LDL. An incubation time of 2 hr was used, providing a fair and quantitative representation of LDL receptor activity. In the absence of LDL, Ro 48–8071 at concentrations of 1–30 nM reduced LDL uptake significantly by ~50% (Fig. 2A). At higher concentrations, Ro 48–8071 strongly stimulated LDL uptake with an increase of 3- and six-fold at 300 nM and 1000 nM, respectively. In the absence of LDL, simvastatin stimulated LDL in a dose-dependent manner with a 2- to 5-fold increase at 10–1000 nM (Fig. 2A). Ro 48–8071 at 100 nM was able to block incorporation of ^{14}C-acetate into cholesterol by ~75% with little alteration of LDL uptake as compared to control cells with no drug (Fig. 2A). In contrast, simvastatin at 1000 nM, which inhibited cholesterol synthesis by the same ~75% strongly stimulated LDL uptake (Fig. 2B). These results suggest that the pools of metabolites regulating cholesterol synthesis and LDL receptors in cells treated with Ro 48–8071 or simvastatin are not identical. As expected, addition of LDL to the medium caused a reduction in basal LDL uptake, i.e. 14–20 ng/mg in the presence of LDL vs 27–40 ng/mg in the absence of LDL. In the presence of LDL, Ro 48–8071 increased LDL uptake slightly at the highest concentration of 1000 nM, whereas simvastatin had no effect (Fig. 3A, B).

Effects of Ro 48–8071 and Simvastatin on HMGR mRNA Levels

Ro 48–8071 mediates the blockade of cholesterol biosynthesis by preventing cyclization of MOS to lanosterol. The MOS formed in response to the inhibition is converted to DOS, which is subsequently cyclized by OSC to 24(S),25-

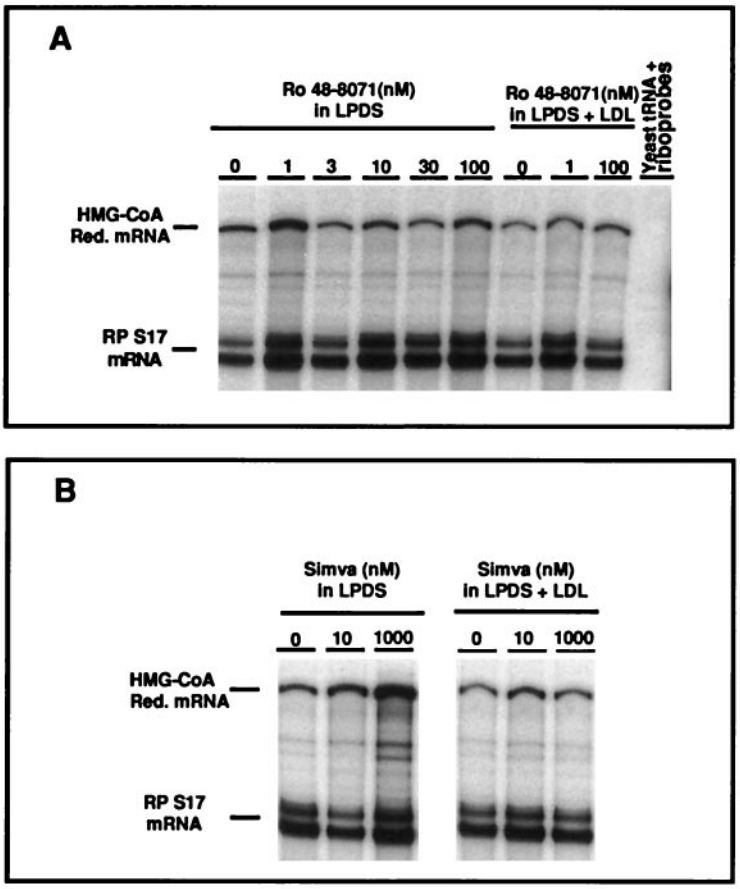

FIG. 4. Autoradiographs of RNase protection assays representing levels of HMGR and RP S17 mRNA levels in cells treated with Ro 48–8071 or simvastatin. Equal amounts (4 μg) of RNA from each treatment condition were used in all RNase protection assays. Cells cultured in medium-LPDS were treated with increasing concentrations of Ro 48–8071 or simvastatin in the absence or presence of LDL (100 μg/mL). Incubations were continued for an additional 18 hr after which cells were lysed directly in the tissue culture plates with 5 M guanidinium thiocyanate: 0.5% sodium lauryl sarcosine as described previously [29]. RNA was subsequently purified from the lysates as described in Materials and Methods. HMGR and RP S17 mRNA levels were determined by RNase protection assays. Effects of Ro 48–8071 and simvastatin on HMGR mRNA levels are shown in panels A and B, respectively.

epoxylanosterol [1, 2, 7, 13]. Both of these effects—reduction of net cholesterol production and formation of 24,25-epoxycholesterol—would affect feedback regulation of cholesterogenic protein levels [2, 6, 16]. Because HMGR is a key determinant in the regulation of cholesterol biosynthesis, we determined the effects of Ro 48–8071 on both mRNA levels and rates of synthesis for this enzyme in C100 cells.

Figure 4A is an autoradiograph illustrating the effects of Ro 48–8071 on HMGR and control RP S17 mRNA levels in C100 cells; this autoradiograph was scanned by laser densitometry for quantification. As expected, HMGR mRNA levels were reduced approximately two-fold in cells cultured in the presence of LDL compared to those of cells cultured in the absence of LDL because of the feedback suppression mediated by LDL-derived cholesterol. Under these conditions, transcription initiated via the SRE in the 5′-promoter region of the HMGR gene is decreased [16, 33].

The addition of 1 nM Ro 48–8071 to cells cultured in LPDS did not change HMGR mRNA levels. However, Ro 48–8071 at concentrations ranging from 3 to 100 nM lowered HMGR mRNA levels by approximately two-fold when added to cells cultured in LPDS, a level comparable to that in control cells cultured in the presence of LDL (Fig. 5A, B). In no case was there an effect on amounts of control RP S17 mRNA attributable to changes in Ro 48–8071 or LDL levels in the medium. The effects of Ro 48–8071 on HMGR mRNA levels were also determined in the presence of LDL (Fig. 4B). Compared to control cells, there was a nonsignificant reduction in HMGR mRNA with 1 nM Ro 48–8071, and a slight increase at 100 nM (Fig. 5B).

The effects of Ro 48–8071 on HMGR mRNA levels were compared to those of the HMGR inhibitor simvastatin as shown in the autoradiograph of Fig. 4B. In contrast to Ro 48–8071, simvastatin at concentrations of 10 and 1000 nM increased HMGR mRNA levels by approximately 3- and 4-fold, respectively, compared to control cells cultured in LPDS (Fig. 5A). In the presence of LDL, simvastatin at 10 and 1000 nM also increased HMGR mRNA levels approximately three-fold compared to control cells (Fig. 5B).

Effects of Ro 48–8071 and Simvastatin on HMGR Synthesis

Fluorographs representing immunoprecipitated HMGR are shown in Fig. 6, and the amounts of ^{35}S-methionine immunoprecipitated from each treatment condition are shown in Fig. 7A and B. The rate of HMGR synthesis in cells cultured in the presence of LDL was reduced approximately two-fold compared to that of cells cultured in the absence of LDL. The magnitude of this decrease was

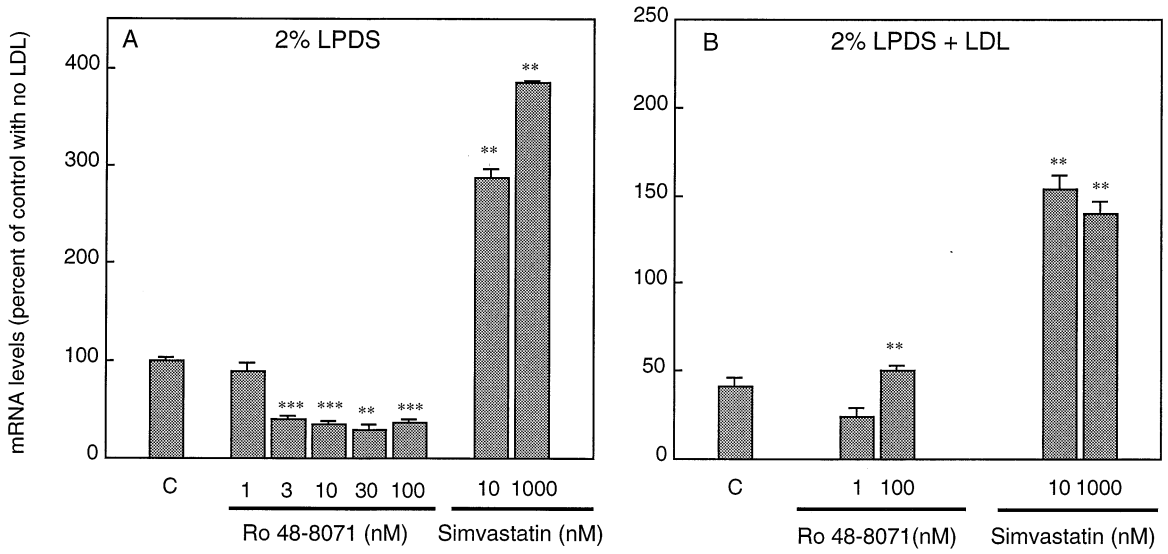

FIG. 5. Quantitative effects of Ro 48–8071 and simvastatin on HMGR mRNA levels in C100 cells. Relative amounts of HMGR and RP S17 mRNAs were quantitated by laser densitometric scanning of autoradiographs shown in panels A and B of Fig. 4, and calculated as a percent of the mRNA content of control cells (no drug added) cultured in the absence (A) or presence (B) of LDL. Each value is the average of three independent determinations ± SD, significantly different from control at **$P < 0.01$ and ***$P < 0.001$.

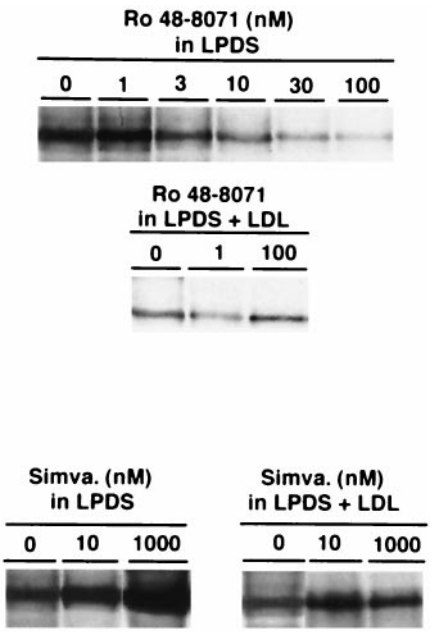

FIG. 6. Fluorographs of representing levels of HMGR protein synthesis in cells treated with Ro 48–8071 or simvastatin. Cells cultured in medium-LPDS were treated with increasing concentrations of Ro 48–8071 or simvastatin in the absence or presence of LDL (100 μg/mL). Incubations were continued for an additional 18 hr after which cells were incubated in methionine-free medium supplemented as described above. Cells were then pulsed for 1 hr with ^{35}S-methionine, and HMGR was solubilized with Triton X-100 and immunoprecipitated with a rat-specific antibody. Immunoprecipitated HMGR was visualized by fluorography on a 10% polyacrylamide gel and appears as a single protein species with a molecular weight of 97.4 kDa.

equivalent to that observed for HMGR mRNA levels under identical experimental conditions.

When 1 nM Ro 48–8071 was added to cells cultured in LPDS, HMGR synthesis was decreased more than two-fold, a result in contrast to that observed for HMGR mRNA levels, which did not change with this drug concentration. Metabolic labeling of C100 cells with ^{35}S-methionine was carried out under conditions that minimize any effect of HMGR degradation on the apparent rate of synthesis [18, 19]. Therefore, this decrease in HMGR synthesis appears to be mediated primarily through a posttranscriptional effect at translational level. Moreover, the HMGR protein is degraded rapidly in C100 cells [18, 34] compared to Chinese hamster ovary cells [32, 35] and exhibits no apparent regulated degradation [18] except under particular conditions with compactin plus high mevalonate and 25-hydroxycholesterol added to the culture medium [34]. When 3 to 100 nM Ro 48–8071 were added to cells cultured in LPDS, HMGR synthesis was also reduced two-fold, an effect equivalent to that observed with a drug concentration of 1 nM. Higher levels of Ro 48–8071 did not increase the degree of posttranscriptional suppression of HMGR synthesis observed with the lowest inhibitor concentration. Lack of additional suppression in HMGR synthesis when the concentration of Ro 48–8071 is increased may indicate that post-transcriptional control is already maximal at the lowest concentrations of OSC inhibitor.

In the presence of LDL, Ro 48–8071 had less pronounced effects on HMGR synthesis. At 1 nM, Ro 48–8071 increased HMGR synthesis slightly as compared to cells cultured in the presence of LDL (Fig. 7B), and there was a decrease in HMGR synthesis at 100 nM.

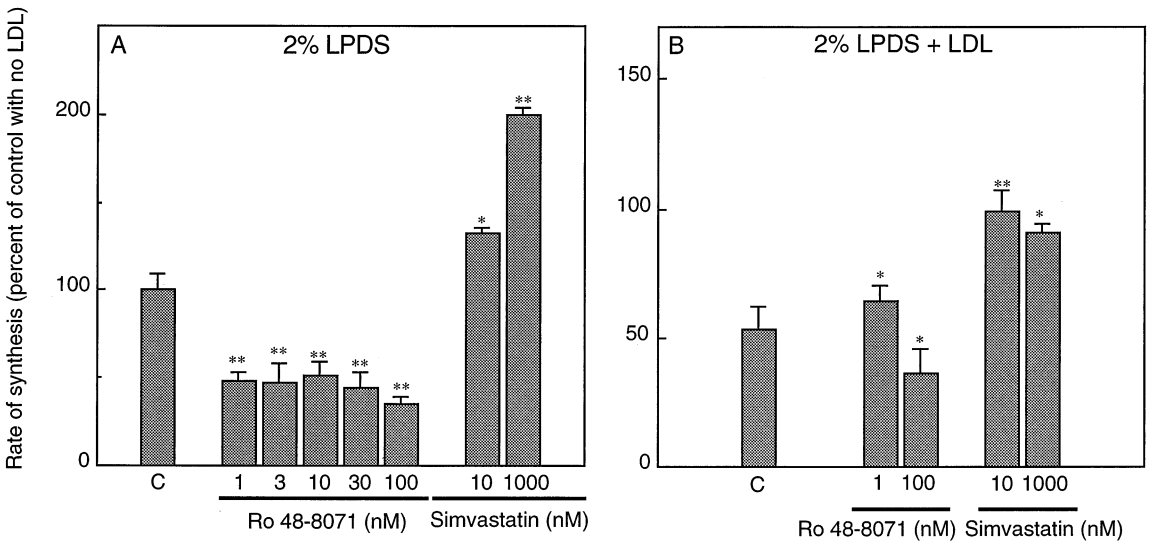

FIG. 7. Quantitative effects of Ro 48–8071 and simvastatin on HMGR protein synthesis in C100 cells. The relative rate of synthesis for HMGR was determined by cutting out the 97.4 kDa species corresponding to HMGR in the fluorographs shown in Fig. 6 and counting ^{35}S-methionine by liquid scintillation spectrometry. Values were corrected for incorporation of ^{35}S-methionine based on the amount of TCA precipitable ^{35}S-labeled protein for each treatment condition. Rates of HMGR synthesis were calculated as a percent of the rate of control cells (no drug added) cultured in the absence (A) or presence (B) of LDL. Each value is the average of three independent determinations ± SD, significantly different from control at *$P < 0.05$ and **$P < 0.01$.

In contrast to Ro 48–8071, addition of 10 and 1000 nM simvastatin to cells cultured in LPDS increased HMGR synthesis by 2.5- and 4-fold, respectively. This magnitude of increase for HMGR synthesis is consistent with that determined for HMGR mRNA levels under identical treatment conditions. In the presence of LDL, simvastatin also increased HMGR synthesis by ~two-fold as compared to control cells. Again, the increase in HMGR synthesis was similar to that for HMGR mRNA levels in the presence of LDL.

DISCUSSION

Presumably, the effects of Ro 48–8071 on feedback regulation of HMGR would be mediated indirectly by a self-regulating circuit sometimes referred to as the alternate dioxidosqualene biosynthetic pathway [15, 17]. In this model, partial inhibition of OSC triggers a regulatory loop whereby metabolites of this alternate pathway decrease HMGR, avoiding overproduction of MOS, DOS, and of potentially harmful nonsterol intermediates. Consistently, another potent OSC inhibitor, BIBX79, led to a decrease in HMGR activity in Hep-G2 cells [2]. Similar effects were described earlier for U-18666A, a much less potent OSC inhibitor [36, 37]; however, U-18666A is also known to: 1) inhibit desmosterol-Δ24-reductase [38–40]; and 2) block intracellular cholesterol trafficking [41], which are two confounding effects likely to interfere with cholesterol homeostasis and HMGR regulation. In the present study, we measured incorporation of ^{14}C-acetate into cholesterol and total nonsaponifiable lipids and LDL uptake, as well as HMGR mRNA levels and synthetic rates of the HMGR protein in order to further delineate the basis of Ro 48–8071-mediated regulation of cholesterol biosynthesis. The effects of the OSC inhibitor Ro 48–8071 on these parameters were compared to those in cells treated with simvastatin. The expression of both HMGR and LDL receptor is regulated by an SRE in the 5′-promoter region of the corresponding genes. The SREs of these two genes differ slightly [42], but they should theoretically respond in a similar fashion to alterations of cellular cholesterol [16]. However, some studies do not fully support this view [43, 44], and it remains to be demonstrated that sterol sensitivity for modulation of HMGR and LDL receptor is always identical.

When cholesterol biosynthesis is reduced, feedback suppression of transcription from SREs in the 5′-promoter region of the HMGR gene should be abolished, consequently increasing HMGR mRNA levels [33, 42]. As expected, HMGR mRNA levels increased in cells treated with the HMGR inhibitor simvastatin. In contrast, a unique effect of Ro 48–8071 was the suppression of HMGR mRNA levels in cells treated with 3–100 nM of the OSC inhibitor. Even though incorporation of ^{14}C-acetate into cholesterol was reduced approximately 10-fold with 100 nM Ro 48–8071, HMGR mRNA levels and rates of synthesis were still reduced approximately two-fold compared to control cells in medium-LPDS (Fig. 5 and 7). Clearly, under these conditions it was expected that transcription from HMGR promoter elements would be increased in a manner analogous to that observed in simvastatin-treated cells. An explanation for this discrepancy is that at 100 nM Ro 48–8071, cyclization of MOS and DOS was only partially blocked, allowing for the synthesis of 24,25-epoxycholesterol in significant amounts (Fig. 1, insert). The latter has previously been found to suppress HMGR levels, in part at the transcriptional level [4–7], an effect that may be mediated through modified interactions

of SRE binding proteins with the SRE in the HMGR gene 5′-promoter region [33, 42]. In this study, 24,25-epoxycholesterol, expressed as a function of drug concentration, followed a bell-shaped curve with high levels over the whole dose-range of Ro 48–8071, consistent with a sustained down-regulation of HMGR which may override the increase in transcription expected for this mRNA when cells are depleted of cholesterol.

Despite this reduction in HMGR mRNA levels and rates of synthesis in cells treated with Ro 48–8071, incorporation of ^{14}C-acetate into total nonsaponifiable lipids was markedly increased at concentrations of Ro 48–8071 greater than 10 nM (Fig. 2A). Most of this incorporation was in the MOS and DOS fractions. This discrepancy indicates that incorporation of ^{14}C-acetate into nonsaponifiable lipids in cells treated with Ro 48–8071 is not an accurate reflection of HMGR activity. Suppression of HMGR mRNA levels and rates of synthesis would be expected only when levels of putative HMGR regulators derived from the alternate cholesterol biosynthetic pathway were sufficient to affect promoter activity of the HMGR gene as well as translational efficiency of HMGR mRNA, respectively. In fact, it was observed in an earlier study [17] that in cells treated with the less potent OSC inhibitor TMD, changes in HMGR mRNA levels and rates of synthesis were observed only after 4 hrs of incubation. Therefore, a delay in suppression of HMGR following addition of ^{14}C-acetate and Ro 48–8071 to cells could result in a significant accumulation of ^{14}C-labeled MOS and DOS. This accumulation would also be enhanced if MOS and DOS were stable intermediates, especially if their conversion to epoxycholesterol was delayed because of partially blocked OSC.

Similarly, LDL receptor-mediated uptake of ^{125}I-LDL was decreased with 1–10 nM Ro 48–8071. Sterol-mediated regulation of LDL receptor levels occurs primarily through transcriptional control, which is mediated by an SRE-1 consensus sequence in the 5′-promoter region of the LDL receptor gene [45]. Because 24,25-epoxycholesterol has also been shown to suppress LDL receptor promoter activity [14], this decrease in LDL uptake may reflect a reduction in LDL receptor mRNA levels analogous to that observed for HMGR mRNA levels. However, increasing Ro 48–8071 concentrations up to 300-1000 nM resulted in a paradoxical increase in LDL uptake, most probably due to a greater inhibition of OSC reducing production of epoxylanosterol, and subsequently of 24,25-epoxycholesterol. The concentration-dependent biphasic effect of Ro 48–8071 on 24,25-epoxycholesterol and LDL receptor was also seen in Hep-G2 cells [1]. Whether the effect on LDL receptor activity with high concentrations of Ro 48–8071 is attributable to a combined reduction of cholesterol and 24,25-epoxycholesterol levels is not clear.

Another important aspect of OSC inhibition is its effect on posttranscriptional regulation of HMGR synthesis. When 1 nM Ro 48–8071 was added to cells in medium-LPDS, there was a decrease in HMGR synthesis with no associated change in mRNA levels. This difference between changes in HMGR synthesis and mRNA levels could be attributed to a translaional effect on HMGR synthesis. The translational control of HMGR synthesis is well documented [6, 18, 19, 46, 47]; it is mediated by nonsterol isoprenoid intermediates of cholesterol biosynthesis [17, 19, 23, 48–50], by intermediates of lanosterol demethylation [47], and by oxylanosterol [6]. Recently, Peffley and Gayen [17] determined that the OSC inhibitor TMD mediated the translational suppression of HMGR synthesis when added to lovastatin-treated C100 cells either in the absence or presence of 10 mM mevalonate. In an earlier study [50], this effect was attributed to a mevalonate-derived nonsterol synthesized between farnesyl diphosphate and MOS. Because both TMD and Ro 48–8071 would have similar effects on levels of putative posttranscriptional regulators of HMGR synthesis, the translational suppression of HMGR synthesis found here could also be attributed to this same mevalonate-derived nonsterol.

In summary, at doses inhibiting cholesterol synthesis by 50–75%, Ro 48–8071 over a 100-fold range did not increase HMGR mRNA and synthesis in the absence of LDL. This is in contrast to simvastatin, which increased HMGR mRNA and synthesis several-fold. Similarly, Ro 48–8071 had little effect on HMGR mRNA and synthesis in the presence of LDL, whereas simvastatin increased both HMGR mRNA and synthesis, consistently with what has been observed *in vivo* in hamsters [1]. Inhibiting OSC is unique as compared to inhibition of other target enzymes of this pathway, which would not trigger an epoxysterol-mediated, self-limited loop downregulating the cholesterol synthetic pathway. Accordingly, squalene synthase inhibitors were shown to trigger an increase in HMGR expression [49, 51] and to prevent the mevalonate-mediated suppression of HMGR [17]. The squalene epoxidase inhibitor NB-598 increases mRNA as well as activity of HMGR and LDL receptor in Hep-G2 cells [52], and increases HMGR mRNA levels in C100 cells in the absence or presence of LDL (Peffley and Morand, unpublished observations).

In conclusion, we have determined that treatment of cells with Ro 48–8071 leads to a decrease in HMGR mRNA levels and the HMGR synthetic rate. Increased 24,25-epoxycholesterol levels in Ro 48–8071-treated cells would account for decreased mRNA levels, whereas an unidentified nonsterol intermediate would enhance translational suppression of HMGR synthesis. Although earlier reports have already shown that OSC inhibition triggers a reduction in HMGR activity, our study, using a potent, selective inhibitor, demonstrates for the first time the level at which such regulation takes place. Current research is being directed toward identifying the mediator of post-transcriptional control and characterizing the mechanism through which intermediates of cholesterol biosynthesis decrease the translational efficiency of HMGR mRNA.

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