Effectors of Rapid Homeostatic Responses of Endoplasmic Reticulum Cholesterol and 3-Hydroxy-3-methylglutaryl-CoA Reductase*

Received for publication, August 20, 2007, and in revised form, November 9, 2007 Published, JBC Papers in Press, November 16, 2007, DOI 10.1074/jbc.M709672000

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The cholesterol content of the endoplasmic reticulum (ER) and the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) imbedded therein respond homeostatically within minutes to changes in the level of plasma membrane cholesterol. We have now examined the roles of sterol regulatory element-binding protein (SREBP)-dependent gene expression, side chain oxysterol biosynthesis, and cholesterol precursors in the short term regulation of ER cholesterol levels and HMGR activity. We found that SREBP-dependent gene expression is not required for the response to changes in cell cholesterol of either the pool of ER cholesterol or the rate of cholesterol esterification. It was also found that the acute proteolytic inactivation of HMGR triggered by cholesterol loading required the conversion of cholesterol to 27-hydroxycholesterol. High levels of exogenous 24,25-dihydrolanosterol drove the inactivation of HMGR; lanosterol did not. However, purging endogenous 24,25-dihydrolanosterol, lanosterol, and other biosynthetic sterol intermediates by treating cells with NB-598 did not greatly affect either the setting of their ER cholesterol pool or the inactivation of their HMGR. In summary, neither SREBP-regulated genes nor 27-hydroxycholesterol is involved in setting the ER cholesterol pool. On the other hand, 27-hydroxycholesterol, rather than cholesterol itself or biosynthetic precursors of cholesterol, stimulates the rapid inactivation of HMGR in response to high levels of cholesterol.

Cellular cholesterol is elaborately controlled by multiple sterol-regulated pathways (1, 2). For example, reducing the level of cell cholesterol shifts the balance between the synthesis and the hydrolysis of cholesterol esters toward the free sterol (2). Cholesterol depletion also initiates gene activation, leading to the elaboration of proteins required for compensatory de novo synthesis and endocytosis of cholesterol. This transcriptional response in cholesterol-depleted cells is mediated by the proteolytic release of a soluble fragment of the integral membrane transcription factor, sterol regulatory element-binding protein (SREBP)2 (1). Cholesterol accretion through gene expression takes many hours. In contrast, perhaps because of its toxicity (3), cells start to offset a rise in free cholesterol within minutes. In particular, excess cholesterol promptly drives the esterification and sequestration of cholesterol by acyl-cholesterol acyltransferase (ACAT) as well as suppressing the activation of SREBP. Concurrently, sterol production is curtailed by the rapid proteolytic inactivation of HMGR, the rate-determining enzyme in the biosynthetic pathway (1, 2, 4).

The aforementioned homeostatic processes are all initiated by proteins integral to the ER membrane. Furthermore, each of these processes appears to be responsive to the level of ER cholesterol, cholesterol precursors, and/or side chain oxysterols such as 27-HC, underscoring the central importance of ER sterols in maintaining an optimal level of cellular cholesterol. The size of the ER cholesterol pool is set by the rapid bidirectional flux of cholesterol between the ER and the plasma membrane; in this way, the magnitude of the latter can be sensed and regulated by the former (4, 5). The level of cholesterol in the ER is normally kept quite low, but it rises sharply as plasma membrane cholesterol is elevated even slightly above its physiological level (6). As a result, the activity of the sterol sensing proteins in the ER correct the plasma membrane excess (4). Whereas a detailed understanding of intracellular sterol transport is lacking, several plausible mechanisms have been described (2, 7–12).

The sharp rise in ER cholesterol with increasing plasma membrane cholesterol loads has recently been explained in terms of a mechanistic model based on the stoichiometric complexation of cholesterol with plasma membrane phospholipids (13). The complexed plasma membrane sterols have a low chemical activity, whereas cholesterol in excess of the complexing capacity of the phospholipids has a high chemical activity (fugacity) that drives it to intracellular and extracellular accep-

2 The abbreviations used are: SREBP, sterol regulatory element-binding protein; ACAT, acyl-cholesterol acyltransferase; GC, gas chromatography; ER, endoplasmic reticulum; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; HPCD, 2-hydroxypropyl-β-cyclodextrin; NB-598, (E)-5-ethyl-2-[bithiophen-5-yl]methoxy]benzenemethanamine; SCAP, SREBP-cleavage activating protein; DMEM, Dulbecco’s modified Eagle’s medium; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; CTX, cerebroside-dinosan xanthomatosis; MS, mass spectrometry; LPDS, lipoprotein-deficient serum.
tor compartments. The sharp rise in intracellular cholesterol above the plasma membrane threshold might stimulate the synthesis of 25-HC in the ER and 27-HC in the mitochondria (14). These side chain oxysterol derivatives appear to serve as signals of cholesterol excess to homeostatic proteins in the ER, nucleus, and elsewhere (1, 2, 15, 16). These various feedback mechanisms would maintain the cholesterol in the plasma membrane at the complexing capacity of its phospholipids.

We seek to understand the control of the short term responses made by the ER to fluctuations in cell cholesterol. For this purpose, we examined M19 cells, which are defective in SREBP activation, and cerebrotendinous xanthomatosis (CTX) cells, which are deficient in the synthesis of 27-HC. Since lanosterol and 24,25-dihydrolanosterol, its reduction product in the cholesterol biosynthetic pathway, have recently been implicated in the short term regulation of HMGR degradation (17), we also examined the role of these precursors in cholesterol homeostasis. We found that neither the setting of the ER cholesterol pool nor the rate of cholesterol esterification was significantly responsive in these three experimental systems. Furthermore, we observed that 27-HC, rather than cholesterol or its biosynthetic precursors, was responsible for stimulating the proteolytic inactivation of HMGR in response to elevated cell cholesterol.

**EXPERIMENTAL PROCEDURES**

**Materials**—HPCD and NB-598 were purchased from Sigma. Sterols were obtained from Sigma and Steraloids. MG-132 was from Calbiochem. We obtained 1a,2a-[3H]cholesterol and [oleoyl-1-14C]coenzyme A from Amersham Biosciences and Moravek Biochemicals, respectively. Sodium [3H]acetate and HMG-coenzyme A, bearing a DL-3-[glutaryl-3-14C] label, were from American Radiolabeled Chemicals, Inc.

**Cells and Culture**—Normal human foreskin fibroblasts were obtained and cultured in DMEM with 10% fetal bovine serum plus 100 units/ml penicillin and 100 µg/ml streptomycin (18). A flask of cultured fibroblasts typically had 200–300 µg of protein and 10–15 µg of cholesterol. M19 and the parent CHO-K1 cell line were a gift from T.-Y. Chang (Dartmouth University). We substituted F-12:DMEM (1:1) for DME when growing these lines. Fibroblasts from three CTX patients were the gift of Prof. Sebastiano Calandra (University of Modena). These lines were: CTX14-BG (male compound heterozygote with the mutations: c.1182C>T (R362C) and c.1213G>A (R372Q)), CTX6-PX (male homozygote with the mutation c.1182C>T (R362C)), and CTX2-BF (female compound heterozygote with the mutations: c.1182C>T (R362C)), and CTX6-PX (male homozygote with the mutation c.1182C>T (R362C)), and CTX2-BF (female compound heterozygote with the mutations. c.645G>C (A183P) and IVS7 + 5G>T (splicing defect)). Two other CTX lines were obtained from Eran Leitersdorf (Hebrew University-Hadassah Medical Center): CTX 205-5 and CTX 205-6, as described (19). CTX cells were grown with or without R362C, and CTX2-BF (female compound heterozygote with the mutations: c.1182C>T (R362C)) and CTX6-PX (male homozygote with the mutation c.1182C>T (R362C)), and CTX2-BF (female compound heterozygote with the mutations. c.645G>C (A183P) and IVS7 + 5G>T (splicing defect)). Two other CTX lines were obtained from Eran Leitersdorf (Hebrew University-Hadassah Medical Center): CTX 205-5 and CTX 205-6, as described (19). CTX cells were grown in DMEM as described above.

**Cell Treatments**—Cell cholesterol was acutely depleted and increased with HPCD as described (6, 20). Basically, cholesterol was reduced to different degrees by incubating flasks of cells for 5–8 min at 37 °C in PBS containing 5–12 mg/ml HPCD. To varyably enrich cell cholesterol (mostly in their plasma membranes), aliquots ranging from 6 to 35 µl of a PBS solution of ~8 mg/ml cholesterol complexed with 300 mg/ml HPCD were added to 2 ml of PBS overlying the cells. The flasks were then incubated for 8 min at 37 °C, and the HPCD-cholesterol removed. To stimulate the expression of HMGR, cells were depleted of cholesterol as described in the figure legends. 25-HC, 27-HC, lanosterol, and 24,25-dihydrolanosterol were added to cultures in ethanol (≥1% by volume, final). Cells were harvested from flasks using a 1-min incubation at 37 °C with 0.05% trypsin and 0.02% EDTA.

**Assays**—ER cholesterol was taken to be that which was esterified by ER ACAT in a run-off assay in which cell homogenates were incubated with [oleoyl-1-14C]CoA (6, 18). These data were expressed as picomole of ER cholesterol/mg of cell protein or % of total cell cholesterol located in the ER. Esterification of [3H]cholesterol by intact cells was assayed as described (6) and expressed as % of total cell label in esters; the rate of esterification was linear over the 2–3 h interval examined. Protein was assayed with a BCA kit (Pierce) using a bovine serum albumin standard. HMGR activity was determined in duplicate in each experiment as described (4); averaged values were expressed as picomole of mevalonate/min/mg of cell protein. Cholesterol mass was determined by HPLC (21).

**GC/MS Identification of Sterols**—Electron impact gas chromatography/mass spectrometry (GC/MS) analyses of the sterols were performed on a Finnigan (San Jose, CA) SSQ-7000 single-stage quadrupole mass spectrometer with a Varian (Walnut Creek, CA) 3400 GC, which is controlled by Finnigan ICIS software operated on a DEC alpha station. The sterol solutions in 1 µl of ethanol were injected in a split mode (split ratio 50/1) and analyzed by GC on a Restek (Bellefonte, PA) RTX-5 column (15 m, 0.33 mm inner diameter, 1-mm film thickness). The initial temperature of GC was set at 200 °C for 1 min, increased to 300 °C at a rate of 15 °C/min, and maintained at 300 °C for another 10 min. The temperatures of the injector, transfer line of the GC column, and the ion source were set at 280, 280, and 240 °C, respectively. The full scan mass spectra (50 to 500 Da) were acquired at a rate of 1 scan/0.25 s.

**Statistics**—Whenever appropriate, results were expressed as mean ± S.E. The statistical significance of differences in mean values was determined by single factor analysis of variance.

**RESULTS**

**Role of SREBP-targeted Genes in the Acute Regulation of ER Cholesterol**

**Response of M19 Cells to Cholesterol Loading**—Activated SREBP promotes the expression of multiple genes that drive the accretion of cell cholesterol (1, 22). We utilized M19 cells to test whether any SREBP-regulated proteins participate in the management of ER cholesterol. In this cholesterol auxotrophic strain of CHO-K1 cells, the Golgi metalloprotease, S2P, fails to cleave and activate SREBP in response to a reduction in cell cholesterol. As a result, the expression of SREBP target proteins is severely impaired in M19 cells. Consequently, the cells do not ingest cholesterol via low-density lipoprotein receptors nor synthesize it but rather take it up through the nonspecific endocytosis of serum lipoproteins (23, 24).

We found that M19 cells contained 22.2 ± 1.3 (n = 13) µg of cholesterol/mg of protein, compared with 25.9 ± 0.8 (n = 14)
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In Vivo Cholesterol Esterification in M19 Cells—The average basal rates of cholesterol esterification in intact M19 cells and wild-type cells were, respectively, 0.29 ± 0.02% (n = 7) of total cell cholesterol, compared with 0.58 ± 0.06% (n = 4) in the wild-type. The low ER cholesterol in M19 cells is consistent with the chronic undersupply of cholesterol that characterizes this auxotroph. We also compared the responses of ER cholesterol in control and M19 cells to increases in cell cholesterol (Fig. 1). In both cell strains, the ER cholesterol increased severalfold with modest increments in cell cholesterol, just as in normal human fibroblasts (6). Thus, the ER pool in the SREBP-inactive M19 cells responded normally to plasma membrane cholesterol loading.

FIGURE 1. Effect of cell cholesterol on ER cholesterol in CHO-K1 and M19 cells. Flasks of cells were depleted of or enriched in cholesterol by incubation with HPCD alone or HPCD complexed with cholesterol as described under "Experimental Procedures." The cells were chased for 45 min in Dulbecco’s PBS at 37 °C and their total cholesterol, ER cholesterol, and protein determined. Each point represents the average of duplicate determinations expressed relative to the unmodified control (filled symbols). The data are from separate experiments, 3 on CHO-K1 and 5 on M19 cells. Panel A, CHO-K1 cells; Panel B, CHO-K1 cells. Note the difference in scales for the two panels. The level of ER cholesterol in the mutant and wild-type lines reached 270 and 673 pmol of cholesterol/mg of protein, respectively; these values amounted to 0.58 and 0.84% of total cell cholesterol.

In Vivo Cholesterol Esterification in M19 Cells—The average basal rates of cholesterol esterification in intact M19 cells and wild-type cells were, respectively, 0.29 ± 0.02% (n = 7) of total cell cholesterol, compared with 0.58 ± 0.06% (n = 4) in the wild-type. The low ER cholesterol in M19 cells is consistent with the chronic undersupply of cholesterol that characterizes this auxotroph. We also compared the responses of ER cholesterol in control and M19 cells to increases in cell cholesterol (Fig. 1). In both cell strains, the ER cholesterol increased severalfold with modest increments in cell cholesterol, just as in normal human fibroblasts (6). Thus, the ER pool in the SREBP-inactive M19 cells responded normally to plasma membrane cholesterol loading.

HMG-CoA Reductase in M19 Cells—The HMGR activity of cultured M19 cells, expressed as picomole of mevalonate/min/mg of protein, was not significantly different from wild-type cells: 15.3 ± 5.1 (n = 3) versus 12.1 ± 3.2 (n = 4), respectively. The HMGR activity in the wild-type cells increased ~3-fold during an overnight incubation in a cholesterol-deficient medium (Fig. 3). The HMGR activity in M19 cells showed no appreciable response to cholesterol deprivation. This result was expected because of the inability of these cells to activate SREBP. The slightly increased basal level of HMGR activity in the mutant may therefore reflect a diminished down-regulation of this enzyme because the cells contain less of the sterols that promote HMGR proteolysis.

FIGURE 2. Effect of cell cholesterol on cholesterol esterification in CHO-K1 and M19 cells. Flasks of cells were depleted of or enriched in cholesterol as described in the legend to Fig. 1. The flasks were rinsed and pulse-labeled with [3H]cholesterol for 10 min at 15 °C and then chased in medium containing 5% LPDS for 2 h at 37 °C. Following dissociation, cell cholesterol, protein, [3H]cholesterol, and [3H]cholesterol esters were determined. The data points are single determinations from 5 separate CHO-K1 and 4 separate M19 experiments. The values represent the fraction of total cell [3H]cholesterol recovered in the ester fraction and are plotted relative to the corresponding unmodified control (filled symbols). Panel A, CHO-K1 cells; panel B, CHO-K1 cells. Note the difference in scales for the two panels. The highest rates of cholesterol esterification in the mutant and wild-type lines were similar: ~4% of cell [3H]cholesterol esterified per hour.

FIGURE 3. Induction of HMG-CoA reductase activity in CHO-K1 and M19 cells. Flasks of cells were incubated overnight in F-12:DMEM (1:1) containing either 10% fetal bovine serum (control, shaded bars) or, for cholesterol depletion, 5% LPDS plus 1 mM mevalonate and 20 μM oleic acid (open bars). HMGR activity was then determined in duplicate. Values (in pmol of mevalonate/min/mg of protein) are mean ± S.E. from four independent experiments. The difference between the two M19 values was not significant (p = 0.6).
cholesterol depletion. These results suggest that the absence of SREBP-driven protein expression caused a depletion of cholesterol in M19 cells but did not otherwise undermine the normal management of their ER cholesterol pool.

**Oxysterol Regulation of ER Cholesterol Homeostasis**

*Side Chain Oxysterol Synthesis in CTX Cells*—In addition to the sterol itself, cholesterol abundance is signaled in different ways by various oxysterol derivatives. Certain oxysterols activate nuclear LXR receptors, stimulating several metabolic responses such as bile acid production (26). Oxysterols are also ligands for Insig proteins in the ER membrane; there, they act to reduce the activities of SREBP and, presumably, HMGR (1, 16). Furthermore, exogenous oxysterols mimic cholesterol excess in intact cells by displacing plasma membrane cholesterol to the ER; this promotes cholesterol esterification and the sequestration of SREBP (1, 6, 15, 27).

We therefore sought to determine whether endogenous side chain oxysterols played a physiologic role in the management of ER cholesterol and HMGR inactivation. For this, we used cells from CTX patients, because they are deficient in sterol 27-hydroxylase (CYP27A1), the mitochondrial enzyme responsible for the synthesis of the major cellular oxysterol, 27-HC (28). Five isolates of CTX fibroblasts were examined. Their average cell cholesterol content was 32.7 μg/mg of protein, close to that of wild-type human fibroblasts (35.0 μg/mg of protein). In contrast to wild-type cells, all five CTX lines lacked endogenous 22(R), 24-, and 27-HC (Table 1). Loading cells with cholesterol stimulated the accumulation and secretion of 27-HC by the wild-type by 1.6-fold but did not evoke the production of 27-HC in CTX cells, thereby confirming the absence of the hydroxylase. 25-HC was found in extracts of the three CTX cell lines grown at Rush University in Chicago but not in the two lines grown and analyzed at Washington University (not shown). It seems likely that, despite all precautions, the 25-HC in the three transported samples was an oxidation artifact; this effect is well understood (29). In any case, the level of 25-HC in the wild-type fibroblasts and did not differ significantly between normal and CTX cells nor did it respond to cellular cholesterol enrichment. Because the 25-HC levels were at the limit of detection of the GC-MS-based assay, it was not possible to determine whether there were significant differences in 25-HC production among the CTX cell lines.

Cholesterol loading resulted in an increase of up to 8-fold in the level of ER cholesterol in the three CTX cell lines tested (Fig. 4A). This response was comparable with that observed previously in normal human fibroblasts (Fig. 4B). Similarly, the stimulation of cholesterol esterification evoked by increasing plasma membrane cholesterol levels in CTX cells was comparable with that in the wild-type (compare Fig. 5, A and B). Thus, the deficiency in 27-HC did not affect the responses of these two indicators of ER cholesterol regulation to cell cholesterol loads. We note in passing, however, that the basal level of cholesterol in the ER of CTX cells was only about one-fifth that in wild-type fibroblasts: 54 versus 284 pmol/mg of cell protein, respectively. Similarly, the rate of cholesterol esterification in the mutant was about one-sixth that of normal: 0.05 versus 0.32% esterified/h, respectively. This preliminary finding is worth further investigation.

**TABLE 1**

| Cells     | Cholesterol enrichment | 24-HC       | 25-HC       | 27-HC       |
|-----------|------------------------|-------------|-------------|-------------|
| WT        | –                      | 3.1 ± 1.7   | 0.43 ± 0.20 | 3.1 ± 1.5   |
| CTX       | +                      | 2.0 ± 1.1   | 0.40 ± 0.06 | 5.0 ± 1.4   |
| CTX       | +                      | 0           | 0.45 ± 0.31 | 0           |
| WT        | –                      | 0           | 0.42 ± 0.29 | 0           |

*WT*, wild type.

**FIGURE 4. Effect of cell cholesterol on ER cholesterol in wild-type and CTX fibroblasts.** Panel A, in four separate experiments, the cholesterol in three CTX cell lines was varied as described in the legend to Fig. 1. The cells were rinsed, chased for 45 min at 37°C in Dulbecco’s PBS, and total cell cholesterol, ER cholesterol, and protein determined. Each point represents the average of duplicate determinations expressed relative to the unmodified control (filled symbols). ○, CTX2-BF cells; △, CTX14-BG cells; ◊, CTX6- PX cells. Panel B, published data from comparable experiments with wild-type human fibroblasts (6). For the unmodified CTX and wild-type cells, the average ER cholesterol values were 54 and 284 pmol/mg of protein, respectively.

**FIGURE 5. Effect of cell cholesterol on cholesterol esterification in wild-type and CTX fibroblasts.** Panel A, in three independent experiments, flasks of CTX6-PX cells were variably enriched in cholesterol as described in the legend to Fig. 1. The cells were rinsed, pulse-labeled with [3H]cholesterol for 10 min at 15°C, rinsed, and chased in medium containing 5% LPDS for 3 h at 37°C. Following dissociation, cell cholesterol, protein, [3H]cholesterol, and [3H]cholesterol esters were determined. Each data point is the average of duplicate determinations. Values represent the fraction of total cell [3H]cholesterol recovered in the ester fraction and are plotted relative to the corresponding unmodified control (filled symbol). ○, CTX2-BF cells; △, CTX14-BG cells; ◊, CTX6-PX cells. Panel B, published data from comparable experiments with wild-type human fibroblasts (6). For the unmodified wild-type cells, the mean cholesterol content was 34.1 μg/mg of protein and the average esterification rate was 0.32%/h.
**Regulation of HMG-CoA Reductase Inhibitory Activity in Commercial Lanosterol**

Panel A, HPLC of Sigma L-1540 lanosterol. 0.5 mg of this preparation was analyzed by HPLC at 30 °C with a mobile phase of acetonitrile:isopropyl alcohol (99:1) and the eluate was collected over 23 min. Early peaks, labeled a–c, were pooled. Peak d was identified as lanosterol. Panels B–D, replicate flasks of wild-type fibroblasts were preincubated overnight in growth medium containing 5% lipoprotein-deficient serum. Each flask then received 2 ml of PBS containing 0.4% ethanol alone (bar 1) or ethanol containing a sterol (bars 2 and 3). After an incubation of 1 h at 37 °C, HMGR activity was determined. Panel B, bar 1, untreated control; bar 2, 33 μg of unfractonated lanosterol (Sigma L-1540); bar 3, pool of peaks a–c (panel A) from 33 μg of lanosterol (Sigma L-1540). Panel C, bar 1, untreated control; bar 2, 48 μg of unfractonated lanosterol (Sigma L-1540); bar 3, lanosterol purified from 48 μg of lanosterol (Sigma L-1540). Panel D, bar 1, untreated control; bar 2, 40 μg of pure lanosterol (Sigma L-5768); bar 3, 40 μg of 24,25-dihydrolanosterol (Steraloids C-8600). Experimental values are means of at least two independent experiments, each performed in duplicate and are expressed relative to their corresponding controls. Duplicate assays agreed to better than 4%. Error bars (S.E.) are too small to be visible.

**An Unfractionated Commercial Preparation of Lanosterol** (Sigma L-1540) caused the rapid inactivation of HMGR (Fig. 7, bars 2 in panels B and C). This was consistent with the previous report (17). However, this material was not homogeneous on HPLC (Fig. 7A). The major component of the mixture (Fig. 7A, peak d) caused only minor HMGR inhibition (Fig. 7C, bar 3), even though it had the mobility and mass spectroscopy signature of pure lanosterol (see below). Likewise, a pure lanosterol preparation from Sigma (L-5768) had no effect on HMGR activity under these conditions (Fig. 7D, bar 2). In contrast, the pool of peaks a–c from Fig. 7A was highly inhibitory of HMGR (Fig. 7B, bar 3). Confirmatory results were obtained for late (inactive) lanosterol and early (active) contaminating material from another commercial lanosterol preparation (Steraloids, C3250).

To characterize the active contaminant, GC/MS total ion current chromatography was performed on the crude lanosterol preparation (Sigma L-1540). The major component eluted at 10.72 min (Fig. 8A), the mobility of a pure lanosterol standard (Sigma L-5768; not shown). Electron impact mass spectra showed that the molecular weight of this compound was 426.4, confirming its identity as lanosterol. A major component of the high-mobility, inhibitory impurity from the Sigma lanosterol preparation, HPLC pool a–c in Fig. 7A, eluted in the gas chromatogram at 10.30 min (Fig. 8B). This retention time corresponded to that of 24,25-dihydrolanosterol (Steraloids C-8600). To confirm the identity of the contaminant, we subjected the crude lanosterol preparation to reductive hydrogenation. The major hydrogenation product eluted at 10.31 min and had a molecular mass of 428.4, identical to that of 24,25-dihydrolanosterol (Fig. 8, C–F). Furthermore, the GC mobility of the inhibitory material that eluted early on HPLC (Fig. 7A, region a–c) coincided with that of a 24,25-dihydrolanosterol standard (not shown). We also showed that a 24,25-dihydro-

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**Figure 6. Effect of sterols on HMG-CoA reductase in wild-type (panel A) and CTX (panel B) fibroblasts.** Replicate flasks were treated as follows. Bars 1, cholesterol was depleted by an overnight incubation in 3 ml of medium containing 5% lipoprotein-deficient serum. Bars 2, control overnight incubation in 3 ml of medium containing 10% whole serum. Bars 3–6, cholesterol was depleted as for bars 1 and the flasks then treated as follows. Bars 3, cells were enriched in cholesterol by incubation with 2 ml of PBS containing 1.8 mg of HPCD + 52 μg of cholesterol for 6 min at 37 °C, then rinsed and chased for 45 min in 2 ml of medium containing 5% lipoprotein-deficient serum. Bars 4–6, flasks were incubated for 45 min at 37 °C with 1 ml of PBS containing, respectively, 12 μM 25-hydroxycholesterol, 12 μM 27-hydroxycholesterol, or 5 μM 24,25-dihydrolanosterol. The values are mean ± S.E. from three experiments using three different CTX cell lines. Some error bars are not visible.

**Role of Cholesterol Precursors in Short Term Cholesterol Homeostasis**

Regulation of HMG-CoA Reductase Activity by Lanosterol and 24,25-Dihydrolanosterol—It was recently reported that commercial preparations of these biosynthetic intermediates, but not cholesterol itself, stimulated the ubiquitination and proteolytic destruction of HMG-CoA reductase (17). It was inferred that, rather than cholesterol, lanosterol (the first sterol intermediate in the biosynthetic pathway) can regulate sterol biosynthesis at the enzyme level (1). Because this premise countered our finding that exogenous cholesterol brought about the rapid inactivation of HMG-CoA reductase in fibroblasts (4), we sought to clarify the role played by sterol intermediates in cholesterol homeostasis at the ER level.
lanosterol preparation free of lanosterol (Steraloids C-8600) potently suppressed HMGR activity (Fig. 7D).

Because the time scale of HMGR inactivation in fibroblasts was only a few minutes, we sought to substantiate that proteasomal degradation was involved. We applied a specific inhibitor, MG-132, for this purpose. Fig. 9 documents that 10 μM MG-132 substantially blocked the loss of HMGR activity induced by loading cells with either cholesterol or 24,25-dihydrolanosterol.

**Effect of Biosynthetic Sterol Intermediates on HMGR**—The action of pharmacologic doses of 24,25-dihydrolanosterol suggests that it and possibly other endogenous cholesterol precursors might play a role in the physiological down-regulation of HMGR (17). We therefore tested whether decreasing the pools of these biosynthetic intermediates caused the level of HMGR to rise. These endogenous cholesterol precursors can be increased by stimulating sterol synthesis through cholesterol depletion. This rise in sterol precursor biosynthesis can be
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In preliminary control experiments, we tested the ability of NB-598, an inhibitor of squalene epoxidase (30). The endogenous cholesterol precursors synthesized prior to the blockade should clear within a few hours of inhibition as the result of their conversion to cholesterol (31). NB-598 was preferred to widely used HMGR inhibitors (statins) because the latter interfere with isoprenoid production, whereas NB-598 allows the continued synthesis of squalene and isoprenoids (30, 32, 33). It is important not to block the synthesis of geranylgeraniol, because it is a putative promoter of the proteolytic inactivation of HMGR (34). Also, because HMGR was not inactivated in these experiments, there was no need to replenish isoprenoids by feeding the cells high levels of their biosynthetic precursor, mevalonate (1). Finally, avoiding statins in these experiments allows for the direct and quantitative determination of HMGR enzymatic activity.

In preliminary control experiments, we tested the ability of NB-598 to block the synthesis of sterol intermediates. For this purpose, we induced biosynthesis by depleting fibroblasts of cholesterol with a 10-min HPCD extraction. This was followed by a 6-h chase in the presence or absence of NB-598. The efficacy of the block was tested by adding [3H]acetate during the last 3 h of the chase. In the absence of NB-598, the depleted cells showed the expected marked increase in the labeling of cholesterol, 7-dehydrocholesterol, desmosterol, and lanosterol, and other cholesterol precursors. In contrast, NB-598 inhibited the synthesis of all detectable sterols by >95%. Instead, essentially all of the incorporated label was found in squalene (not shown). We also performed this control in another way: cholesterol biosynthesis was stimulated by an overnight incubation without serum lipids. This was followed by 1-h preincubation of the cells with [3H]acetate ± NB-598. The outcome was essentially the same as with the acute cyclodextrin depletion experiment described above (not shown).

Do Endogenous Cholesterol Precursors Play a Role in the Regulation of ER Cholesterol?—To test this hypothesis, the sterol biosynthetic pathway was blocked with NB-598 for 4 h. Cell cholesterol levels were then modified with cyclodextrin ± cholesterol and the response of the ER pool determined. As shown in Fig. 10A, NB-598 treatment reduced the basal ER pool size by 13%; the ER pool in cholesterol-depleted cells was reduced by 15%; and the ER pool in cholesterol-enriched cells was 30% lower than its matched control. Whereas these reductions were statistically significant, these data nevertheless suggest that eliminating sterol intermediates like 24,25-dihydrolanosterol and lanosterol does not have an important effect on the size or physiological responses of the ER pool.

Next, the effect of endogenous cholesterol precursors on HMGR activity was examined. Normal fibroblasts were depleted of cholesterol by means of a 10-min HPCD extraction. (The use of an acute HPCD extraction rather than overnight starvation of serum lipids bypassed the slow and variable lag during which cellular cholesterol stores must be exhausted physiologically before cholesterol levels fall sufficiently to activate SREBP-dependent expression of the HMGR.) The depleted cells were then incubated for 6 h in the presence or absence of NB-598. As seen in Fig. 10B, cholesterol depletion caused the level of HMGR activity to increase about 10-fold during the 6-h chase. Blocking sterol biosynthesis with NB-598 caused a ~22% increase in the induced HMGR activity compared with its control. This modest increment was significant; *p* = 0.025 in a two-tailed *t* test.
DISCUSSION

According to a simple mechanistic model, the size of the pool of ER cholesterol is set in proportion to the excess of plasma membrane sterols over the phospholipids with which they complex stoichiometrically (see Introduction). Cholesterol exceeding this equivalence point has a high chemical activity (fugacity) and readily redistributes to intracellular compartments such as the ER and, perhaps, the mitochondria (4). The dependence of ACAT activity on cellular cholesterol loads also conforms to this mechanism, although the high-order substrate dependence of this enzyme might also contribute to the acute inflection of its dose-response curve at the physiological set-point (Figs. 2 and 5 and Ref. 2). Despite the cogency of this hypothesis, other physiological mechanisms might also affect the size of the ER pool. For example, various protein kinase C-directed agents evoke either increases or decreases in the ER cholesterol compartment (35). The present study therefore undertook an examination of three possibilities for other kinds of control of cell cholesterol at the ER level.

The ER is a central nexus for both short term and long term regulation of sterol homeostasis. In the simplest case, cholesterol feeds back negatively upon its own abundance both through the immediate stimulation of its esterification by ACAT in the ER and by sequestering SREBP (also in the ER), thereby down-regulating cholesterol levels. A second mode of regulation comes from the conversion of cholesterol to side chain oxysterols, such as 25-HC and 27-HC. Such oxysterol derivatives then cause Insig to sequester SREBP and, presumably, to promote the proteolysis of HMGR, two additional rapid ER feedback responses (16). Recently, a third kind of homeostatic sterol signal was proposed: biosynthetic intermediates such as lanosterol and 24,25-dihydrolanosterol were reported to stimulate the inactivation of HMGR (17). In the present study, we examined the effects of oxysterols, sterol intermediates, and sterol-regulated gene expression on the cholesterol level in the ER and the level of activity of two of its homeostatic constituents, ACAT and HMGR. Our results argue against a major role for SREBP-targeted proteins and biosynthetic sterol intermediates in setting the ER pool size. On the other hand, we provide evidence that endogenous 27-HC, derived from and representing cellular cholesterol, is an important short term regulator of HMGR activity. To examine the role of SREBP-dependent gene expression in the regulation of ER cholesterol homeostasis, we studied the M19 cell line, a CHO-K1 mutant that fails to respond to alterations in cellular cholesterol because of defective SREBP processing. Because HMGR expression is positively controlled by SREBP, it is not surprising that the activity of this enzyme did not increase when M19 cells were depleted of cholesterol (Fig. 3). In contrast, the expression of SREBP-dependent genes was not required for cellular adjustments of ER cholesterol or the rate of cholesterol esterification in response to changes in cell cholesterol (Figs. 1 and 2). In particular, the steep inflections of these dose-response curves were qualitatively normal, as was the positioning of their thresholds at their physiological set point. The prompt responses of ER cholesterol pool size and the rate of cholesterol esterification in M19 cells also suggest that at least some regulatory mechanisms are not required for cellular adjustments of ER cholesterol or the rate of cholesterol esterification in response to changes in cell cholesterol.

3 M. H. Lanier and D. S. Ory, unpublished results.
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HMGR levels by only about half over 6 h. We therefore suggest that the exogenous cholesterol in that study acted on SREBP-dependent gene expression, known to be slowly down-regulated by cholesterol at the ER level (1), rather than on the rapid (<1 h) proteolytic inactivation of the enzyme studied here.

Our data suggest that 25-HC is not likely to be the physiological mediator of the acute, cholesterol-driven down-regulation of HMGR even though, like 27-HC, exogenous 25-HC at high levels is a strong effector of HMGR inactivation (e.g. Fig. 6). For one thing, Table 1 shows it to be, on average, an order of magnitude less abundant than 27-HC in human fibroblasts. Given that these oxysterols are of comparable potency, 3 27-HC should then play the dominant role. Furthermore, 27-HC but not 25-HC increased in response to cholesterol loading in the wild type (Table 1), again suggesting greater participation of 27-HC than 25-HC. In addition, it is likely that the 25-HC we detected was an artifactual oxidation product of cholesterol, because none was detected under the most stringent preparative conditions (see “Results”). Also note that the 25-HC level, even taken at face value, was not affected by the CTX mutation and would therefore not seem to be the effector of the CTX phenotype. Indeed, the sterol 25-hydroxylase is a distinctly different ER enzyme, so that 25-HC should not be perturbed by the mitochondrial CTX mutation.

We also examined the hypothesis that sterol intermediates promote HMGR inactivation (1, 17). This premise seemed problematical, given that HMGR activity increases dramatically in cholesterol-starved cells in which biosynthetic sterol intermediates should rise far above their normal physiological levels. Indeed, we observed that adding lanosterol (the predominant sterol intermediate) at doses greatly exceeding physiological did not affect either the size of the ER pool (not shown) or the activity of HMGR (Fig. 7). Likewise, treating cells with NB-598, a squalene cyclase inhibitor that blocks the endogenous production of sterols such as lanosterol, only slightly reduced the size of the ER cholesterol pool (Fig. 10A) and only slightly increased HMGR activity (Fig. 10B). (The mechanism underlying these minor effects is not clear.) Thus, neither pharmacologic nor physiologic tests revealed a significant effect of lanosterol in this system. The failure of lanosterol to alter HMGR levels here is consistent with the absence of a physically demonstrable interaction of lanosterol with this enzyme in vitro (1).

Unlike lanosterol, pharmacologic levels of exogenous 24,25-dihydrolanosterol strongly promoted the inactivation of HMGR (Fig. 7). (The effect of exogenous 24,25-dihydrolanosterol on ER cholesterol pool size and the rate of cholesterol esterification could not be assessed because the high concentrations of this sterol interfered with the activity of ACAT, which is required for both of those assays.) On the other hand, blocking the biosynthesis of 24,25-dihydrolanosterol and other sterols with NB-598 had only minor effects on ER cholesterol and HMGR (Fig. 10). Our results therefore suggest that lanosterol and 24,25-dihydrolanosterol do not normally play a major role in cholesterol homeostasis. This inference is supported by the remarkably unperturbed sterol metabolism of cultured cells in which almost all of the cholesterol has been replaced by these sterol intermediates (41). Also supporting this conclusion is the report that incubating cells with commercial lanosterol (that presumably also contains 24,25-dihydrolanosterol) did not suppress the proteolytic activation of SREBP (17), as would be the case if either of these sterol intermediates were able to increase ER cholesterol.

That the inhibition of sterol production with NB-598 did not greatly affect the ER cholesterol pool size (Fig. 10A) supports another, unrelated premise: namely, that the size of the ER pool is not sustained by cholesterol biosynthesis, even though the ER is the site of its elaboration. Rather, it seems that the ER pool is flushed out and set by the rapid bidirectional flux of cholesterol between the ER and plasma membrane. This mechanism enables the cholesterol level in the ER membrane to signal the needs of the plasma membrane to the homeostatic elements associated with it (4, 6).

The literature on the regulation of HMGR by sterols is complex (17, 37, 40, 42, 43). First, there are large differences in the aqueous solubility of various exogenous sterol effectors. Consequently, neither the relevant membrane concentration nor the degree of saturation of sterol-binding proteins is known in such studies. Second, HMGR is regulated by a variety of sterols at multiple metabolic levels over differing time spans. In particular, cholesterol down-regulates the expression of HMGR over hours, whereas oxysterols stimulate proteolysis in a few minutes. Another level of complexity, suggested by Fig. 6, is that cholesterol itself may be unable to promote the short term inactivation of HMGR but apparently gains potency through side chain hydroxylation. This effect can explain why cholesterol inactivates HMGR quite well in intact fibroblasts (4) but does not stimulate ubiquitination in permeabilized cells (17, 44) in which cholesterol may not reach the mitochondrial sterol 27-hydroxylase or the oxysterol product may not reach the HMGR. Third, high levels of exogenous oxysterols might have “off target” effects not elicited in vivo by their endogenous counterparts. For example, exogenous 25-HC might act upon the plasma membrane as an intercalating amphipath, displacing cholesterol from phospholipid complexes (45). This effect can send some of the plasma membrane cholesterol to intracellular compartments where it can exert multiple homeostatic effects (4). Thus, high levels of exogenous oxysterols might act pharmacologically and not be proper mimics of the physiologic effects of their endogenous counterparts (1).

That sterols act homeostatically at multiple sites adds further complexity to the setting of HMGR levels. It might be, for example, that the sterol-sensing domain of HMGR has specificity for sterols, whereas the binding site on Insig, the activator of its ubiquitination, is restricted to oxysterols. This seems to be the case for the association of Insig with SCAP: either cholesterol (a SCAP ligand) or 25-HC (an Insig ligand) can apparently cause the retention of SREBP in the ER by the SCAP-Insig complex (1, 16). The binding of a sterol to the sterol-sensing domain of HMGR and an oxysterol to Insig might synergistically promote HMGR ubiquitination. Such a concerted action might contribute to the hyper-sharp dependence on cell cholesterol loading observed for the destruction of HMGR in fibroblasts (4). This would certainly be the case if both the level of cholesterol in the ER (6) and the activity of mitochondrial sterol 27-hydroxylase were acutely inflected functions of plasma membrane cholesterol.
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On the other hand, there is evidence that only one of the two proteins, HMGR and Insig, need be occupied by its respective sterol or oxysterol to stimulate ubiquitination. In particular, exogenous 24,25-di-4-hydrolanosterol inactivates HMGR in CTX cells even though those cells lack the sterol 27-hydroxylase required for its hydroxylation (Fig. 6). In that case, the binding of 24,25-di-4-hydrolanosterol to the sterol-sensing domain of HMGR and not to Insig may suffice to stimulate ubiquitination. Similarly, depleting cells of cholesterol so as to minimize their ER cholesterol pool does not reduce their susceptibility to HMGR inactivation by exogenous 25-HC or 27-HC (Fig. 6). It follows that only the oxysterol site and not a sterol site need be occupied for their action.

Among the modifications of the sterol backbone thus far reported to promote HMGR inactivation are the 3β-hydroxylation of ring A; dimethylation at carbon 4 of sterol ring A; and/or hydroxylation at carbons 25 or 27 of the iso-octyl side chain (17, 46). The findings shown in Fig. 6 extend the structure-activity analysis of the promotion of HMGR inactivation by sterols by demonstrating the following: 1) 3β-hydroxylation is not sufficient, because cholesterol is inactive in CTX cells. 2) Dimethyl substitution at carbon 4 may also not be sufficient, because high levels of lanosterol were without effect. 3) Hydroxylation of the side chain of the sterol is not essential, because 24,25-di-4-hydrolanosterol is effective both in CTX and wild-type fibroblasts and hydroxylation at carbon 25 seems unimportant in this system. Because 27-hydroxylanosterol is particularly potent in promoting the ubiquitination of HMGR (17) and sterol 27-hydroxylase enables exogenous cholesterol to promote the inactivation of HMGR (Fig. 6B), it is puzzling that exogenous lanosterol did not also inactivate the enzyme in wild-type fibroblasts. Perhaps the side chain of the exogenous lanosterol was not hydroxylated because this very water-insoluble sterol was not taken up well by the cells or was not delivered to the mitochondrial hydroxylation.

As we were preparing to submit this study for publication, a possible physiological role for lanosterol and/or 24,25-di-4-hydrolanosterol in the regulation of HMGR was reported (33). In that study, imposing several hours of extreme hypoxia on a line of CHO cells evoked both high levels of biosynthetic sterol intermediates and high levels of INSIG. This response promoted the proteolytic destruction of HMGR. The authors suggested that the consequent down-regulation of HMGR could serve the function of limiting sterol biosynthesis (with its high demand for oxygen) when scant intracellular oxygen was needed for more vital metabolic processes. Our results suggest that this adaptive mechanism is not operative under normal conditions. Indeed, the extreme conditions required for sterol intermediates to stimulate HMGR destruction contrast with the small shifts in plasma membrane cholesterol that elicit major changes in HMGR activity (4).

Acknowledgments—We thank T.-Y. Chang (Dartmouth University) for the generous gift of M19 and parental CHO-K1 cells; Sebastiano Calandro (University of Modena) and Eran Leitersdorf (Hebrew University-Hadassah Medical Center) for providing us with the CTX cell lines; and Emily Westover for performing chemical hydrogenation of sterol compounds.

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