Endogenous 24(S),25-Epoxycholesterol Fine-tunes Acute Control of Cellular Cholesterol Homeostasis*

Received for publication, August 3, 2007, and in revised form, October 11, 2007 Published, JBC Papers in Press, November 2, 2007, DOI 10.1074/jbc.M706416200

Jenny Wong†1, Carmel M. Quinn§2, Ingrid C. Gelissen†3, and Andrew J. Brown‡4

From the †School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney 2052, Australia and the §Centre for Vascular Research, University of New South Wales, and the Department of Haematology, Prince of Wales Hospital, Sydney 2032, Australia

Certain oxysterols, when added to cultured cells, are potent regulators of cholesterol homeostasis, decreasing cholesterol synthesis and uptake and increasing cholesterol efflux. However, very little is known about whether or not endogenous oxysterol(s) plays a significant role in cholesterol homeostasis. 24(S),25-Epoxycholesterol (24,25EC) is unique among oxysterols in that it is produced in a shunt of the mevalonate pathway which also produces cholesterol. We investigated the role of endogenously produced 24,25EC using a novel strategy of over-expressing the enzyme 2,3-oxidosqualene cyclase in Chinese hamster ovary cells to selectively inhibit the synthesis of this oxysterol. First, loss of 24,25EC decreased expression of the LXR target gene, ABCA1, substantiating its role as an endogenous ligand for LXR. Second, loss of 24,25EC increased acute cholesterol synthesis, which was rationalized by a concomitant increase in HMG-CoA reductase gene expression at the level of SREBP-2 processing. Therefore, in the absence of 24,25EC, fine-tuning of the acute regulation of cholesterol homeostasis is lost, supporting the hypothesis that 24,25EC functions to protect the cell against the accumulation of newly synthesized cholesterol.

In 1978 Kandutsch, Chen, and Heiniger (1) first proposed that the suppressive effect of cholesterol on its own synthesis may be mediated by endogenously produced oxysterols. This led to the formulation of the Oxysterol Hypothesis of Cholesterol Homeostasis, which over time has acquired both its champions and its detractors (1–3). The ability of cholesterol to participate in its own feedback regulation has since become well established (4–6). The recent discovery that the cholesterol biosynthetic precursors, lanosterol (7, 8) and desmosterol (9), are involved in particular aspects of cholesterol homeostasis has added to an already complex picture, highlighting the multiple levels of control. However, oxysterols are the only regulatory molecules described so far to elicit effects on all major levels of cholesterol homeostasis. Most of the physiological oxysterols are tissue-specific, so the idea of a global oxysterol regulator that is present in all cell types is enticing.

The oxysterol, 24(S)25-epoxycholesterol (24,25EC),5 is a strong candidate as its synthesis is unique among other oxysterols in that it is produced de novo in the mevalonate pathway which simultaneously synthesizes cholesterol (Fig. 1A). Hence, any cell type capable of synthesizing cholesterol also has the capacity to produce 24,25EC. 24,25EC has been characterized as one of the most potent physiological oxysterols to elicit effects on multiple levels of cellular cholesterol homeostasis (10–16).

When added to cultured cells, 24,25EC is a potent feedback regulator of cholesterol synthesis and has been shown to reduce the activity of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (10, 11, 17) as well as to stimulate HMG-CoA reductase degradation (18). Moreover, 24,25EC is a potent suppressor of the transcription factor sterol regulatory element-binding protein-2 (SREBP-2), the master regulator of cholesterol biosynthetic and uptake genes, including HMG-CoA reductase and the low density lipoprotein receptor (13, 19, 20).

The nuclear receptor, the liver X receptor (LXR), is a key regulator of genes involved in cellular cholesterol export (21). The LXR target gene, the ATP binding cassette transporter, ABCA1, is the main facilitator of active transport of cholesterol from the cell to extracellular acceptors (22, 23). Oxysterols are natural ligands for LXR (24), and 24,25EC has been demonstrated to be the most potent oxysterol activator of LXR and ABCA1 target gene expression (12, 15, 25, 26). Most of these effects have been gleaned from studies in which 24,25EC was added to in vitro systems and/or cultured cells. The precise role(s) of endogenously produced 24,25EC remains to be elucidated.

Synthesis of 24,25EC is dependent on the activity of the enzyme, 2,3-oxidosqualene cyclase (OSC) (17, 27–30). OSC is a monotopic integral membrane protein which possesses a dual substrate recognition function that enables it to convert the
Selective Inhibition of 24,25EC Synthesis

cholesterol precursor 2,3(S)-monooxidosqualene (MOS) to lanosterol or the 24,25EC precursor 2,3(S):22(S):23-dioxidosqualene (DOS) to 24(S):25-epoxylanosterol (31, 32). Subsequently, lanosterol and 24(S):25-epoxylanosterol are efficiently converted into cholesterol and 24,25EC, respectively, as the end products of this pathway (33). It is important to note, however, that OSC preferentially cyclizes DOS over MOS (27). One way that we (15, 34) and others (17, 28, 35, 36) have augmented endogenous production of 24,25EC is through partial pharmacological inhibition of OSC. Under normal conditions, cholesterol synthesis is favored (Fig. 1A). Under partial inhibition of OSC, the build up of MOS is converted to DOS by the enzyme squalene epoxidase, explaining how cholesterol precursors can be channeled into 24,25EC synthesis at the expense of cholesterol synthesis (Fig. 1B). However, this approach is not ideal because it artificially raises levels of 24,25EC and also decreases cholesterol synthesis, which may be a confounding factor. In addition, there is the usual concern of specificity of effect associated with the use of pharmacological inhibitors. Moreover, conventional gene silencing approaches would also be problematic since synthesis of 24,25EC and cholesterol require the same enzymes; thus, production of both sterols would be inhibited. In the present study we tested a novel way to selectively inhibit 24,25EC production. Overexpression of OSC should have the opposite effect of partial inhibition, i.e. the abundance of OSC should efficiently convert all MOS into lanosterol before any MOS can be converted into DOS and, hence, into 24,25EC (Fig. 1C). This approach is more selective than using a pharmacological agent and should not inhibit cholesterol synthesis.

In the current study we have used this approach to determine the precise role of endogenous 24,25EC in cellular cholesterol homeostasis. We previously hypothesized that 24,25EC protects the cell from accumulation of newly synthesized cholesterol rather than exogenous cholesterol, since both sterols are synthesized in parallel under a variety of conditions (34). Here, we show that loss of endogenous 24,25EC removes the fine-tuning in the control of cholesterol synthesis, indicating an important role for this oxysterol in cellular cholesterol homeostasis.

EXPERIMENTAL PROCEDURES

Reagents—Chemicals and reagents used are listed below with the supplier. From GE Healthcare: [1-14C]acetic acid, sodium salt (specific activity, 56 mCi/mmol); Hybond C nitrocellulose membrane; Hyperfilm. From Invitrogen: Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1 mixture); l-glutamine; Lipofectamine 2000; new born calf serum; penicillin/streptomycin; Lipofuscin 2000; new born calf serum (37). The Chinese hamster ovary cell line CHO-7 was generously provided by Drs. Michael S. Brown and Joseph L. Goldstein (UT Southwestern, Dallas).

Generation of Plasmid Construct—pCMV4-OSC-Myc was constructed by annealing full-length human OSC into pcDNA3.1-V5-His using the TOPO TA cloning kit (Invitrogen). The OSC gene was then excised by restriction endonuclease digestion with NotI and KpnI and ligated into pCMV4-Myc-His.

Generation of OSC-overexpressing Cell Lines—CHO-7 cells were transfected with pCMV4-OSC-Myc (1 μg) for 24 h using Lipofectamine 2000 transfection reagent (4 μl/well in a 6-well plate), and stable transfectants were selected for zeocin (500 μg/ml) resistance by limiting dilution and screened for OSC-Myc expression by Western blotting.

Cell Culture—All cell lines were grown at 37 °C in a 5% CO2 atmosphere. CHO-7 cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1 mixture) containing 5% (v/v) lipoprotein-deficient serum supplemented with penicillin/streptomycin (100 units/100 μg/ml) and l-glutamine (2 mM). Empty vector and OSC-overexpressing cells were maintained as CHO-7 cells but always in the presence of 100 μg/ml zeocin. Treatments were conducted for 24 h in media containing 5% (v/v) lipoprotein-deficient serum. All treatments were added to cells either in absolute ethanol or dimethyl sulfoxide and compared with vehicle-only controls. No cell toxicity was observed for any treatment at the concentrations employed.

Reverse Transcriptase PCR and Quantitative Real-time PCR—Cells were harvested for total RNA using Tri Reagent according to the manufacturer’s instructions. Concentrations of total RNA were measured by spectrophotometry (Nanodrop ND-100 Spectrophotometer, Thermo). Reverse transcriptase-PCR was performed according to the manufacturer’s protocol.
Selective Inhibition of 24,25EC Synthesis

for SuperScript III First Strand cDNA synthesis kit. Quantitative (real-time) reverse transcriptase-PCR (QRT-PCR) was performed using SensiMix dT on a Corbett Rotorgene 3000 and analyzed using Rotor-Gene 6 Version 6.0 (Build 27) (Corbett Research). Primer pairs used for the amplification of various genes from cDNAs were described previously (19, 38). Primer pairs for human/hamster OSC were: forward, ACCTAT-GAGACCAAGCCTTG; reverse, TCCAGGCCAACCAGGTG. PCR products were verified by sequencing. Melting curve analysis was performed to confirm production of a single product in each reaction. The change in gene expression levels was determined by normalizing mRNA levels of the gene of interest to the mRNA level of the housekeeping gene, porphobilinogen deaminase (19).

Western Blotting—To measure OSC-Myc protein expression, total cell lysates were prepared as previously described (5). Briefly, cells in 6-well plates or 60-mm dishes were washed in phosphate-buffered saline, and the cell pellet was resuspended in 1% SDS or 10% SDS lysis buffer containing protease inhibitors. Cell pellets were lysed by passing through a syringe needle (22 gauge, 25 times) and vortexed for 20 min at room temperature. Protein concentrations were determined by the bicinchoninic acid method. Samples of equal protein were analyzed by SDS-PAGE (10% for SREBP-2, OSC-Myc, and β-actin). Protein was transferred onto nitrocellulose membrane and incubated with blocking solution (5% w/v nonfat milk, 0.1% v/v Tween 20 in phosphate-buffered saline; 1 h). SREBP-2 was probed first using IgG-7D4, a mouse monoclonal antibody against hamster SREBP-2 (amino acids 32–250 (39); prepared from a mouse hybridoma cell line), ATCC CRL2198, followed by stripping and reprobing for β-actin and, last, OSC-Myc. Antibodies were incubated with the membranes (4 °C, overnight). After washing, the membranes were incubated for 1 h with peroxidase-conjugated affinity-purified secondary antibodies (anti-mouse (IgG-7D4, Myc) or anti-rabbit (β-actin)). After further washing, bound antibodies were visualized by chemiluminescence and exposed to film at room temperature for 2 s to 1 min.

Cholesterol and 24(S),25-Epoxycolesterol Synthesis Assay—Cells were metabolically labeled with [1-14C]acetate acid for 24 h during treatments as previously described (15). For the acute cholesterol synthesis assay, cells were pretreated with compactin for 24 h, washed, and labeled with [1-14C]acetate acid for 2 h. Cells were harvested and saponified, and the neutral lipid extracts were separated by thin-layer chromatography. Bands corresponding to cholesterol and 24(S),25-epoxycolesterol were visualized by phosphorimaging (18-h exposure). Positive identification of the band corresponding to 24(S),25-epoxycolesterol has previously been performed chemically and by mass spectrometry (see the supplemental data in Wong et al. (15)).

Human ABCA1 Promoter Activity Assay—Promoter analysis was performed as previously described (19). Briefly, reporter plasmids (250 ng/well) were transfected for 24 h into cells using Lipofectamine 2000 (1 µl/well). The phRL-TK Renilla internal control plasmid (25 ng/well) was co-transfected for normalization of transfection efficiency. After treatment (24 h), cells were washed and resuspended in 100 µl of 1 × passive lysis buffer. Luciferase assays were performed using the Dual Luciferase Assay Reporter System according to the manufacturer’s instructions in a Veritas luminometer (Turner Designs). Results were normalized to the renilla control and expressed as percent changes in luciferase activity relative to the wild-type ABCA1 vehicle-treated control condition.

Cholesterol Mass Assay—Total cellular cholesterol mass was determined using an enzymatic fluorometric assay, employing the Amplex Red Cholesterol assay kit according to the manufacturer’s instructions.

Data Analysis and Statistics—All results shown are representative of at least two separate experiments. Data are presented either as mean ± half-range or means ± S.E. unless otherwise stated. Where appropriate, statistical differences were determined by analysis of variance or Student t tests. A p value less than 0.05 (two-tailed) was considered statistically significant.

RESULTS

Screening and Selection of Stable Cell Lines Over-expressing Human OSC—The enzyme OSC is key to the activity of the shunt pathway that produces 24,25EC. We cloned the human OSC cDNA into a mammalian expression vector containing a C-terminal c-Myc epitope tag and stably transfected it into CHO-7 cells. Stable cell lines with empty vector (EV) or varying levels of OSC expression were created and screened alongside the wild-type CHO-7 cells. In Fig. 2A, a conserved set of primers was employed that detected both the human and hamster gene, showing that overexpression of human OSC was as much as 20-fold above endogenous levels. In all three OSC overexpressing cell lines, protein expression (Fig. 2B) reflected gene expression (Fig. 2A). Synthesis of 24,25EC was then examined, and in line with our prediction, 24,25EC was substantially decreased with increased OSC expression (by >95% in OSC3 cells) (Fig. 2C). No change was observed in cholesterol synthesis or cellular cholesterol mass in all cell lines (Fig. 2, C and D).

To confirm that overexpression of OSC specifically inhibited 24,25EC synthesis in the OSC-overexpressing cell lines, we treated the EV control cell line and the highest OSC-overexpressing cell line, OSC3, with a specific inhibitor of OSC, GW534511X. Similar to our previous observations in CHO cells (34), 0.1–1 nM GW534511X greatly enhanced 24,25EC synthesis in EV cells (Fig. 2E), in line with the scheme depicted in Fig. 1B. In OSC3 cells, a 100-fold higher concentration of inhibitor was required to force the synthesis of 24,25EC, reflecting the higher relative expression of OSC in these cells (Fig. 2A). This result clearly demonstrates that the reduced ability of OSC-overexpressing cells to synthesize 24,25EC was a direct consequence of their overexpression of OSC. The cell lines with the highest expression of OSC, OSC2 and particularly OSC3, together with the empty vector control cell line, EV, were selected for use in subsequent experiments.

ABCA1 Gene Expression Is Decreased in Cells Lacking the LXR Ligand 24,25EC—24,25EC is one of the most potent oxysterols for LXR (15, 25), which regulates expression of a number of genes involved in cholesterol efflux, including ABCA1. When OSC is overexpressed, the decrease in 24,25EC synthesis led to a corresponding decrease in ABCA1 gene expression (Fig. 3A). This is consistent with our previous find-
greatly reduced to levels observed in OSC3 cells (Fig. 3B), consistent with inhibited synthesis of an LXR ligand produced in the mevalonate pathway.

When ABCA1 gene expression was examined over time after compactin pretreatment, we observed that ABCA1 mRNA levels increased in the EV control cells (Fig. 3C). In contrast, ABCA1 mRNA expression remained unchanged in OSC3 cells over the first 8 h but increased by 24 h. This suggests that other sterol ligands are insufficient to initially compensate for the lack of 24,25EC with respect to ABCA1 expression. Indeed, when increasing concentrations of 24,25EC were added to EV, OSC2, and OSC3 cell lines, ABCA1 gene expression was restored to control levels at 0.1 μM in the two cell lines lacking 24,25EC (Fig. 3D). All three cell lines responded similarly to higher concentrations of 24,25EC.

To confirm that this effect was mediated transcriptionally at the level of LXR activation, ABCA1 promoter activity was then analyzed by transfecting cells with a luciferase reporter construct driven by a ~1 kilobase fragment of the human ABCA1 promoter (from −928 to +101 bp) (40). OSC3 cells and compactin-treated EV cells both displayed reduced activity of the ABCA1 promoter to levels comparable to those seen when the DR4 motif (where LXR binds) was mutated (Fig. 3E). The decreased ABCA1 promoter activity of OSC3 cells was restored by the addition of 0.1 μM 24,25EC, an effect that was not apparent with the DR4-mutated construct. Taken together, these data indicate that the decrease in ABCA1 expression in OSC3 cells is principally due to the loss of 24,25EC and confirms the importance of this endogenously produced LXR ligand in LXR target gene expression, notably ABCA1.

Inhibition of 24,25EC Synthesis Leads to an Increase in Acute Cholesterol Synthesis—We have proposed that 24,25EC functions as a feedback regulator to protect all cholesterogenic cells against the accumulation of newly synthesized cholesterol (34). Because little difference was observed between the relative synthesis of cholesterol in EV and OSC3 cells over an extended (24-h) period (Fig. 2C), we then examined what effect the loss of 24,25EC had on acute cholesterol synthesis. To investigate this, de novo cholesterol synthesis was stimulated by pretreatment with compactin. The inhibitory effect of compactin on cholesterol synthesis leads to a compensatory increase in expression of several key cholesterol biosynthetic enzymes, including HMG-CoA reductase, which are regulated by SREBP-2 (41, 42).

Hence, removal of compactin results in a concentration-dependent surge in cholesterol synthesis (34), which can then be monitored by acute (2-h) metabolic labeling with [14C]acetate. Using this approach, OSC3 cells, lacking 24,25EC, showed an approximate doubling in acute cholesterol synthesis for all statin pretreatment concentrations when compared with EV cells (Fig. 4, A and B). Next, we followed the feedback inhibition of cholesterol synthesis over time, subsequent to compactin pretreatment (5 μM). EV and OSC3 cells showed comparable rates of decrease over time (Fig. 4C), although the initial increase in relative cholesterol synthesis in OSC3 cells after 2 h of statin pretreatment was maintained for the first 8 h.

As mentioned, relative cholesterol synthesis over an extended (24-h) period was similar between OSC3 and EV cells (Figs. 2C and 4D). The addition of 24,25EC decreased chole-
Selective Inhibition of 24,25EC Synthesis

**FIGURE 3.** ABCA1 gene expression is decreased in cells lacking the LXR ligand 24,25EC. For A–D, ABCA1 mRNA levels were determined by QRT-PCR. A, EV, OSC2, and OSC3 cell lines were cultured under standard conditions (see “Experimental Procedures”). B, EV and OSC cells were incubated in the absence or presence of compactin (5 \( \mu \)M). C, EV and OSC cells were preincubated with compactin (5 \( \mu \)M) for 24 h, which was then removed, and media without compactin was added. Cells were harvested at the time points indicated. D, EV, OSC2, and OSC3 cells were incubated in the indicated concentrations of 24,25EC for 24 h. For E, EV and OSC cells were transiently transfected for 24 h with phRL-TK Renilla internal control plasmid together with either pGL3-hABCA1 wild-type (wt) or DR4 mutant (mut). After transfection, cells were incubated for 24 h in the absence or presence of compactin (5 \( \mu \)M) or 24,25EC (0.1 \( \mu \)M). For A and B, values are presented as the mean ± S.E. relative to the EV vehicle-treated control condition and are averaged from \( n = 3 \) separate experiments (each performed in triplicate). For C, values are the mean ± S.E. (\( n = 3 \) replicate cultures) relative to EV cells incubated without compactin and are representative of \( n = 2 \) separate experiments (each performed in triplicate). For D, values are mean the ± S.E. relative to the EV vehicle-treated control condition from \( n = 3 \) replicate cultures. For E, values are presented as the percentage change relative to the pGL3-hABCA1 wild-type construct of the EV vehicle-treated control condition and are the mean ± S.E. averaged from \( n = 3 \) separate experiments (each performed in triplicate). *, ABCA1 gene expression was significantly lower for the OSC2 or OSC3 cells relative to the EV control cells (\( p < 0.01 \) by paired t tests for A and B, by two-way analysis of variance for C, and by two-sample t tests for D).

SREBP-2 Increase Acutely—Added 24,25EC has been shown to inhibit cholesterol synthesis by decreasing HMG-CoA reductase activity (11, 17, 43). We determined if the acute increase in cholesterol synthesis in OSC3 cells was the result of increased HMG-CoA reductase gene expression. Basal levels were similar between EV and OSC3 cells, as were the increases in HMG-CoA reductase gene expression levels observed after statin pretreatment (0 h) (Fig. 5A). However, 2 h after removing the statin, HMG-CoA reductase mRNA expression increased significantly, by \( \sim 40\% \) in the OSC3 cells relative to the EV cells (Fig. 5B). Whereas EV cells displayed a smooth and gradual decrease over time, OSC3 cells showed after the initial increase an abrupt fall in HMG-CoA reductase mRNA levels up to 8 h and subsequent rebounding of levels by 24 h (Fig. 5C). These results show that with loss of 24,25EC, acute regulation of HMG-CoA reductase gene expression is erratic and suggests that the fine-tuned control of HMG-CoA reductase gene regulation is perturbed.

The transcription factor SREBP-2 is the primary regulator of HMG-CoA reductase gene expression (41, 42). SREBP-2 is synthesized as an inactive precursor that requires proteolytic release of the active N-terminal (mature) transcription factor (44). In both cell types, SREBP-2 processing as assessed by Western blotting was stimulated by compactin treatment and suppressed by the addition of 24,25EC (Fig. 6A). To determine whether the acute increase in HMG-CoA reductase mRNA levels in OSC3 cells (Fig. 5B) was the result of increased SREBP-2 activation, SREBP-2 processing was measured under the same experimental conditions in which the increased HMG-CoA reductase mRNA expression was observed. Processing of SREBP-2 to the mature form was similar for both EV and OSC3 cells after compactin pretreatment (0 h) (Fig. 6, B and C). However, after removal of the statin, a significant increase in the mature form of SREBP-2 was detected in OSC3 cells over 2 h when compared with EV cells (Fig. 6, B and C). This difference in acute processing between EV and OSC3 cells was observed in four separate experiments.
Selective Inhibition of 24,25EC Synthesis

When combined, our results suggest that with the loss of 24,25EC, acute suppression of SREBP-2 processing does not occur. In fact, levels of mature SREBP-2 increased, and as a consequence, SREBP target genes such as HMG-CoA reductase are acutely up-regulated. Therefore, acute feedback regulation of cholesterol synthesis is perturbed without 24,25EC.

DISCUSSION

We previously proposed that 24,25EC functions as a safety valve to protect the cell against the accumulation of newly synthesized cholesterol, since 24,25EC is produced in parallel with cholesterol, and syntheses of both sterols are subject to feedback regulation in response to added cholesterol (34). In the current study, we tested this hypothesis using a novel approach of overexpressing OSC to selectively inhibit 24,25EC synthesis.

Our current work shows that endogenous 24,25EC has an important role in the acute modulation of cholesterol synthesis. Interestingly, no difference was observed in basal levels of cholesterol synthesis or mass between EV cells and OSC3 cells despite the lack of 24,25EC synthesis (Fig. 2, C and D). However, under stimulated conditions where a burst of cholesterol synthesis was induced by statin pretreatment, OSC3 cells showed an approximate doubling in cholesterol synthesis compared with EV cells (Fig. 4, A and B). Although the subsequent rate of decrease in cholesterol synthesis was the same for both OSC3 and EV cells, the initial increase in cholesterol biosynthetic activity of OSC3 cells persisted for at least 8 h (Fig. 4C). Considering that the difference in cholesterol biosynthesis was observed at the earliest time point after statin pretreatment, our data indicates that 24,25EC is important for acute feedback regulation of cholesterol synthesis. Our findings substantiate previous proposals that 24,25EC serves as a feedback regulator of hepatic cholesterol synthesis (28, 45) and helps to generalize it to all cholesterogenic cells.

In terms of mechanism, loss of 24,25EC appears to stimulate acute cholesterol synthesis at the level of SREBP-2 processing.
Selective Inhibition of 24,25EC Synthesis

Analysis of HMG-CoA reductase gene expression and SREBP-2 processing revealed that acute feedback regulation of these processes was lost in OSC-overexpressing cells (Figs. 5B and 6C). Thus, a relative increase in the levels of mature SREBP-2 could account for the increase observed in the expression of the SREBP-2 target gene, HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis.

It was recently shown that the suppressive effect of oxysterols (including 24,25EC) on SREBP-2 processing was mediated by direct binding to the ER retention protein Insig (20). By contrast, cholesterol is sensed by SCAP in the ER (4–6). Binding of oxysterols to Insig or cholesterol to SCAP causes Insig to retain the SCAP-SREBP complex in the ER, thereby inhibiting SREBP processing. Loss of endogenous 24,25EC leads to impaired feedback of SREBP-2 processing acutely (Fig. 6, B and C) despite the increase in cholesterol synthesis (Fig. 4, A and B). In light of our findings and those of Radhakrishnan et al. (20), we propose that 24,25EC, produced parallel to cholesterol in the mevalonate pathway, may represent an early warning system for newly synthesized cholesterol. 24,25EC allows retention of the SCAP-SREBP complex by Insig in the ER before newly synthesized cholesterol accumulates to sufficient levels to be sensed by SCAP. In contrast to other oxysterols such as 27-hydroxycholesterol, which is synthesized in the mitochondria by CYP27A1, the ER localization of the mevalonate pathway (including HMG-CoA reductase) responsible for 24,25EC synthesis could enable 24,25EC to rapidly inhibit cholesterol synthesis by promoting the retention of the SCAP-SREBP complex. This would, thus, limit accumulation of potentially cytotoxic levels of free cholesterol in the ER (46).

Another way by which 24,25EC could potentially affect acute cholesterol synthesis is by post-translational regulation of HMG-CoA reductase. Indeed, Song and DeBose-Boyd (18) have shown that 24,25EC accelerates HMG-CoA reductase degradation when added to cells. Therefore, it is likely that the increase in cholesterol synthesis observed in cells lacking 24,25EC is due to a combination of increased gene expression and decreased protein degradation of HMG-CoA reductase.

Our results indicate that endogenously produced 24,25EC acts at multiple points in facilitating the control of cholesterol homeostasis. In addition to its effects on acute cholesterol synthesis, it is also likely to stimulate cholesterol efflux by serving as a ligand for LXR. Indeed, selective inhibition of 24,25EC synthesis was associated with reduced expression and transcriptional activity of the LXR target gene, ABCA1 (Fig. 3). This result provides the best evidence to date for previous assertions that 24,25EC is an important natural LXR ligand based on in vitro addition studies (12, 25). Moreover, this work further substantiates our previous studies indicating that statins down-regulate ABCA1 expression by inhibiting synthesis of 24,25EC (15, 47). Considering that loss of 24,25EC increased SREBP-2 processing, it is likely that 24,25EC affects a multitude of SREBP target genes. For example, we found that gene expression of the low density lipoprotein receptor increased acutely after removal of statin, similar to HMG-CoA reductase (Fig. 5B), suggesting that 24,25EC probably also reduces receptor-mediated cholesterol influx into the cell.

Further studies are needed to determine precisely how loss of 24,25EC will affect cellular cholesterol homeostasis in vivo. Chen et al. (26) recently showed that certain oxysterols are important ligands for LXR in vivo. They reported that overexpression of a mouse oxysterol-catabolizing enzyme, SULT2B1b, or a triple knock-out of 24-, 25-, and 27-hydroxylase in mice leads to impaired LXR-mediated effects in response to dietary cholesterol, whereas stimulation with the non-sterol LXR agonist TO901317 showed regular increases. Considering the unique nature of 24,25EC synthesis, overexpressing OSC to selectively inhibit its synthesis is a promising approach to study the importance of this potent oxysterol in vivo.

In conclusion, this is the first study to investigate the role of endogenous 24,25EC by selectively inhibiting its synthesis in cells. 24,25EC, as with many oxysterols derived from cholesterol, acts at multiple points in cholesterol homeostasis. However, our work indicates that 24,25EC has a special role in protecting the cell against the accumulation of newly synthesized cholesterol. Thus, we propose that endogenously produced 24,25EC protects against surges in cholesterol synthesis by smoothing the cholesterol homeostatic responses during acute feedback regulation. In the absence of 24,25EC, the controls which fine-tune this acute feedback regulation are lost, and

6 J. Wong and A. J. Brown, unpublished data.
these responses tend to be more exaggerated and erratic, which may be deleterious to the cell.

Acknowledgments—We thank several researchers for sharing their valuable tools: Drs. Michael S. Brown and Joseph L. Goldstein for the CHO-7 cell line and Dr. Alan Tall for the pGL3-hABCA1 wild-type plasmid. We are also grateful to Dr. Lijuan Xie for preparing the IgG-7D4 anti-SREBP-2 antibody from the hybridoma cell line.

REFERENCES

1. Kandutsch, A. A., Chen, H. W., and Heiniger, H. J. (1978) Science 201, 498–501
2. Schroepfer, G. J., Jr. (2000) Physiol. Rev. 80, 361–554
3. Bjorkhem, I. (2002) J. Clin. Investig. 110, 725–730
4. Brown, A. J., Sun, L., Feramisco, J. D., Brown, M. S., and Goldstein, J. L. (2002) Mol. Cell 10, 237–245
5. Adams, C. M., Reitz, J., De Brabander, J. K., Feramisco, J. D., Li, L., Brown, M. S., and Goldstein, J. L. (2004) J. Biol. Chem. 279, 52772–52780
6. Radhakrishnan, A., Sun, L. P., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2004) Mol. Cell 15, 259–268
7. Song, B. L., Javitt, N. B., and DeBose-Boyd, R. A. (2005) Cell Metab. 1, 179–189
8. Nguyen, A. D., McDonald, J. G., Bruick, R. K., and DeBose-Boyd, R. A. (2007) J. Biol. Chem. 282, 27436–27446
9. Yang, C., McDonald, J. G., Patel, A., Zhang, Y., Umetsu, M., Xu, F., Westover, E. J., Covey, D. F., Mangelsdorf, D. J., Cohen, J. C., and Hobbs, H. H. (2006) J. Biol. Chem. 281, 27816–27826
10. Saucier, S. E., Kandutsch, A. A., Taylor, F. R., Spencer, T. A., Phirwa, S., and Gayen, A. K. (1985) J. Biol. Chem. 260, 14571–14579
11. Taylor, F. R., Kandutsch, A. A., Gayen, A. K., Nelson, J. A., Nelson, S. S., Phirwa, S., and Spencer, T. A. (1986) J. Biol. Chem. 261, 15039–15044
12. Lehmann, J. M., Kiewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J. L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A., and Willson, T. M. (1997) J. Biol. Chem. 272, 3137–3140
13. Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kiewer, S. A., Corey, E. J., and Mangelsdorf, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 259–268
14. Telford, D. E., Lipson, S. M., Barrett, P. H., Sutherland, B. G., Edwards, J. Y., Aebi, J. D., Dehmlow, H., Morand, O. H., and Huff, M. W. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 2608–2614
15. Dollis, D., and Schuber, F. (1994) Biochem. Pharmacol. 48, 49–57
16. Song, B. L., and DeBose-Boyd, R. A. (2004) J. Biol. Chem. 279, 28798–28806
17. Wong, J., Quinn, C. M., and Brown, A. J. (2006) Biochem. J. 400, 485–491
18. Radhakrishnan, A., Ikeda, Y., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 6511–6518
19. Zelcer, N., and Tontonoz, P. (2006) J. Clin. Investig. 116, 607–614
20. Schnitz, G., and Langmann, T. (2005) Biochim. Biophys. Acta 1735, 1–19
21. Soumian, S., Albrecth, C., Davies, A. H., and Gibbs, R. G. (2005) Vasc. Med. 10, 109–119
22. Ory, D. S. (2004) Circ. Res. 95, 660–670
23. Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kiewer, S. A., Corey, E. J., and Mangelsdorf, D. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 266–271
24. Chen, W., Chen, G., Head, D. L., Mangelsdorf, D. J., and Russell, D. W. (2007) Cell Metab. 5, 73–79
25. Coutaud, O., Dolis, D., and Schuber, F. (1992) Biochem. Biophys. Res. Commun. 188, 898–904
26. Mark, M., Muller, P., Maier, R., and Eisele, B. (1996) J. Lipid Res. 37, 148–158
27. Eisele, B., Budzinski, R., Muller, P., Maier, R., and Mark, M. (1997) J. Lipid Res. 38, 564–575
28. Morand, O. H., Aebi, J. D., Dehmlow, H., Ji, Y. H., Gains, N., Lengsfeld, H., and Himber, J. (1997) J. Lipid Res. 38, 373–390
29. Bloch, K. (1965) Science 150, 19–28
30. Nelson, J. A., Steckbeck, S. R., and Spencer, T. A. (1981) J. Biol. Chem. 256, 1067–1068
31. Panini, S. R., Sexton, R. C., Gupta, A. K., Parish, E. J., Chitrakorn, S., and Rudney, H. (1986) J. Lipid Res. 27, 1190–1204
32. Wong, J., Quinn, C. M., and Brown, A. J. (2007) Lipids Health Dis. 6, e10
33. Rowe, A. H., Argmann, C. A., Edwards, J. Y., Sawyer, C. G., Morand, O. H., Hegele, R. A., and Huff, M. W. (2003) Circ. Res. 93, 717–725
34. Bunza, M., Neslep, C. L., Sawyer, C. G., Edwards, J. Y., Markle, J. G., Hegele, R. A., and Huff, M. W. (2007) J. Biol. Chem. 282, 5207–5216
35. Goldstein, J. L., Bassa, S. K., and Brown, M. S. (1983) Methods Enzymol. 98, 241–260
36. Kielar, D., Dietmaier, W., Langmann, T., Aslanidis, C., Probst, M., Nasruszewicz, M., and Schmitz, G. (2001) Clin. Chem. 47, 2089–2097
37. Yang, J., Sato, R., Goldstein, J. L., and Brown, M. S. (1994) Genes Dev. 8, 1910–1919
38. Costet, P., Luo, Y., Wang, N., and Tall, A. R. (2000) J. Biol. Chem. 275, 28240–28245
39. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) J. Clin. Investig. 101, 2331–2339
40. Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W., Brown, M. S., and Goldstein, J. L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12027–12032
41. Saucier, S. E., Kandutsch, A. A., Gayen, A. K., Swahn, D. K., and Spencer, T. A. (1989) J. Biol. Chem. 264, 6863–6869
42. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) Cell 124, 35–46
43. Spencer, T. A., Gayen, A. K., Phirwa, S., Nelson, J. A., Taylor, F. R., Kandutsch, A. A., and Erickson, S. K. (1985) J. Biol. Chem. 260, 13391–13394
44. Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) Nat. Cell Biol. 5, 781–792
45. Wong, J., Quinn, C. M., Gelissen, I. C., Jessup, W., and Brown, A. J. (2007) Atherosclerosis, in press