Olate Potentiates Oxysterol Inhibition of Transcription from Sterol Regulatory Element-1-regulated Promoters and Maturation of Sterol Regulatory Element-binding Proteins*

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Activation of genes containing SRE-1 (sterol regulatory element 1) sequences is known to be under the regulation of sterols through modulation of the proteolytic maturation of SREBPs (SRE-1-binding proteins). Previous work has demonstrated SREBP-mediated transcriptional activation of genes encoding enzymes of sterol and fatty acid biosynthesis. Because synthesis of both sterols and C18 fatty acids are required for cell growth, in the absence of exogenous supplements of these lipids, we examined the hypothesis that fatty acid can also be regulatory in SREBP maturation. Our data indicate that C18 fatty acids can potentiate the biological activities of a typical, regulatory sterol: 25-hydroxycholesterol. Inhibition of C18 fatty acid synthesis in cells cultured in serum-free medium renders them resistant to killing by 25-hydroxycholesterol. Repression of expression of reporter constructs driven by promoters bearing SRE-1 element(s) by 25-hydroxycholesterol is increased by C18 fatty acid supplementation. C18 fatty acids also increase the inhibitory effect of 25-hydroxycholesterol on proteolytic maturation and nuclear localization of SREBPs. Furthermore, we also show that C18 fatty acid supplementation can enhance the inhibitory effect of 25-hydroxycholesterol on sterol and fatty acid biosynthesis. These results demonstrate that maximal down-regulation of SREBP maturation and the consequent repression of SRE-1 promoters occurs in response to both a regulatory sterol and fatty acid.

Oxygenated sterols, such as 25-hydroxycholesterol, are down-regulators of cholesterol biosynthesis (1). 25-Hydroxycholesterol down-regulates the rate-limiting step in this pathway, 3-hydroxy-3-methyl-glutaryl (HMG) coenzyme A reductase, through several mechanisms but also has a pleiotropic effect on transcription of all the genes of the cholesterol biosynthetic pathway that have been studied to date (2). This transcriptional control is mediated through the regulated two-step proteolysis (3) of transcription factors called SREBPs, which are synthesized as proteins localized to the endoplasmic reticulum. SREBP proteolysis to the soluble, mature transcription factors is inhibited by 25-hydroxycholesterol at the first cleavage. Genes that are transcriptionally activated by SREBPs possess specific recognition elements for these proteins, called SREs (2). These include many of the genes of the cholesterol biosynthetic pathway and the gene encoding the LDL receptor (4). In addition, the genes encoding two of the enzymes of fatty acid biosynthesis (4, 5) and HMG-CoA reductase (6) also contain sequences recognized by SREBPs that differ from the classically defined SRE.

At least under some experimental conditions, it is possible to demonstrate regulation of promoters for these genes encoding enzymes of fatty acid biosynthesis, as well as those for cholesterol biosynthesis, by expression of SREBPs. Such studies, utilizing promoter reporter constructs or measurement of mRNA levels for specific enzymes of fatty acid biosynthesis, have been performed both in cultured cells (4, 5, 7, 8) and transgenic mice (7, 9) with truncated, soluble SREBP-1a and SREBP-1c. However, these observations create something of a metabolic paradox because they suggest that fatty acid biosynthesis can be inhibited by sterols, a regulatory mechanism that would lead to inhibition of cell growth when exogenous sources of sterols were high but fatty acids were poor.

Somatic cell mutants of CHO cells, resistant to killing by 25-hydroxycholesterol in the absence of lipoprotein cholesterol, have been extremely useful in further defining the role of SREBPs in the regulation of cholesterol biosynthesis (10, 11). The mutants characterized to date, fall into two classes (3): class 1 mutants are sterol-resistant because they produce a truncated SREBP-2 that serves as a constitutive, mature transcription factor, and class 2 mutants are defective in a protein called SCAP (SREBP-cleavage activating protein), which appears to transduce the oxysterol-mediated inhibition of SREBP proteolysis. Class 2 mutants carry out the normally oxysterol-regulated first proteolytic cleavage constitutively.

Many oxysterol-resistant mutants were isolated long before the mechanism of transcriptional regulation of cholesterol biosynthesis, particularly the role of the SREs and SREBPs, was understood. Early somatic cell genetic characterization indicated that there was genetic diversity in such mutants and that some were recessive (13, 14). The class 1 and 2 mutants, whose defects are now known, are dominant (or co-dominant) mutations. Our laboratory reported (15) a novel, recessive 25-hydroxycholesterol-resistant mutant (crB). This mutant had a genetic defect in fatty acid elongation that was required for both resistance to killing and down-regulation of mRNA levels transcribed from cholesterol biosynthetic genes. Lack of regulation of the activity of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, as well as its mRNA, was...
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Supplementation of these cells with a mix of stearate and oleate restored cell growth to normal, but most of the regulation of HMG-CoA reductase by 25-hydroxycholesterol and partial restoration of cell growth could be produced by oleate supplementation alone. These results suggest a solution to the metabolic paradox outlined above, which is that oxysterols can lose activity as regulators of SRE promoters when cells are starved for C18 fatty acids, particularly oleate.

In this report we further explore the molecular basis for these observations and test the hypothesis that oleate can modulate the transcriptional regulatory activity of 25-hydroxycholesterol. The crB mutant turned out to be difficult to analyze for such initial studies because, as will be described elsewhere, its genotype appears to be complex. Instead, we have made use of a potent inhibitor of fatty acid elongation, hexadecynoic acid, in the current study.

EXPERIMENTAL PROCEDURES

Materials—IgG-2A4 and IgG-7D4 cells, which produce monoclonal antibodies SREBP-1 and SREBP-2, respectively, were purchased from American Type Culture Collection (catalog numbers CRL-2121 and CRL-2122). All cell culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum albumin (FBS- BSA; catalogue number A6003) was from Sigma. Stearate and oleate were obtained from NuChek Prep (Elysian, MN), and 25-hydroxycholesterol was from Steraloids Inc. (Wilton, NH). 3-Threo-dichloroacetylt-1,2,14C]chloro-phenic acid (catalog number NEC408A) was from NEN Life Science Products. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Pierce. The plasmids pLDLRCAT-6500 and pTK-Kx3-CAT were provided by Dr. David Russell (University of Texas, Dallas, TX). 2-Hexadecynoic acid (HDA) was a kind gift of Dr. J. M. Lowenstein (Brandeis University, Waltham, MA).

Cell Culture and Transfection—CHO-K1 cells were grown in Ham's F12 medium containing 5% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (F12FC5) at 37 °C and 5% CO2. Stably transfected cells were produced by co-transfecting cells with 2 μg of pWLneo containing 300 μg/ml G418 (Life Technologies, Inc.) in F12FC5. Resistant colonies were then fixed and stained with crystal violet as described previously (15). Protein concentration was determined using a BCA protein assay (Pierce) using BSA as standard.

RESULTS

Effect of Inhibition of Fatty Acid Elongation on the Cytotoxicity of 25-Hydroxycholesterol—We have previously shown that CHO-K1 cells treated with HDA, an inhibitor of endogenous fatty acid elongation, synthesize very little stearate and no detectable oleate compared with untreated controls (15). We have also previously shown that a somatic cell mutant defective in elongation of palmitate is resistant to the cytotoxic effects of 25-hydroxycholesterol in lipid-free medium (15). Therefore, we reasoned that if C18 fatty acid synthesis was necessary for 25-hydroxycholesterol cytotoxicity, HDA treatment might render cells resistant to 25-hydroxycholesterol. Indeed, when CHO-K1 cells were incubated with HDA, the cytotoxicity of 25-hydroxycholesterol was diminished compared with cells not treated with HDA (Fig. 1). HDA supplementation by itself was without effect on the growth rate of the cells. These results complement our prior observation that C18 fatty acid synthesis has a synergistic role in 25-hydroxycholesterol cytotoxicity.

Effect of Fatty Acid Supplementation on Sterol Regulation of SRE Promoter Activity—Because inhibition of C18 fatty acid synthesis with HDA renders CHO-K1 cells resistant to killing by 25-hydroxycholesterol in lipid-poor medium, it seemed plausible that HDA treatment might also produce resistance of genes containing SREs to down-regulation by 25-hydroxycholesterol, as well. This has been the general pattern observed with 25-hydroxycholesterol-resistant somatic cell mutants.

To determine whether cellular C18 fatty acid levels affect sterol regulation, we examined the effects of HDA treatment and fatty acid supplementation on transcription. For this, we utilized an LDL receptor promoter construct, pLDLRCAT-6500, which contains the entire LDL receptor promoter and has
been demonstrated to be regulated by sterols (19). When stably transfected into CHO-K1 cells, transcription of this construct is up-regulated when incubated in delipidized medium and down-regulated when the cells are treated with 25-hydroxycholesterol (Fig. 2). Supplementation of the delipidized medium with fatty acids (stearate and oleate) complexed to FAF-BSA or with FAF-BSA alone had little or no effect on the expression of the reporter gene. However, when the delipidized medium was supplemented with 25-hydroxycholesterol and fatty acids, expression of the reporter gene was reduced by approximately 2-fold compared with cells incubated in the same medium without fatty acids (Fig. 2). On the other hand, treatment of CHO-K1 cells with HDA partially inhibited the down-regulation of the LDL receptor promoter by 25-hydroxycholesterol (Fig. 2). HDA treatment caused a decrease in the down-regulation produced by 25-hydroxycholesterol from 7.2-fold to 2.5-fold. Furthermore, the effect of HDA on the response to 25-hydroxycholesterol could be nearly abolished by supplementing the delipidized medium with 10 mM stearate and 10 mM oleate (S.O.) as indicated. All fatty acids were presented complexed to FAF-BSA, and the final concentration of the culture medium was adjusted to contain 0.03% FAF-BSA and 0.05% ethanol (vehicle for 25-hydroxycholesterol). The cells were then harvested and assayed for CAT activity as described under “Experimental Procedures.”

We next examined whether the SRE-1 alone was sufficient to confer fatty acid augmentation of sterol down-regulation or if additional sequences outside of the SRE-1 were necessary for this effect. For this we utilized an artificial promoter construct, pTK-Kx3-CAT, which contains three tandemly repeated LDL receptor SRE-1 sequences within the thymidine kinase promoter and has been shown to be regulated by sterols (20). CHO-K1 cells stably transfected with pTK-Kx3-CAT showed a response to 25-hydroxycholesterol similar to that of cells transfected with the full-length LDL receptor promoter construct (Fig. 3). The sterol-mediated down-regulation of CAT activity was further enhanced by C18 fatty acids. CHO-K1 cells stably
transfected with a control construct lacking a SRE showed no down-regulation when incubated in delipidized medium supplemented with 25-hydroxycholesterol alone or C18 fatty acids and 25-hydroxycholesterol (data not shown; also see Ref. 20). These results indicate that the SRE-1 element is the region of the LDL receptor promoter that is responsible for the observed effect of C18 fatty acids on the down-regulation of transcription by 25-hydroxycholesterol. We also noted that most, if not all, of the effect of fatty acid supplementation on proteolytic processing of SREBP is most readily demonstrated in cultures in which fatty acid elongation had been inhibited with HDA, where the proteolytic maturation of SREBPs was most readily demonstrated in cultures in which fatty acid supplementation would enhance the lowering of nuclear SREBP levels produced by 25-hydroxycholesterol. To test this hypothesis, nuclear extracts were prepared from CHO-K1 cells incubated in delipidized media supplemented with or without fatty acids in the presence or absence of oxysterols. Aliquots of nuclear extract were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis using monoclonal antibodies to SREBP-1 and SREBP-2. As expected, the nuclei of cells cultured in the absence of sterols contained the mature form of SREBP-1 and SREBP-2, whereas the nuclei of cells incubated in medium supplemented with sterols contained significantly less of the mature form of either SREBP (Fig. 5). Supplementation with fatty acids alone did not obviously alter the nuclear levels of the mature SREBP-1 or BP-2. Consistent with the results from the reporter constructs, incubation of CHO-K1 cells with C18 fatty acids significantly enhanced the activity of 25-hydroxycholesterol in lowering the levels of SREBP-1 and BP-2 found in nuclear extracts. Because the major reported mechanism regulating nuclear SREBP levels is through sterol-mediated inhibition of their proteolytic maturation, these data are consistent with C18 fatty acids or a metabolite thereof, having a co-regulatory role in the proteolytic processing of SREBPs in response to sterols. We found that the effects of fatty acid supplementation on proteolytic processing of SREBPs was most readily demonstrated in cultures in which fatty acid elongation had been inhibited with HDA, where the proteolytic processing is expected to be very efficient. The results of such an experiment (Fig. 6), confirms that C18 fatty acid supplementation enhances the activity of 25-hydroxycholesterol in lowering nuclear SREBP levels. Furthermore, the large decrease in the nuclear levels of the mature SREBPs in cells treated with the combination of fatty acids and 25-hydroxycholesterol is accounted for by a large increase in the accumulation of SREBP precursors in the membrane fractions. This result is consistent with a co-regulatory effect of fatty acid supplementation and 25-hydroxycholesterol on SREBP proteolytic maturation. Examination of stearate and oleate for specificity of regulation of nuclear SREBP levels indicates that the regulatory response is specific for oleate (Fig. 7).

**Fig. 4.** Oleate is sufficient for increasing sterol regulation of the LDL receptor promoter in CHO-K1 cells. CHO-K1 cells stably transfected with pLDLRCAT-6500 were incubated for 16 h in F12DIPE5 supplemented with and without sterols and either 10 \( \mu \)M stearate, 10 \( \mu \)M oleate, or 10 \( \mu \)M stearate plus 10 \( \mu \)M oleate as described for the upper panel of Fig. 2.

**Fig. 5.** Effect of C18 fatty acids on sterol regulation of nuclear SREBP-1 and SREBP-2 levels. Nuclear proteins were isolated from CHO-K1 cells cultured for 24 h in F12DIPE5 in the presence or absence of sterols (1 \( \mu \)g/ml 25-hydroxycholesterol and 10 \( \mu \)g/ml cholesterol) and C18 fatty acids (10 \( \mu \)M stearate and 10 \( \mu \)M oleate [S.O.]) as indicated. Samples of nuclear protein (50 \( \mu \)g) were subjected to electrophoresis on denaturing SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Immunoblots were prepared using 5 \( \mu \)g/ml of a monoclonal antibody to either SREBP-1 (IgG-2A4) or SREBP-2 (IgG-7D4). The immunocomplexes were visualized by ECL. Films were exposed for 2 min.

**Fig. 6.** C18 fatty acids enhance sterol inhibition of SREBP-1 and SREBP-2 proteolytic maturation. Nuclear and membrane proteins were isolated from CHO-K1 cells cultured for 24 h in F12DIPE5 containing 20 \( \mu \)M HDA and supplemented with and without sterols and C18 fatty acids as described for Fig. 5. 100-\( \mu \)g samples of membrane proteins (upper panels) and 50-\( \mu \)g samples of nuclear proteins (lower panels) were analyzed by immunoblotting as described in the legend to Fig. 5.

**Fig. 7.** Oleate supplementation potentiates the reduction of nuclear SREBP-1 and SREBP-2 levels in response to sterols. Nuclear proteins were prepared from CHO-K1 cells cultured for 24 h in F12DIPE5 supplemented with and without sterols (1 \( \mu \)g/ml 25-hydroxycholesterol and 10 \( \mu \)g/ml cholesterol) and 10 \( \mu \)M stearate, 10 \( \mu \)M oleate, or 10 \( \mu \)M stearate plus 10 \( \mu \)M oleate as indicated in the figure. Nuclear protein samples (75 \( \mu \)g) were subjected to immunoblotting with monoclonal antibodies IgG-7D4 and IgG-2A4 as described in the legend to Fig. 5.
lipids. Differential regulation, presumably based on differential activity of residual mature SREBP in fatty acid starved cells, would permit fatty acid synthesis to proceed under conditions where exogenous sources of sterol, but not fatty acids, were present in sufficient quantity to permit cell growth. When both lipids are present, it would be expected that both pathways would be significantly down-regulated.

To test these hypotheses, we examined the effect of 25-hydroxycholesterol treatment on incorporation of labeled acetate into sterols (as nonsaponifiable lipid) and fatty acids (as saponifiable lipid) in CHO-K1 cells in the presence or absence of exogenous oleate. The results (Table I) indicate that treatment with 1 μg/ml 25-hydroxycholesterol, a condition that partially suppresses SREBP processing (Fig. 5) and suppresses transcription from the LDL receptor promoter approximately 5-fold (Fig. 2), inhibits sterol synthesis approximately 3-fold, whereas fatty acid synthesis is inhibited only 15%. Oleate supplementation enhances the inhibition of both sterol and fatty acid synthesis by 25-hydroxycholesterol.

**DISCUSSION**

Because fatty acids are precursors to membrane phospholipids, they constitute, along with cholesterol, the major structural constituents of the plasma membrane. It is therefore plausible that the biosynthetic pathways for these membrane components should be coordinately regulated. Several studies have appeared (4, 5, 7, 8) demonstrating that sterols can be regulatory for fatty acid synthesis through transcriptional control mediated by SREBP-1. A role for SREBP-1 in whole animal fatty biosynthesis is also consistent with reduction in fatty acid synthesis in liver of SREBP-1 knockout mice (21). These studies are good evidence for coordination of the cholesterol and fatty acid biosynthetic pathways by sterols.

The opposite side of the cross-talk between fatty acid synthesis and cholesterol synthesis would be regulation of cholesterologenesis by fatty acids. Some suggestion that this can occur was indicated in a prior study from our laboratory (15) of a somatic cell mutant blocked in elongation of palmitate to stearate, which was defective in down-regulation of mRNAs encoding enzymes of sterol biosynthesis. We found that this mutant required both stearate and oleate for optimal growth, suggesting that cells could be sensitive to supplementation with both fatty acids. In the course of these studies, we also found that the regulatory responses to fatty acids could only be observed in a basal medium that is scrupulously free of C18 fatty acids. The organic solvent delipidized serum supplements or serum-free medium described above must be used to see these effects. Many of the previously published studies on regulation of SREBP processing by oxysterols have been performed in a basal medium with a lipoprotein-deficient serum supplement (see, for example, Ref. 17). Such medium still contains large amounts of fatty acids bound to serum albumin, which would mask the fatty acid component of the response of SREBP processing or LDL receptor promoter regulation to oxysterol treatment that we describe in the current report.

In the current study, we demonstrate that oxysterol inhibi-

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**TABLE I**

Incorporation of $[^3]H$/acetate into lipids of CHO-K1

| Lipid class          | Control    | 25OH     | Oleate    | 25OH + Oleate |
|----------------------|------------|----------|-----------|---------------|
| Nonsaponifiable      | 120826 ± 983 | 34619 ± 1294 | 93273 ± 5105 | 11740 ± 647  |
| Fatty acids           | 120866 ± 2135 | 83612 ± 5899 | 58721 ± 6452 | 32746 ± 1866 |

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**Fatty acids 120866**

Cells were seeded at $1 \times 10^5$/100-mm culture dish and incubated in F12FC5 for 24 h. The medium was changed to F12DIP5 ± 1.0 μg/ml 25-hydroxycholesterol (25OH) and ± 50 μm oleate complexed to 0.15% fatty acid free BSA for 16 h prior to the addition of $[^3]H$/acetate (10 μCi/ml). Following a 24-h incubation, the incorporation into cellular lipids was determined as described under “Experimental Procedures.” The results are the average of triplicate determination ± S.D.
SREBP-mediated regulation. It has also been suggested that regulation of fatty acid synthesis in whole animals by ADD1/SREBP1, particularly in adipose tissue, is mediated primarily by insulin (8).

It is entirely possible that a metabolite of oleate rather than oleate itself is the second regulator. One possibility is the intracellular form of cholesteryl ester, which is cholesterol esterification. It is intriguing to speculate that LDL that is more active than control LDL in lowering endogenous cholesterol synthesis. It is believed to be involved in sensing cellular sterols through its membrane attachment domain. Perhaps, the membrane attachment domain of SCAP can sense such acyl-lipids as well as sterols.

The differential response of SREBP maturation to saturated and monounsaturated fatty acids is somewhat reminiscent of nutritional studies on the effect of oleate-enriched diets on serum cholesterol levels. Studies in both human subjects (32) and monkeys (26) indicate when oleate is the major source of dietary fat LDL, cholesterol levels are lowered and HDL cholesterol levels are raised. It has been observed (12) that human subjects on oleate-enriched diets produce an oleate enriched intracellular form of cholesteryl ester, which is cholesterol esterification. It is intriguing to speculate that these physiological effects of dietary oleate might be correlated with its down-regulatory effects on SREBP processing and endogenous cholesterol synthesis.

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