In Situ Accumulation of 3β-Hydroxylanost-8-en-32-aldehyde in Hepatocyte Cultures

A PUTATIVE REGULATOR OF 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE ACTIVITY*

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Biphasic modulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) has been demonstrated in primary hepatocyte cultures treated with the lanosterol 14α-methyl demethylase inhibitor miconazole. At concentrations of the drug which lead to suppressed levels of reductase activity, the appearance of a polar, mevalonate-derived sterol is noted. Cochromatography of the identified sterol with 3β-hydroxylanost-8-en-32-aldehyde tentatively identified the metabolite as a lanosterol 14α-methyl demethylatice intermediate. Subsequent isolation and characterization of the metabolite by gas chromatography/mass spectroscopy confirmed this structural assignment. When the lanosterol 14α-methyl demethylase-deficient mutant, AR45, was treated with authentic metabolite, a suppression of HMG-CoA reductase was observed. These results demonstrate that metabolism of the oxygenated biosynthetic intermediate is not required to suppress reductase activity. The results also strongly support the hypothesis that oxygenated 14α-methyl demethylase intermediates are endogenously generated modulators of HMG-CoA reductase activity.

Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) by inhibitors of lanosterol 14α-methyl demethylation has been demonstrated in cultured cell systems of various origins (1–4). The observed regulation is biphasic with lower concentrations of the demethylase inhibitor producing suppression of HMG-CoA reductase activity, whereas higher levels of the agents result in inhibition or even stimulation of the enzyme (3, 4). In the previous report (4), we demonstrated that mevalonate-derived sterols were required for the observed regulatory effects of the two 14α-demethylase inhibitors, miconazole and ketoconazole, upon HMG-CoA reductase activity. In addition, Gupta et al. (3) have demonstrated accumulation of the mevalonate-derived product 24,25-epoxylanosterol in ketoconazole-treated cells. This observation led these authors to propose that 24,25-epoxylanosterol was a precursor to some yet unidentified oxysterol metabolite which was responsible for the observed regulation of HMG-CoA reductase activity by ketoconazole. We have also demonstrated that ketoconazole-treated cells accumulate 24,25-epoxylanosterol (4). Miconazole-treatment of cultured cells, however, results in the same biphasic regulation of HMG-CoA reductase without the obligate formation of the epoxidized sterol. These results demonstrate that side chain epoxidation is not a prerequisite for mevalonate-derived endogenous oxysterol regulators of HMG-CoA reductase as has been proposed (3). To date, the identification of a regulated sterol metabolite which reciprocally correlates with HMG-CoA reductase activity has not been demonstrated.

In this report, we describe the effects of miconazole treatment upon HMG-CoA reductase activity and sterol profiles in rat hepatocytes. Our data demonstrate a biphasic regulation of HMG-CoA reductase activity which inversely correlates with the appearance of a lanosterol 14α-demethylase oxysterol intermediate. Purification and characterization of the metabolite by GC/MS have allowed identification of the putative oxysterol regulator of cellular HMG-CoA reductase activity as 3β-hydroxylanost-8-en-32-aldehyde.

EXPERIMENTAL PROCEDURES

Methods

Preparation of Primary Hepatocyte Cultures—Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) weighing 150–250 g were used exclusively during the course of this study. Animals were maintained on a diet containing 3% cholestyramine and were housed under reverse light cycle as previously described (5). Animals were killed at the midpoint of the dark cycle.

Hepatocytes were isolated by a modification of the collagenase perfusion technique described by Seglen (6). All perfusion buffers were bubbled with 5% CO2, 95% O2, before use and were constantly maintained at 37°C during the perfusion. Animals were anesthetized by intraperitoneal injections of pentobarbital (5 mg/100 g of body weight). The liver was perfused in situ with 150 ml of Hank’s balanced salt solution supplemented with 0.5 mM EGTA, 10 mM Hepes, followed by 250 ml of Williams’ Medium E supplemented with 0.5 mg/ml collagenase (Sigma, Type IV), 10 mM Hepes, 2 mM L-glutamine. Hepatocyte viability was determined by trypan blue exclusion (0.4%) and was greater than 95%. Cells were plated on 100 × 20-mm tissue culture dishes (Falcon) at a density of 5 × 105 in 5 ml of Williams’ Medium E supplemented with 10% fetal calf serum, 25 mM sodium pyruvate, 10 mM Hepes, 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium, 50 μg/ml gentamicin, 300 units/ml penicillin and incubated for 3–4 h at 37°C under 5% CO2, 95% air atmosphere. Cells were then washed twice with a total volume of 10 ml of serum-free medium before the addition of 5 ml of Williams’ Medium E containing 4 mg/ml delipidated serum (7), 10 mM Hepes, 25 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml gentamicin,

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1 The abbreviations used are: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Hepes, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; EGTA, [ethylenebis(oxymethylene)tetraacetic acid]; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; CHO, Chinese hamster ovary.

2 Cholestyramine is a bile acid sequestrant resin obtained from Mead Johnson & Co.
and 300 units/ml penicillin. Experiments were performed on 21-h cultures. Fresh medium was added at the start of each experiment and after 24 h during longer treatments. Duplicate dishes were used for each condition.

**Isolation of Microsomes and Determination of HMG-CoA Reductase Activity.** Rat liver homogenates were harvested by aspirating culture medium and washing the cells twice with a 10 mM buffered saline. The cells were collected into 4.5 ml of buffer (160 mM Hepes, 250 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol, pH 7.0) and vortexed vigorously for 30 s. The broken cells were centrifuged at 13,000 rpm for 5 min to remove large granules and cell debris. The supernatant was removed and centrifuged at 100,000 rpm for 6 min in a Beckman Airfuge to sediment the microsomes. The microsomal pellet thus obtained was assayed immediately for HMG-CoA reductase activity or frozen at −80 °C until use. HMG-CoA reductase activity was determined by modification of the procedure described by Philipp and Shapiro (10). The assay mixture (35 μl) consisted of approximately 75 μg of microsomal protein/assay suspended in assay buffer (160 mM Hepes, 200 mM KCl, 1 mM EDTA, 15 mM dithiothreitol, 0.2% Triton N-101 with 80 mM isocitric acid. 1.72 units/ml isocitric dehydrogenase, 9 mM MgCl₂, pH 7.5) was preincubated at 37 °C for 30 min before the addition of substrates: 2 mM NADPH with 181 μM [3-14C]-3-hydroxy-3-methylglutaryl CoA (10,000 dpm/ml) (Du Pont-New England Nuclear). The reaction was terminated after 30 min at 37 °C by addition of 6 N HCl containing recovery standard of (R,S)-[5-3H]mevalonolactone (90,000 dpm/assay) (Du Pont-New England Nuclear). Lactonization was allowed to proceed for an additional 30 min at 37 °C. Assay mixtures were centrifuged at 13,000 rpm for 5 min before spotting the whole supernatant on a silica gel HL strip (Analtech, Inc.). TLC strips were developed in an unsaturated environment employing acetonitrile:benzene (3:2) as developing solvent. The appropriate area containing mevalonolactone was scraped into counting vials and counted in 10 ml of liquid scintillation cocktail (Biofluor (Du Pont-New England Nuclear). Under these conditions, the recovery of [3H]mevalonolactone ranged between 75 and 100%. All assays were performed in duplicate and were corrected for recovery and background measurements.

**Incorporation of Radiative Precursors into Nonsaponifiable Lipids.** Nonsaponifiable lipids were extracted from the microsome fraction by homogenization (51, v/v) in 0.1 M potassium phosphate buffer (also containing 30 mM nicotinamide, 5 mM MgCl₂, 1 mM glutathione, 0.125 M sucrose, pH 7.4). Homogenates were centrifuged at 700 × g for 5 min to remove larger granules and cell debris. The supernatant fraction was then centrifuged at 30,000 × g for 15 min to sediment mitochondria and lysosomes, which left a preparation referred to as S-30, enriched in microsomes and cytosol. The S-30 preparation was used in the incorporation studies described below.

A similar protocol was used to assess the effect of miconazole on mevalonate incorporation into nonsaponifiable lipids generated with rat liver homogenate preparations. Livers were prepared by removal of 56.7% by weight of the supernatant fraction at 37 °C. The supernatant fraction was then centrifuged at 37,000 × g for 10 min to remove mitochondria and lysosomes, which left a preparation referred to as S-30, enriched in microsomes and cytosol. The S-30 preparation was used in the incorporation studies described below.

**Protein Determinations.** Protein was determined by the BioRad dye binding assay according to the manufacturer’s directions employing bovine serum albumin as a standard.

**Materials.**

Sterol standards were prepared as described previously (9). The isomeric mixture of lanost-8-ene-3β,23-diol was resolved by HPLC (Scribner Instrument Co., Inc.) column (2.5 × 40 cm) employing a mobile phase of chloroform:methanol (97:3, v/v). The resolved Δ⁷- and Δ⁵-isomers were further converted to the corresponding C-32 aldehydes as described (9). The desired 3S-
droxylanost-8-en-32-aldehyde showed: m.p. 177-179 °C (recrystallized from acetone), [α]D = −290.6 ± 2.0 (C = 1.03, CHCl3); NMR (300 MHz, CDCl3): 9.45 (s, 1H, 33-CHO), 3.23 (dd, J = 11.3 Hz, 4.7 Hz, 1H, 3α-H), 1.06 (s, 3H, 19-H), 0.98 (s, 3H, 4α-CH, 4β-CH3), 0.89 (d, J = 6.6 Hz, 3H, 21-H), 0.86 (d, J = 6.5 Hz, 6H, 25, 27-H), 0.83 (s, 3H, 4α-CH), 0.76 (s, 3H, 18-H); IR (KBr): 3440 (bs), 2960 (s), 2935 (d, J = 6.5 Hz, 3H, 21-H). 1.06 (s, 3H, 19-H), 0.98 (s, 3H, 4α-CH3), 0.89 (d, J = 6.6 Hz, 3H, 21-H), 0.86 (d, J = 6.5 Hz, 6H, 25, 27-H), 0.83 (s, 3H, 4α-CH), 0.76 (s, 3H, 18-H); IR (KBr): 3440 (bs), 2960 (s), 2935 (d, J = 6.5 Hz, 3H, 21-H). 1.06 (s, 3H, 19-H), 0.98 (s, 3H, 4α-CH3), 0.89 (d, J = 6.6 Hz, 3H, 21-H), 0.86 (d, J = 6.5 Hz, 6H, 25, 27-H), 0.83 (s, 3H, 4α-CH), 0.76 (s, 3H, 18-H); IR (KBr): 3440 (bs), 2960 (s), 2935 (d, J = 6.5 Hz, 3H, 21-H).

All cofactors, enzymes, and other biochemicals were from Sigma as described (5). Culture medium was obtained from GIBCO. Fetal calf serum was from Sterile Systems (Logan, UT). All other reagents and supplies were of the best grade commercially available.

**RESULTS**

**Miconazole Treatment of Hepatocyte Cultures**—We were initially interested in the ability of miconazole to inhibit lanosterol demethylation in cultured hepatocytes and to mimic the biphasic effect upon HMG-CoA reductase activity observed in other cell types (3, 4). To test the drug’s effectiveness, we monitored microsomal HMG-CoA reductase activity and [14C]mevalonolactone incorporation into non-saponifiable lipids.

Fig. 1A shows the changes observed in the labeling pattern of the major cellular sterols in hepatocyte cultures in response to miconazole treatment. It can be seen that a precipitous drop in mevalonate incorporation into cholesterol occurred as a result of increasing miconazole exposure. Complete inhibition was seen at 32 μM. This decrease in cholesterol biosynthesis was accompanied by a reciprocal increase in the amount of labeled lanosterol observed in treated cells. Similarly, an accumulation of dihydrolanosterol was noted, with an obvious peak of accumulated sterol occurring at low miconazole concentrations (16 μM). This dihydrolanosterol peak decreased with increasing miconazole exposure. Under these same conditions of inhibition, little or no effect upon squalene accumulation was observed. Miconazole treatment of hepatocyte cultures also caused a biphasic response in measured HMG-CoA reductase activity (Fig. 1C). Exposure of cells up to 8 μM miconazole resulted in a reduction in measured microsomal HMG-CoA reductase activity, reaching a minimum of 50% of control levels. As the concentration of the agent was increased beyond this point, a return to control values was observed for the measured HMG-CoA reductase activity. Direct exposure of isolated hepatic microsomes to concentrations of miconazole in the range tested were without effect upon enzymatic activity (data not shown). Thus, miconazole was capable of inhibiting HMG-CoA reductase activity in cultured cells, but was without effect when assayed directly upon the enzyme. This was analogous to the CHO cell system (4), although the concentration of miconazole required to suppress HMG-CoA reductase activity was somewhat higher in hepatocyte cultures.

The most interesting observation noted during treatment of hepatocyte cultures with miconazole is presented in Fig. 1B. Shown is the behavior of a novel polar mevalonate-derived product as a function of miconazole concentration. The polar product termed Peak A displayed an interesting biphasic profile. Substantially larger amounts of Peak A material accumulated at low miconazole concentrations; whereas at higher levels of the inhibitor, the detectable amounts of the mevalonate-derived product decreased. Previous results in CHO cells (4) demonstrated that miconazole suppression of HMG-CoA reductase activity required production of a mevalonate-derived sterol. The reciprocal nature of Peak A and HMG-CoA reductase activity measured in miconazole-treated cells suggested a potential connection between the two parameters. Tentative identification of Peak A as 3β-hydroxylanost-8-en-32-aldehyde could be made based upon cochromatography with authentic standard. Further support for the relationship between Peak A and HMG-CoA reductase activity was obtained during a time course experiment.

**Time Course of Miconazole Effect in Cultured Hepatocytes**—The time course of the miconazole effect upon sterol synthetic profiles and inhibition of HMG-CoA reductase activity are shown in Fig. 2. Note that the inhibition of cholesterol synthesis and accumulation of lanosterol in treated cells was quite rapid (Fig. 2A). Total inhibition of cholesterol synthesis was seen after 1 h of exposure to the drug. This high level of inhibition was maintained for several hours; but by 24 h, the effect of the agent was overcome with sterol labeling profiles returning to those observed in untreated cells. Refeeding cultures with fresh medium containing the inhibitor resulted in a second phase of inhibition, showing that the process was repeatable and indicating that metabolism of the agent had occurred. This would be consistent with the known metabolic behavior of miconazole, which is broken down quite rapidly in the liver to inactive metabolites (13). The precursor/produ
The reciprocal nature of the Peak A metabolite and the decrease in measured HMG-CoA reductase activity shown in Figs. 1B and 2B again suggested that the observed mevalonate-dependent modulation of HMG-CoA reductase activity by miconazole was due to the presence of this metabolite. However, in order to establish that Peak A was indeed the proposed 3β-hydroxylanost-8-en-32-aldehyde, it was necessary to purify the metabolite generated in response to miconazole treatment and determine its structure. Similarly, it was necessary to demonstrate suppression of HMG-CoA reductase activity in cultured cells by the identified metabolite, thereby establishing modulation of HMG-CoA reductase activity as proposed.

The transient nature of the Peak A metabolite and the limited flux of mevalonate through the sterol pathway in hepatocyte preparations during the time frame of our experiments prevented sufficient mass accumulation required for identification from this source. We have, however, established a second means to produce Peak A, starting with mevalonate utilizing a liver preparation. S-30 preparations of hepatic origin yield mevalonate-labeled sterol profiles analogous to those obtained with hepatocyte cultures upon exposure to miconazole (Fig. 3). In addition, S-30 preparations have the distinct advantage of allowing sufficiently greater mass accumulation than hepatocytes, thus permitting characterization by mass measurements in addition to cochromatography. Therefore, we have used S-30-generated Peak A metabolite for characterization purposes.

**Characterization of Peak A Generated by Hepatic S-30 Preparations**—Resolution of Peak A from the major sterol components present in the cellular extract can be accomplished by mass measurements in addition to cochromatography. The reciprocal nature of the Peak A metabolite and the decrease in measured HMG-CoA reductase activity shown in Figs. 1B and 2B again suggested that the observed mevalonate-dependent modulation of HMG-CoA reductase activity by miconazole was due to the presence of this metabolite. However, in order to establish that Peak A was indeed the proposed 3β-hydroxylanost-8-en-32-aldehyde, it was necessary to purify the metabolite generated in response to miconazole treatment and determine its structure. Similarly, it was necessary to demonstrate suppression of HMG-CoA reductase activity in cultured cells by the identified metabolite, thereby establishing modulation of HMG-CoA reductase activity as proposed.

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by preparative chromatography as described under "Experimental Procedures." Radiopurity of Peak A following the HPLC separation was confirmed by rechromatography in the same chromatographic system used for purification (Fig. 4). Mass purity of the compound as determined by GC/MS showed that although sufficient material for radioactive measurements was present, no material was detectable by conventional scan-mode analysis (data not shown). However, when GC/MS analysis was performed by monitoring selected ions of the standard derivatized 3β-hydroxylanost-8-en-32-aldehyde, confirmation of Peak A structure was obtained (Fig. 5). Cochromatography and fragmentation ions expected for the derivatized C32 aldehyde standard as well as the unknown were obtained: 455 (M+CH2-OTMS) and 395 (M+CH2-OTMS-CH,COOH). By employing a 13C-enriched mevalonate source, mass labeling of Peak A was also obtained. Upon selective ion monitoring analysis, this appears as an altered carbon abundance about the major fragmentation ions seen for the derivatized Peak A metabolite (Table I). For comparison, the fragmentation pattern observed for synthetic standard 3β-hydroxylanost-8-en-32-aldehyde carried through comparable derivatization procedures is shown. The same alteration in ion abundance was seen for [13C/14C]mevalonate-derived lanosterol obtained from the S-30 preparation when compared to a natural abundance standard analyzed by this technique (Fig. 6 and Table I). Thus, the anomaly of ion abundance is confirmed in a second mevalonate-derived metabolite generated under the same conditions.

**Suppression of HMG-CoA Reductase Activity in the Lanosterol 14α-Methyl Demethylase Mutant, AR45, by 3β-Hydroxylanost-8-en-32-Aldehyde.** Proof that the isolated Peak A, 3β-hydroxylanost-8-en-32-al, could itself suppress HMG-CoA reductase activity without further metabolism was obtained with the lanosterol 14α-methyl demethylase-deficient mutant AR45. In this cell, cholesterol synthesis is blocked at the point of lanosterol demethylation; thus, metabolism of the oxylanosterol demethylation intermediate is prevented. Treatment of AR45 cells with the C32 oxysterol causes a suppression of HMG-CoA reductase activity (Table II). The decrease is due to a loss of immunoreactive HMG-CoA reductase protein (Fig. 7). Thus, the endogenously generated cholesterol biosynthetic intermediate can suppress HMG-CoA reductase activity without further metabolism, thereby establishing its role as a putative modulator of cholesterol biosynthesis.

**DISCUSSION**

In this report, we have demonstrated that the antifungal miconazole is a very effective inhibitor of cholesterol biosynthesis in cultured hepatocytes. The inhibitor acts at the site of lanosterol demethylation during cholesterol biosynthesis as shown by the accumulation of substantial amounts of lanosterol and its side chain saturated analogue, dihydrolanosterol, in treated cells. Similar observations have been made for the effect of the drug when studied in microorganisms (14) and other cell systems (4).

During exposure of hepatocyte cultures to the drug, it was

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Comparison of mass fragmentation ion abundance for sterol standards and \[^{13}C/^{14}C\]mevalonate-derived sterols

Sterol samples were prepared as described under "Experimental Procedures." Derivatization was performed also as described prior to GC/MS analysis. Ion abundance of each ion is presented as a percentage of the major ion seen in the mass spectrum for a given fragment.

| Compound | Ion m/e | Ion abundance |
|----------|---------|---------------|
| 3\(^\beta\)-Hydroxylanost-8-ene-3\(a\)-al \[^{13}C/^{14}C\]-Labeled Peak A | 395.40 | 100.00 |
| 3\(^\beta\)-Hydroxylanost-8-ene-3\(a\)-al \[^{13}C/^{14}C\]-Labeled Peak A | 455.40 | 100.00 |
| Lanosterol \[^{13}C/^{14}C\]-Lanosterol | 393.40 | 100.00 |
| Lanosterol \[^{13}C/^{14}C\]-Lanosterol | 411.40 | 100.00 |
| Lanosterol \[^{13}C/^{14}C\]-Lanosterol | 426.40 | 100.00 |

**TABLE II**

Effect of Peak A metabolite, 3\(^\beta\)-hydroxylanost-8-en-3\(a\)-aldehyde, upon measured HMG-CoA reductase activity in the lanosterol 14\(a\)-methyl demethylase mutant, AR45

Cells were grown and treated, and HMG-CoA reductase activity was determined on cellular homogenates as described (4). Sterols were given to cells as a suspension in 2.5% bovine serum albumin in 100% ethanol. HMG-CoA reductase activity was determined after a 6-h treatment period. Results are the average of duplicate assays done in two cultures.

| Addition | Control HMG-CoA reductase activity |
|----------|------------------------------------|
| None     | 100                                |
| 2.5 \(\mu\)g Peak A                 | 45                                 |
| 0.1 \(\mu\)g/ml 25-hydroxycholesterol | 24                                 |

\(^a\) 100% activity = 558 pmol/min/mg.

**FIG. 7.** Immunoblot of HMG-CoA reductase-reactive protein in AR45 cells treated with Peak A metabolite, 3\(^\beta\)-hydroxylanost-8-en-3\(a\)-aldehyde. AR45 CHO cells were grown and treated as described in the legend to Table II. Immunoreactive HMG-CoA reductase protein was detected in homogenates of treated cultures by Western blot analysis employing anti-HMG-CoA reductase antisera as described (4). Lane 1, Bethesda Research Laboratories molecular weight marker phosphorylase b (M, 97,400); lane 2, blank; lane 3, control; lane 4, 0.1 \(\mu\)g/ml 25-hydroxycholesterol; lanes 5-8, 1.25, 2.5, 5, and 10 \(\mu\)M 3\(^\beta\)-hydroxylanost-8-en-3\(a\)-aldehyde, respectively.

also noted that substantial quantities of polar sterols accumulated in response to increasing miconazole treatment. The level of the accumulated sterols, however, was reduced at higher concentrations of the drug. Comparable effects of miconazole upon the accumulation of polar 14\(a\)-methyl demethylation intermediates have been observed when studied directly upon the isolated demethylase enzyme (15). Accumulation of polar sterols in hepatocyte cultures in response to miconazole treatment therefore was not an entirely unexpected event. Interestingly, miconazole treatment of cultured cells also led to the inhibition of the rate-limiting enzyme of the cholesterol biosynthetic pathway, HMG-CoA reductase. Direct exposure of microsomal HMG-CoA reductase to the drug, however, did not produce any inhibitory effect upon enzymatic activity. These results were intriguing as they suggested that miconazole was capable of modulating HMG-CoA reductase activity through a mechanism other than direct inhibition of enzymatic activity.

An observed correlation between the level of polar sterol metabolite accumulation and the inhibition of HMG-CoA reductase activity in response to miconazole treatment has been noted (Figs. 1 and 2). The isolated metabolite has been identified as 3\(^\beta\)-hydroxylanost-8-en-3\(a\)-aldehyde, an intermediate in the C-32 demethylation sequence of dihydrolan-
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This particular oxygenated sterol has been shown to suppress HMG-CoA reductase activity in a variety of cell types (16). It has also been demonstrated that the C-32 aldehyde is converted to C15 sterols including cholesterol (16). Thus, its suppressive effect upon HMG-CoA reductase could be due to this effect. In the present report, we have demonstrated that the C-32 aldehyde suppresses HMG-CoA reductase in the lanosterol 14α-methyl demethylase-deficient mutant, AR45. With this mutant, metabolism of lanosterol and the 14α-methyl demethylation intermediates to the immediate demethylation product, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, and subsequently cholesterol is blocked.

Therefore, it is demonstrated that the C-32 aldehyde suppresses HMG-CoA reductase without conversion to other sterol products. Cross-resistance of 25-hydroxysterol-resistant mutants with the C-32 aldehyde also indicates that both sterols act through a common mechanism (16). Similarly, other functionalized 14α-methyl sterols inhibit HMG-CoA reductase activity in a manner analogous to 25-hydroxycholesterol in cultured cell systems (17, 18). Taylor et al. (18) have demonstrated that a positive correlation exists between suppression of HMG-CoA reductase activity in cultured cells and binding to a cytosolic oxysterol-binding protein. Functionalized 14α-methyl sterols fit the correlation established in this extensive investigation. It appears therefore that the mode of action of the endogenously generated oxysterol correlates with the proposed mechanism of action of exogenously added oxysterols in cultured cells (19).

The present data raise the possibility that the 3β-hydroxy-lanost-8-en-32-aldehyde may indeed be an endogenous modulator of HMG-CoA reductase activity and cholesterol biosynthesis. However, to function as a modulator, production of the metabolite must be correlated with decreased levels of HMG-CoA reductase activity in untreated cells. To date, such studies are limited. Tabacik et al. (20) isolated the 14α-oxygenated sterol from normal human lymphocytes under conditions of altered sterol flux in this cell type. These authors concluded that the isolated dihydrolanosterol C-32 aldehyde was a natural regulator of HMG-CoA reductase activity. In the present report, the same oxysterol intermediate accumulated as a result of inhibiting lanosterol 14α-methyl demethylation with coincident suppression of HMG-CoA reductase. Similarly, under cell culture conditions with increased carbon flux through the latter stages of cholesterol synthesis obtained with mevalonate loading (21), a comparable accumulation of lanosterol and dihydrolanosterol occurs with HMG-CoA reductase suppression. Dissociation of oxygenated demethylation intermediates would be predicted under these conditions based upon previous in vitro results (15). It should be pointed out, however, that lower HMG-CoA reductase activity is observed in the lanosterol 14α-methyl demethylase-deficient mutant, AR45 (4). Thus, the modulation of HMG-CoA reductase activity may be influenced by factors other than oxysterols of the type described in this report.

Our results demonstrate that alternate pathways need not exist for the generation of oxygenated sterol regulators of cholesterol biosynthesis. Significant quantities of regulatory metabolites can be generated during the synthesis of cholesterol and accumulate as a result of the kinetic properties of particular enzymes in the biosynthetic sequences (14). Our results and those of Tabacik et al. (20) demonstrate that the level of side chain saturation may be a key and significant factor in the generation of regulatory metabolites of the cholesterol biosynthetic pathway. It is the side-chain-saturated, C-32 functionalized sterol which correlates with an inhibition of HMG-CoA reductase activity even though substantially larger amounts of the side-chain-unsaturated precursor accumulates. In CHO cells, the situation is quite different. Under comparable conditions with lanosterol demethylase inhibitors, the appearance of a side chain-unsaturated lanosterol-derived oxysterol has been noted (4). Definitive structure confirmation of this sterol, however, has not been made. It is well established that both unsaturated as well as saturated C30 sterol analogues are converted to cholesterol (22, 23). However, the relative efficiency of conversion of various sterols is dependent upon side chain saturation status. Lanosterol is the preferred substrate for the C-32 demethylation when compared to 24,25-dihydrolanosterol (15). Most important is the viewpoint of generation of regulatory sterols is the ability of rather low levels of lanosterol to promote the accumulation of oxygenated dihydrolanosterol derivatives when the latter are used as the primary substrate for the demethylase enzyme (15). This latter situation appears to correlate most closely with the observations made with cell culture systems (20) and the present report.

Unfortunately, little is known about the enzymology of the steroid 24-reductase enzyme which is responsible for the formation of dihydrolanosterol from lanosterol. Avigan et al. (24) have shown that a single enzyme species is responsible for side chain reduction of lanosterol or desmosterol in membrane preparations. The enzyme-catalyzed reaction has been shown to be quite slow (25), and Pozetti et al. (26) have demonstrated that the preferred substrate for the reaction is the 4,4,14-trinormethyl sterol, desmosterol. Data to support the importance of side chain saturation status in the generation of cholesterol biosynthesis regulators and a demonstration of increased amounts of C30 oxysterols under conditions of decreased sterol synthesis are necessary to substantiate their regulatory role. Studies are currently underway in this laboratory aimed at understanding this mechanism of oxysterol generation.

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