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D-*ERYTHRO*-SPHINGOSINE LOWERS 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE ACTIVITY IN CHINESE HAMSTER OVARY CELLS

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Summary: D-*Erythro*-sphingosine lowered the levels of HMG-CoA reductase activity in CHO-K1 cells. Significant suppression of reductase activity was observed at 5 μM, 10 μM, and 15 μM concentrations of the sphingolipid base and ~50% lowering was found at 10 μM. In contrast, L-*threo*-sphingosine had no effect on the levels of reductase activity under the conditions studied. Direct addition of D-*erythro*-sphingosine, at concentrations up to 100 μM, to rat liver microsomes had no effect on the levels of HMG-CoA reductase activity. The concentrations of D-*erythro*-sphingosine required to lower reductase activity in CHO-K1 cells correspond to reported levels of sphingosine in mammalian cells.

Sterols and sphingolipids represent two classes of unique constituents of eukaryotes. In the cells of these organisms they are localized almost exclusively in membranous components, and the bulk of each is found in plasma membranes. Both cholesterol and sphingolipids occur in high concentrations in brain and spinal cord, both cholesterol (and its esters) and sphingomyelin are particularly abundant in atherosclerotic lesions and both increase with the severity of the lesions (1). Certain complex sphingolipids are recognized to have important antigenic properties, both in normal cells (e.g., blood group determinants) and in tumor cells (2). Some complex sphingolipids have been implicated in receptor functions for toxins, hormones, viruses, and other ligands and may play roles in differentiation and oncogenic transformation (3). A great deal of attention has recently been focussed upon free sphingolipid bases (4-6) and their N-alkyl (7,8), N-acyl (9,10), and 1-phosphate derivatives (11-13) as potential modulators of cellular physiology, metabolism, and replication.

We report here a previously undescribed action of a sphingolipid base on a critical enzyme in sterol and isoprenoid biosynthesis and in the regulation of cell function and replication. D-*Erythro*-sphingosine (SPH), but not its L-*threo* analog, is shown to lower the levels of HMG-CoA reductase activity in CHO-K1 cells.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; SPH, sphingosine; TLC, thin layer chromatography; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; GC, gas chromatography; IR, infrared; TMS, trimethylsilyl; MPLC, medium pressure liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol.

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MATERIALS AND METHODS

The sources for silica gel G plates for TLC, CHO-K1 cells, (3*RS*)-[3-^{14}C]HMG-CoA, (3*RS*)-[2-^{3}H]mevalonolactone, Lux tissue culture plasticware, trypsin, Ham's F12 medium, PBS, FCS, and BSA (fatty-acid free) have been described previously (14). ^{1}H and ^{13}C NMR spectra were measured on a Bruker AMX 500 (500 MHz for ^{1}H) or an IBM AF300 spectrometer (300 MHz for ^{1}H, 75.5 MHz for ^{13}C) in $CDCl_3$ solution and referenced to tetramethylsilane (^{1}H) or $CDCl_3$ at 77.0 ppm (^{13}C). TMS derivatives were prepared by a modification of the method of Carter and Gaver (15) or by treatment with N,O-bis(trimethylsilyl)trifluoroacetamide-pyridine (1:1) for 4 h at 20°C. GC analysis on packed 3% OV-17 or 3% OV-1 columns was carried out as described previously (16) and capillary GC analyses were made on a 15-m Rt_x 1701 column (Restek Corp.; 0.25 mm i.d., 0.1 μm film thickness) with splitless injection and temperature programming from 100°C to 240°C at 20°C per min. Under the latter conditions the tris-TMS derivatives of *erythro*- and *threo*-SPH showed retention times of 9.90 and 9.89 min, respectively, whereas the N-acetyl-TMS derivatives of the *erythro*- and *threo*-SPH, prepared by a modification of the method of Gaver and Sweeley (17), showed retention times of 11.89 and 11.56 min, respectively. The solvent system used for TLC of the bases was $CHCl_3$-methanol-concentrated aqueous ammonia, 100:25:2.5).

D-*Erythro*-SPH and L-*threo*-SPH were isolated from beef brain sphingolipids by a modification (16) of a procedure described by Gaver and Sweeley (18). Briefly, bovine brain sphingolipids (50 g), prepared according to Carter *et al.* (19), were heated under reflux with a mixture of 5 N HCl and methanol (1:4; 1000 ml) for 18 h. After cooling to room temperature, the resulting mixture was extracted with petroleum ether (3 x 500 ml) to remove fatty acids. The pH of the residual aqueous methanol mixture was, after cooling in an ice bath, adjusted to 12 by the addition of 15 N KOH. $CHCl_3$ and water were added to give a ratio of $CHCl_3$-methanol-water of 8:4:3. After thorough mixing the lower layer was removed and washed with an equal volume of $CHCl_3$-methanol-water (6:94:96; 2 x 1800 ml) and then evaporated to dryness under reduced pressure to give a mixture of crude sphingolipid bases (10.9 g). The bases were dissolved in a mixture of $CHCl_3$ and methanol (9:1; 40 ml) and subjected to MPLC on a silicic acid (32-63 μm; ICN Pharmaceuticals, Inc.) column (100 cm x 2.5 cm) using operating conditions described previously (16). Using a mixture of $CHCl_3$ and methanol (9:1) as the eluting solvent, fractions ~20 ml in volume were collected. The contents of fractions 83-148, showing a single component on TLC, were crystallized twice from ethyl acetate to give D-*erythro*-SPH as white crystals. This material showed a single component on TLC and, as the tris-TMS derivative, one major component on GC analysis (3% OV-17; 220°C) with a small amount (3%) of a contaminant which, in previous studies (16) had the same retention time as the corresponding derivative of authentic dihydrosphingosine. A portion of the D-*erythro*-SPH was further purified by two recrystallizations from ethyl acetate with charcoal to give white crystals. GC (3% OV-17; 220°C) of the N-acetyl-bis-O-TMS derivative showed a single component which was easily distinguished from the corresponding derivative of an authentic sample of L-*threo*-SPH. Just prior to use in the experiments described herein, the D-*erythro*-SPH was repurified by chromatography on a Unisil (100-200 mesh; Clarkson Chemical Company) column (18 cm x 1 cm) by successive elution with $CHCl_3$ (50 ml), $CHCl_3$-methanol (98:2; 100 ml), and $CHCl_3$-methanol (95:5). The contents of fractions showing a single component on TLC with the mobility of D-*erythro*-SPH were pooled and recrystallized from ethyl acetate. The identity of this material was confirmed by its NMR spectra: ^{13}C NMR ($CDCl_3$, 75 MHz, 22°C, δ), C-1, 63.49; C-2, 56.15; C-3, 74.91; C-4, 129.25; C-5, 134.47; C-6, 32.35; C-7, 29.20; C-8, 29.3; $(CH_2)_n$, 29.6; C-15, 29.3; C-16, 31.88; C-17, 22.65; C-18, 14.08 and ^{1}H NMR ($CDCl_3$, 500 MHz, 30°C, δ), H-1_A, 3.64 (dd, 10.8, 5.9 Hz); H-1_B, 3.70 (dd, 10.8, 4.2 Hz), H-2, 2.91 (br q, 5 Hz); NH and OH, 2.1 (br s); H-3, 4.09 (t, 6.1 Hz); H-4, 5.48 (dd, 15.4, 7.0 Hz); H-5, 5.77 (dt, 15.3, 6.8 Hz); H-6_A and H-6_B, 2.05 (m); H-7, 1.36 (m); $(CH_2)_n$, 1.26 (m); H-16, 1.24 (m); H-17, 1.29 (m); H-18, 0.88 (t, 7.0 Hz). Considering different solvent and operating conditions, these data are compatible with reported ^{1}H data (20) and ^{13}C data (21), although the assignments of C-4 and C-5 are reversed in the latter

work.* The purity was estimated to be 98% on the basis of TLC, capillary GC analysis of the tris-TMS ether derivative, ^{1}H NMR (500 MHz), and ^{13}C NMR (75 MHz) analyses. No contamination with *threo*-SPH was detected by ^{1}H NMR (estimated detection limit of ~1%). D-*Erythro*-SPH, purified in the same manner, was further characterized in the form of its triacetate derivative: MP, 101.5-102.5°C [lit.: 103.5-104°C (22), and 101-102°C (23,24)]; $[\alpha]_D^{22}$ -13.6° (c 1.1, $CHCl_3$) [lit.: -11.7° (24), -12.8° (22,23); IR, ν_{max} 3290, 1736, 1687, 1553, 1267, 1233, and 1030 cm^{-1}. ^{1}H NMR (500 MHz) indicated no impurities (estimated detection limit, ~2%).

L-*Threo*-SPH was isolated in the following manner. In the MPLC of the crude sphingolipid bases (see above), further elution of the column gave a mixture composed chiefly (on the basis of TLC analyses of the free base and GC analyses of the tris-TMS derivative and the N-acetyl-bis-O-TMS derivative) of L-*threo*-SPH and D-*erythro*-SPH along with small amounts of material with the behavior of authentic dihydrosphingosine. A mixture of the *threo*- and *erythro*-SPH from the initial MPLC separation of the bases was subjected to repeated MPLC on a silicic acid column (100 cm x 1.5 cm) as described above. Using the same solvent mixture for elution, fractions 12 ml in volume were collected. The contents of fractions 96-190 (496 mg) were pooled and crystallized from ethyl acetate to give L-*threo*-SPH; ^{13}C NMR ($CDCl_3$:CD_3OH ~9:1, 75 MHz, 22°C, δ), C-1, 63.77; C-2, 56.17; C-3, 73.30; C-4, 129.31; C-5, 133.91; C-6, 32.22; C-7, 29.1; $(CH2)_n$ 29.5; C-16, 31.77; C-17, 22.53; C-18, 13.93 and ^{1}H NMR, ($CDCl_3$, 500 MHz, 30°C, δ), H-1_A, 3.56 (dd, 10.9, 6.3 Hz); H-1_B, 3.70 (dd, 10.9, 4.2 Hz), H-2, 2.85 (br q, 5 Hz); NH and OH, 2.0 (br s); H-3, 4.01 (t, 6.3 Hz); H-4, 5.46 (dd, 15.4, 6.9 Hz); H-5, 5.76 (dt, 15.3, 6.9 Hz); H-6_A and H-6_B, 2.05 (m); H-7, 1.37 (m); $(CH_2)_n$, 1.26 (m); H-17, 1.30 (m); H-18, 0.88 (t, 7.0 Hz). After consideration of differences in solvent and operating conditions, the ^{13}C data are compatible with reported values (21), although the assignments of C-4 and C-5 are reversed in ref. 21.* The purity of the L-*threo*-SPH was estimated to be at least 98% on the basis of TLC, ^{1}H NMR (500 MHz), and ^{13}C NMR (75 MHz) analyses. No contamination with *erythro*-SPH was detected by ^{1}H or ^{13}C NMR (estimated detection limits of ~1% and 3%). L-*Threo*-SPH, purified in the same manner, was further characterized by conversion to its dihydrosphingosine derivative. Hydrogenation (1.5 atm of hydrogen) overnight over a platinum oxide catalyst in ethanol followed by removal of the catalyst and two recrystallizations from ethyl acetate-methanol gave L-*threo*-dihydrosphingosine. This material was characterized as the tris-TMS derivative by GC (3% OV-17, 220°C) and as the N-acetyl-bis-O-TMS derivative by GC (3% OV-1, 222°C). The optical rotation ($[\alpha]_D^{27}$; methanol) of the *threo*-dihydrosphingosine was -8.6° and the rotation ($[\alpha]_D^{26}$; methanol) of D-*erythro*-dihydrosphingosine was +8.1° [lit.: $[\alpha]_D^{22}$ +10.1° (methanol) (16)].

For cell culture studies, the sphingolipid bases were added as ethanolic solutions (sterilized by filtration) to Ham's F12 medium (25) supplemented with 5% delipidated (26) FCS (lipid-deficient medium) and allowed to equilibrate for at least 6 h at room temperature prior to storage for 48 h at 4°C. The ethanol concentration in all of the experimental media was constant. In some experiments, D-*erythro*-SPH was added to the culture media in the form of an equimolar complex of the base with BSA. To each mixture, Ham's F12 medium supplemented with 5% delipidated FCS (1.0 ml) was added, and after thorough mixing by swirling, transferred to tubes containing additional lipid-deficient medium. After incubation for 6 h at room temperature, the media were stored at 4°C. In experiments involving the SPH-BSA complex, control experiments were carried out using media containing the SPH alone or BSA alone.

The CHO-K1 cells were maintained in Ham's F12 medium supplemented with 5% FCS (lipid-rich medium) in a humidified atmosphere of 5% CO_2-95% air at 37°C. Each experiment was initiated by inoculating 3.75 x 10^5 cells into 100 mm dishes containing the lipid-rich medium (10 ml), followed by incubation for 48 h. The medium was aspirated and, after rinsing of the plates with PBS (10 ml), the cells were incubated for 18 h in lipid-deficient media (10 ml). The cells then were incubated for 4 h with fresh lipid-deficient media (10 ml) containing various concentrations of

*The assignments of C-4 and C-5 were reversed in the initial paper on ^{13}C NMR of sphingolipids (28), and this error has been carried over into subsequent papers. The basis of the assignments of C-4 and C-5 will be discussed elsewhere: W.K. Wilson, M. Tsuda, R.J. Kulmacz, A. Kisic, and G.J. Schroepfer, Jr., manuscript in preparation.

the sphingolipid bases. The experimental medium was aspirated, and the plates were rinsed with ice cold PBS (10 ml). The cells were harvested by scraping into ice cold PBS (5 ml) containing DTT (5 mM), and detergent-solubilized cell preparations were assayed for HMG-CoA reductase activity as described previously (27).

The effect of D-*erythro*-SPH on the increase in HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient medium was measured as follows. After incubation of the cells in lipid-rich medium for 18 h as described above, the lipid-rich medium was aspirated. The plates were rinsed with PBS (10 ml), and lipid-deficient medium (10 ml) containing the indicated concentration of SPH was added to each plate. After incubation of the cells for 6 h, the experimental media were aspirated. The plates were rinsed with ice cold PBS, and the cells were isolated and processed as described above for assay of HMG-CoA reductase activity. Microscopic examination of cells incubated for 4-6 h in the presence of the bases revealed no morphological alterations in the cells.

The effect of direct addition of D-*erythro*-SPH on the levels of rat liver microsomal HMG-CoA reductase activity was studied by a minor modification of conditions used previously (29). Rat liver microsomes, isolated from chow-fed male Sprague-Dawley rats, were suspended in Tris-Cl buffer (50 μM; pH 7.44) containing EDTA (1 mM), EGTA (1 mM), DTT (1 mM), and leupeptin (50 μM) at a concentration of 5 mg protein per ml. Aliquots (0.1 ml) of the microsomal suspension were preincubated with propylene glycol (10 μl) alone or with solutions of D-*erythro*-SPH in propylene glycol (10 μl) for 15 min at 37°C. The enzyme reaction was initiated by the addition of potassium phosphate buffer (50 μl; 0.1 M; pH 7.4) containing DTT (10 mM), NADPH (2 mM), and (3*RS*)-[3-^{14}C]HMG-CoA (450 μM; 3,000 dpm per nmol). After incubation for 15 min at 37°C, the reaction was terminated by the addition of 10 N HCl (25 μl).

RESULTS

D-*Erythro*-SPH caused a concentration-dependent decrease in the levels of HMG-CoA reductase activity in CHO-K1 cells (Table 1). This sphingolipid base suppressed the rise in HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media. Clearly significant

Table 1. Effect of D-*erythro*-sphingosine on the levels of HMG-CoA reductase activity in CHOK1 cells

	HMG-CoA Reductase Activity (% of Control Activity)	
Sphingosine Concentration (μM)	Suppression of Rise Induced by Transfer of Cells to Lipid-deficient Media (n = 4)[a]	Suppression of Elevated Levels Induced by Transfer of Cells to Lipid-deficient Media (n = 8)
0	100.0 ± 5.4[b]	100.0 ± 4.7[b]
1	88.7 ± 8.9[c]	87.4 ± 3.7[c]
5	74.9 ± 6.2[c]	67.5 ± 5.7[c]
10	48.7 ± 7.3[c]	51.8 ± 6.0[c]
15	35.9 ± 14.2[d]	42.2 ± 6.2[c]

[a] Except for 15 μM sphingosine in which only 2 experiments were carried out. [b] Variation expressed as average of standard deviations for triplicate assays of HMG-CoA reductase activity for the individual experiments (n = 4 or n = 8). [c] Variation expressed as S.E.M. from values obtained from replicate (n = 4 or n = 8) individual experiments in which triplicate assays of HMG-CoA reductase activity were made for each concentration of sphingosine. [d] Variation expressed as average deviation from mean (n = 2).

lowering of the levels of reductase activity was observed at 5 μM (-25%), 10 μM (-51%), and 15 μM (-64%) concentrations of the sphingolipid base in the medium. In addition, D-*erythro*-SPH lowered the elevated levels of HMG-CoA reductase induced by transfer of the cells to lipid-deficient media. In these experiments, clearly significant lowering of the levels of reductase activity was observed at 5 μM (-33%), 10 μM (-48%), and 15 μM (-58%) concentrations of the sphingolipid base in the medium. In contrast to the case of D-*erythro*-SPH, L-*threo*-SPH had no significant effect on the elevated levels of HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media. Seven separate experiments were completed (with concentrations of 1.0 μM to 15 μM) as in the experiments listed in Table 1. Cells incubated in the presence of L-*threo*-SPH showed mean values of reductase activity which were indistinguishable from those of control cells (data not shown).

Equimolar complexes of D-*erythro*-SPH and BSA were similar in potency to D-*erythro*-SPH alone in suppressing the rise in HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media (Table 2) and they were also similar in potency to D-*erythro*-SPH alone in reducing the elevated levels of HMG-CoA reductase activity induced by transfer of the cells to lipid-deficient media (data not shown). BSA alone had no significant effect on HMG-CoA reductase activity under either condition except at a concentration of 15 μM in the former assay system, wherein a 29% lowering of reductase activity was observed.

Whereas D-*erythro*-SPH was highly active in lowering the levels of HMG-CoA reductase activity in CHO-K1 cells, direct addition of D-*erythro*-SPH (at concentrations of 1 μM to 100 μM) in propylene glycol to rat liver microsomes had no effect on HMG-CoA reductase activity (Table 3).

DISCUSSION

D-*Erythro*-SPH has been shown to lower the levels of HMG-CoA reductase activity in CHO-K1 cells. Significant lowering was observed at 5 μM and ~50% lowering was observed at

Table 2. Effect of D-*erythro*-sphingosine, D-*erythro*-sphingosine complexed to bovine serum albumin (1:1), and bovine serum albumin alone on suppression of the elevation of the levels of HMG-CoA reductase activity induced by incubation of CHO-K1 cells in lipid-deficient medium for 6 hours

Sphingosine Concentration (μM)	HMG-CoA Reductase Activity (% of Control Activity)[a]		
	Sphingosine	Sphingosine-BSA	BSA alone
0	100.0 ± 2.7[b]	100.0 ± 3.4[c]	100.0 ± 2.2[d]
1	83.1 ± 2.2	79.8 ± 7.3	98.9 ± 2.0
5	72.4 ± 2.1	67.6 ± 3.6	95.1 ± 0.8
10	48.6 ± 1.3	53.5 ± 3.1	105.5 ± 4.9
15	21.7 ± 2.3	34.3 ± 2.8	70.6 ± 5.0

[a] Variation expressed as ± S.D. of triplicate assays of HMG-CoA reductase activity. [b-d] Mean values for controls were 1676, 1846, and 1458 pmol/min per mg of protein, respectively.

Table 3. Lack of effect of direct addition of D-*erythro*-sphingosine to rat liver microsomes on the level of HMG-CoA reductase activity

Sphingosine Concentration (μM)	HMG-CoA Reductase Activity (% of Control Activity)[a]
0	100.0 ± 5.0
1	101.9 ± 4.9
10	99.3 ± 3.0
15	105.2 ± 1.7
25	109.7 ± 3.3
50	103.7 ± 1.7
75	104.5 ± 0.9
100	100.0 ± 3.3

[a] The mean control activity was 267 pmol/min/mg protein. Variation is expressed as S.E.M. from values obtained from replicate (n = 3) incubations.

10 μM.** These concentrations correspond to those estimated to exist in some human cells. For example, Merrill *et al.* (31) have estimated that the 12.3 pmol of free SPH per 10^6 cells reported for HL-60 cells corresponds to an intracellular concentration of ~10 μM. It is also important to note that the concentrations found to affect HMG-CoA reductase activity are considerably lower than those required to affect many of the enzyme activities or cellular processes reported to be regulated by free sphingolipid bases or their derivatives.

The mechanism(s) involved in the lowering of HMG-CoA reductase activity in the cells by D-*erythro*-SPH is not known. It is important to note that direct addition of this sphingolipid base, at concentrations of 1 μM to 100 μM, to rat liver microsomes had no effect on reductase activity.

D-*Erythro*-SPH has been reported to inhibit its own synthesis in cultured cerebellar neurons (32), presumably by its action in lowering the levels of serine palmitoyltransferase activity (33). The IC_{50} values for these actions of SPH appear to be somewhat higher (~25 μM) than those reported here for lowering of HMG-CoA reductase activity in CHO-K1 cells. Thus, SPH appears to be a candidate as a regulator not only of sphingolipid synthesis but also of sterol and isoprenoid synthesis.

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**Our results with CHO-K1 cells differ from those of Gupta and Rudney (30) who reported (as a part of a larger study of the effects of sphingomyelinase on cells) the results of one experiment with commercial SPH in which increases of reductase activity were noted at 16.7 μM (+26%) and 33 μM (+48%) concentrations of SPH.

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