Cholesterol metabolism

Effect of 26-thiacholesterol and 26-aminocholesterol, analogues of 26-hydroxycholesterol, on cholesterol synthesis and low-density-lipoprotein-receptor binding

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1. The effects of 26-aminocholesterol and 26-thiacholesterol on cholesterol synthesis and LDL (low-density lipoprotein)-receptor activity were compared with naturally occurring 26-hydroxycholesterol utilizing both human fibroblasts and hepatoma (Hep G2) cells. 2. At equimolar concentrations (0.625 μM), down-regulation of LDL-receptor activity and cholesterol synthesis was greater with human fibroblasts than with Hep G2 cells. 3. At much higher concentrations (5–20 μM) the 26-thia analogue had little effect on either cholesterol synthesis or LDL-receptor activity.

INTRODUCTION

The finding that 26-hydroxycholesterol is a naturally occurring component of human plasma lipoproteins [1–3] and is a potent inhibitor of HMG-CoA reductase [4,5] has led us to evaluate its effects on human hepatoma cells (Hep G2) and human fibroblasts. The 26-amino and 26-thia analogues of 26-hydroxycholesterol were prepared to define further the significance of the primary alcohol group in these biological effects.

EXPERIMENTAL

Materials

Carrier-free Na^{125}I was purchased from Amersham Corp. $^2\text{H}_2\text{O}$ was purchased from MSD Isotopes (St. Louis, MO, U.S.A.). Diosgenin was obtained from Sigma, and all agent-grade chemicals were purchased from Aldrich Chemical Co. Tissue-culture flasks (T-75, 80 cm, 260 ml) were supplied from Nunc Inc. and Falcon tissue-culture dishes (60 mm diameter) were obtained from Becton-Dickinson Labware. Bovine serum albumin and deoxyribonuclease I from bovine pancreas was purchased from Sigma Chemical Co. Eagle's minimum essential medium with Earle's salts (MEM) and all media supplements were purchased from Gibco Laboratories. Normal LDLR(+) human fibroblasts (GM3440) were obtained from the National Institute of General Medical Sciences Human Genetic Cell Repository, Camden, NJ, U.S.A. Hep G2 cells were a gift from Dr. Barbara Knowles of the Wistar Institute, Philadelphia, PA, U.S.A.

Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. N.m.r. spectra [^2H]chloroform] were run on a Nicolet QE-300 spectrometer. I.r. spectra were obtained on a Matteson Polaris FT-IR instrument.

Preparation of 26-hydroxycholesterol analogues

25(*R*),26-Aminocholesterol [6] was prepared by lithium aluminium hydride reduction of 25(*R*),26-azidocholesterol 3-acetate synthesized from 26-tosylcholesterol 3-acetate [7]. 26-Tosylcholesterol 3-acetate (397 mg, 0.663 mmol) was dissolved in 25 ml of DMF containing 1.5 equiv. of NaN_3 and 1 ml of water. The solution was heated to between 90 and 100 °C for 2 h, cooled to room temperature and then worked up as usual. Rotary evaporation yielded 254 mg (yield = 81.6%) of 26-azidocholesterol 3-acetate as a white powder, which was subsequently recrystallized (with ethanol) to yield 126 mg of white waxy crystals (m.p. 90–90.5 °C). N.m.r.: δ (p.p.m.) [^2H]chloroform] 0.701 (3, s, C-18 methyl), 0.941 (3, d, C-27 methyl, *J* 6.6 Hz), 0.970 (3, d, C-21 methyl, *J* 6.6 Hz), 1.042 (3, s, C-19 methyl), 2.058 (3, s, methyl of acetate on C-3), 3.1765 (3, multiplet ABX, *J* 38.17 Hz, *J* 11.89 Hz, *J* 5.95 Hz), 4.626 (1, m, C-3), 5.398 (1, d, C-6, *J* 4.50 Hz). I.r. (cm^{-1}): 2950 (C–H), 2100 (N–N), 1725 (C=O), 1250 (N–N), 1050 (C–O).

Lithium aluminium hydride (20 mg, 527 mmol) reduction of 26-azidocholesterol 3-acetate (100 mg, 0.213 mmol) in diethyl ether, followed by usual work-up, afforded 70 mg (yield 81.9%) of 25(*R*),26-aminocholesterol crude powder that was purified by flash chromatography [chloroform/ethanol/29% (w/v) NH_4OH , 93:7:1 (by vol.)] [m.p. 149–152 °C (literature value 148–151 °C [6])]. N.m.r.: δ (p.p.m.) [^2H]chloroform]: 0.669 (3, s, C-18 methyl), 0.875 (3, d, C-27 methyl, *J* 6.6 Hz), 0.906 (3, d, C-21 methyl, *J* 6.6 Hz), 0.999 (3, s, C-19 methyl), 2.528 (2, m, C-26), 3.499 (1, m, C-3) and 5.342 (1, d, C-6 proton, *J* 5.1 Hz). I.r. (cm^{-1}): 3600 (N–H), 2950 (C–H), and 1050 (C–O).

25(*R*),26-Thiacholesterol was prepared from the lithium aluminium hydride reduction of 25(*R*),26-thiacholesterol 3,26-diacetate synthesized from 26-tosylcholesterol 3-acetate [7]. Potassium thiocyanate (600 mg,

Abbreviations used: LDL, low-density lipoprotein; HMG-CoA, hydroxymethylglutaryl-CoA; MEM, Eagle's minimum essential medium with Earle's salts; FBS, fetal-bovine serum; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; DMF, *N,N*-dimethylformamide.

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5.148 mmol) was added to 20 ml of dry ethanol contained in a 50 ml three-neck flask; 393 mg (0.656 mmol) of 26-tosylcholesterol 3-acetate was added, and the solution was refluxed for 3 h, cooled to room temperature and then concentrated by rotary evaporation. Subsequent workup afforded 340 mg of crude yellow-green foul-smelling oil, which was purified by flash chromatography [hexane/ethyl acetate, 9:1 (v/v)], then recrystallized (with ethanol) to afford 215 mg (yield 65.16%) of the diacetate as white needles (m.p. 97.5–100 °C). N.m.r.: δ (p.p.m.) [(^2H)chloroform]: 0.696 (3, s, C-18 methyl), 1.039 (3, s, C-19 methyl), 2.055 (3, s, methyl of acetate at C-3), 2.353 (3, s, C-26 methyl of thioacetate), 2.852 (2, multiplet ABX, C-26 protons, J 50.03 Hz, J 13.24 Hz, J 5.77 Hz), 4.608 (1, m, C-3 proton) and 5.396 (1, d, C-6 proton, J 4.2 Hz). I.r. (cm^{-1}): 2950 (C–H), 1725 (C=O of acetate), 1690 (C=O of thioacetate), 1260 (C–S) and 1090 (C–O).

Pure 25(R),26-thiacholesterol 3,26-diacetate (130 mg, 0.258 mmol) was reduced quantitatively by the procedure described above. The resulting product was recrystallized (with ethanol) to afford shiny white crystals that gave a positive nitroferricyanide test for thiol functionality (m.p. 128.5–129.5 °C). N.m.r.: δ (p.p.m.) [(^2H)chloroform]: 0.693 (3, s, C-18 methyl), 0.929 (3, d, C-21 methyl, J 6.52 Hz), 0.975 (3, d, C-27 methyl, J 6.63 Hz), 1.023 (3, s, C-19 methyl), 2.476 (2, multiplet ABXY, C-26 protons), 3.534 (1, m, C-3 proton), and 5.360 (1, d, C-6 proton, J 4.2 Hz). I.r. (cm^{-1}): 3600 (O–H), 2950 (C–H), 1100 (C–O) and 680 (C–S).

Incubation of the 26-hydroxycholesterol analogues

Stock fibroblast monolayers were maintained in T-75 disposable Nunc flasks in 10 ml of MEM, pH 7.4, supplemented with 10% (v/v) FBS, 2% (v/v) non-essential and essential amino acids, 1% (v/v) MEM vitamins, 1% L-glutamine (292 $\mu\text{g}/\text{ml}$), 1% penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37 °C in a humidified atmosphere of CO_2/air (1:19). Hep G2 stock monolayers were maintained under identical conditions with MEM supplemented with 10% (v/v) heat-inactivated FBS, and 1% (v/v) each of penicillin/streptomycin, non-essential amino acids and L-glutamine. The medium was routinely changed every 3–4 days, and the cells were passaged (1:3) every 7 days. For experiments utilizing fibroblasts, confluent (5–18 passages) stock cultures were washed once with Dulbecco's PBS, dissociated with 0.05% trypsin/0.02% EDTA, and seeded (3.0×10^5 cells) into 60 mm-diameter tissue-culture dishes containing 3 ml of the above medium for overnight attachment (18–24 h; day 0). The growth medium was aspirated and the dishes were washed once with Dulbecco's PBS before addition of 3 ml aliquots of experimental MEM medium containing 25% (v/v) $^2\text{H}_2\text{O}$, 9% (v/v) delipidated FBS, 1% FBS (v/v), 1% MEM vitamins and L-glutamine, 2% of essential and non-essential amino acids, and 20 mM-Hepes. For experiments involving Hep G2 cells, stock confluent monolayers were washed once with Dulbecco's PBS, dissociated with 0.05–0.02% trypsin/EDTA, followed by treatment with deoxyribonuclease I (0.1%), and then seeded (3.0×10^6) under identical conditions. The experimental MEM medium used contained 25% $^2\text{H}_2\text{O}$, 9% delipidated FBS and 1% (v/v) each of heat-inactivated FBS, penicillin/streptomycin, non-essential amino acids and L-glutamine,

as well as 20 mM-Hepes. As described in detail previously [8], the cells were cultured in media containing 25% $^2\text{H}_2\text{O}$ and 1% FBS for the purpose of determining cholesterol synthesis *de novo* under conditions that permit receptor-mediated cholesterol transport. In preliminary experiments not reported here it was determined that the presence of $^2\text{H}_2\text{O}$ (25%) in the medium does not affect the LDL-receptor binding.

The sterols, dissolved in DMF (9 $\mu\text{l}/\text{dish}$; 0.3%, v/v), were added to each dish. The cells were then grown to confluency (96 h, day 5). At the maximum concentrations studied, no effects on cell growth were noted. In all experiments, duplicate or triplicate dishes containing 9 μl of DMF were used as controls. The effects of the analogues on LDL-receptor binding at 4 °C, cholesterol synthesis *de novo* and the distribution of total and esterified cholesterol were determined at this stage.

^{125}I -LDL total binding to culture cells

LDL [relative density (d) 1.019–1.03] was isolated by sequential preparative ultracentrifugation of normal human plasma [9], solid KBr being used for density adjustment [10]. The isolated LDL was then iodinated with Na^{125}I by the monochloride procedure [11] as modified for lipoproteins [12]. To remove free iodine, the LDL preparation was subjected to column chromatography on a Sephadex G-25M disposable mini-column (Pharmacia PD-10) and was dialysed against 0.15 M-NaCl/0.3 mM-EDTA, pH 7.4. The specific radioactivity of all LDL preparations ranged between 160 and 300 c.p.m./mg of LDL proteins. ^{125}I -LDL was stored at 4 °C and was sterilized by filtration through a 0.45 μm -pore-size membrane just before incubation with the cells. Human LPDS was prepared as previously described [13].

For LDL-receptor binding studies [13], the dishes were placed on ice and stored in a cold-room (4 °C) for 30 min. The growth medium was aspirated off and the monolayers were washed with ice-cold PBS. Portions (2 ml) of ice-cold MEM medium containing 10% human LPDS, 20 mM-Hepes and 10 μg of ^{125}I -LDL/ml in the presence or absence of a 50-fold excess of unlabelled LDL were added to each dish. Specific binding was measured after 4 h incubation at 4 °C. The medium was removed and the cell monolayers were washed as described [14], then dissolved in 1 ml of 0.4 M-NaOH. Cell-associated radioactivity was determined in a Packard Autogamma liquid-scintillation spectrometer (model 5160), and cell protein was measured by the method of Lowry *et al.* [15], with bovine serum albumin as a standard. ^{125}I -LDL-receptor binding is expressed as the mass of ^{125}I -LDL (ng) per mg of total cell protein.

Cholesterol assays

After 96 h incubation, the medium was removed and the cells were washed with Dulbecco's PBS and harvested with 0.05% trypsin/0.02% EDTA. The proteinase was de-activated with an equal volume of soybean trypsin inhibitor and the resulting cell suspension was centrifuged for 5 min at 500 g . An internal standard of 5 β -cholestan-3 β -ol was added to the pellets before extraction with chloroform/methanol/water (8:4:3, by vol.; Folch mixture) [16]. A portion of the extract dried in a current of nitrogen and silylated [*N*-methyl-*N*-(trimethylsilyl)tri-fluoroacetamide; Pierce)/toluene/pyridine (2:1:1, by

Table 1. Effect of 26-amino-, 26-thia- and 26-hydroxy-cholesterol on total cell cholesterol, the percentage esterified and synthesis *de novo*, and specific ^{125}I -LKL receptor binding (4 °C) to LDLR (+) human fibroblasts

Values are means $\pm$ S.D. for three independent studies; significance: * $P < 0.01$; ** $P < 0.02$; *** $P < 0.05$.

Sterol analogue	Cholesterol:			^{125}I -LDL binding (ng of LDL protein/mg of cell protein)
	Total (μg of cholesterol/mg of cell protein)	Percentage esterified	Percentage synthesized <i>de novo</i>	
None	32.1 $\pm$ 1.2	28.5	19.0	55.7 $\pm$ 2.6
26-Hydroxy 0.625 μM	24.7 $\pm$ 2.8	28.7	3.9	50.3 $\pm$ 2.9
26-Amino 0.156 μM	27.9 $\pm$ 5.3	26.4	16.7	47.4 $\pm$ 2.4***
0.312 μM	31.5 $\pm$ 6.1	18.6	10.0	41.3 $\pm$ 1.7*
0.625 μM	20.3 $\pm$ 6.4	6.4	2.7	29.9 $\pm$ 1.4*
26-Thia 5.0 μM	26.0 $\pm$ 1.7	13.6	27.8	50.5 $\pm$ 3.2
10.0 μM	31.3 $\pm$ 4.5	31.1	21.9	47.5 $\pm$ 2.7***
20.0 μM	26.1 $\pm$ 1.0	33.1	13.6	47.8 $\pm$ 1.2**

Table 2. Effect of 26-amino-, 26-thia- and 26-hydroxy-cholesterol on total cell cholesterol, percentage synthesis *de novo* and specific ^{125}I -LDL receptor binding (4 °C) to HepG2 cells

Values are means $\pm$ S.D. for three independent studies; significance: * $P < 0.01$.

Sterol	Cholesterol:		
	Total (μg of cholesterol/mg of cell protein)	Percentage synthesized <i>de novo</i>	^{125}I -LDL binding (ng of LDL protein/mg of cell protein)
None	28.7 $\pm$ 0.2	55.8	12.5 $\pm$ 0.3
26-Hydroxy 0.625 μM	24.7 $\pm$ 2.8	57.1	12.3 $\pm$ 2.0
26-Amino 0.156 μM	30.6 $\pm$ 3.7	38.3	12.0 $\pm$ 1.2
0.312 μM	25.0 $\pm$ 0.6	45.1	10.9 $\pm$ 0.1*
0.625 μM	28.8 $\pm$ 1.8	42.0	8.3 $\pm$ 1.2*
26-Thia 5.0 μM	25.5 $\pm$ 4.3	49.7	11.0 $\pm$ 2.4
10.0 μM	27.4 $\pm$ 4.0	47.9	9.4 $\pm$ 2.4
20.0 μM	23.2 $\pm$ 1.2	41.9	12.0 $\pm$ 2.0

vol.)). Free cholesterol was then quantified by g.l.c. on a Perkin-Elmer capillary gas-liquid chromatograph (model 8320) fitted with a 20 m De-1 methylsilicone glass column (Supelco). The remaining extract was dried and saponified with 2% ethanolic KOH at 80 °C for 1 h. The sterols were then re-extracted, and total cholesterol was measured by g.l.c. after silylation. Cholesterol ester was calculated as the difference between total and free cholesterol determinations. Newly synthesized cholesterol was quantified by a g.l.c.-m.s. isotope mass-ratio method using a Hewlett-Packard g.c.-m.s. spectrometer (model 5890) fitted with DB-1 methylsilicone 0.18 m (internal diameter) $\times$ 20 m long glass column (Supelco) according to a previously described method [8].

RESULTS

Effects of 26-amincholesterol

Incubation of fibroblasts with 26-amincholesterol at concentrations ranging from 0.156 to 0.625 μM caused a progressive decrease in total cell cholesterol (Table 1). The decrease in total cell cholesterol is attributable to both a diminution in cholesterol synthesis and a decrease in LDL-receptor binding, which implies a decrease in LDL-mediated transport. As the total amount of cell cholesterol decreased, a decrease in the proportion of the esterified fraction occurred.

The effects of 26-amincholesterol on cholesterol synthesis and LDL-receptor activity that were found to

occur in fibroblasts are smaller for Hep G2 cells (Table 2).

Effects of 26-thiacholesterol

The 26-thia analogue of 26-hydroxycholesterol, when added to fibroblasts and Hep G2 cell cultures, had little effect on either cholesterol synthesis or LDL-binding activity (Tables 1 and 2). In preliminary studies (results not included), 26-thiacholesterol did not effect cell growth at the highest concentration tested (50 μ M). In contrast, both 26-aminocholesterol and 26-hydroxycholesterol at concentrations of 1.25 mM or greater were found to inhibit cell growth.

DISCUSSION

The synthesis of 26-hydroxycholesterol from cholesterol in human liver can be attributed to a mitochondrial cytochrome *P*-450 enzyme, designated as 'C₂₇ sterol 26-hydroxylase' because of its broad substrate specificity [17]. Because initial studies using Chinese-hamster ovary cells [4] and L cells [5] demonstrated that 26-hydroxycholesterol is a potent inhibitor of HMG-CoA reductase, it was assumed that its major biological role might be to regulate enzyme activity in the liver.

26-Hydroxycholesterol, however, is an intermediate in bile acid synthesis and therefore is normally present together with other steroid intermediates as a small pool that turns over rapidly [18–20]. Entry of 26-hydroxycholesterol from the medium into hepatocytes can thus be expected to result in mixing with the endogenous steroid-intermediate pool. Some of these intermediates, particularly those that have undergone side-chain oxidation, modulate HMG-CoA reductase activity [8], and therefore no added effect of the exogenous 26-hydroxycholesterol was detected at the relatively low concentrations used. In addition, since Hep G2 cells are constantly synthesizing bile acids [21], some of the 26-hydroxycholesterol underwent further metabolism. Because of these differences between fibroblasts and hepatocytes, it is reasonable that the major role of 26-hydroxycholesterol as a constituent of the plasma lipoproteins [1–3] may be in the regulation of cholesterol metabolism in non-hepatic tissues.

The findings in the present study together with a recent report that 26-hydroxycholesterol down-regulates LDL-receptor activity in fibroblasts [22] are consonant with studies of the promoter region of the genes regulating both LDL receptor and HMG-CoA reductase synthesis [23]. It has been shown that sterol-dependent repression of both these genes resides in their promoter regions, segments of which have a similar nucleotide sequence. Thus down-regulation of both HMG-CoA reductase and LDL-receptor activity would be expected to occur. In non-hepatic tissues these effects should protect against the accumulation of cholesterol and cholesterol ester in the cell.

Sterol-dependent repression is thought to be mediated

by the binding of an oxysterol to a specific protein [5] that has been shown to bind a number of different oxygenated sterols. Our findings indicate that it is not the oxygen function that determines binding, but rather the polarity of the sterol. Thus 26-aminocholesterol is at least as potent as 26-hydroxycholesterol, which is equipotent to 25-hydroxycholesterol [5]. By contrast, the 26-thiacholesterol, which is less polar than either the 26-hydroxy or 26-amino derivative, has lower sterol-dependent repressor activity. These new derivatives should prove useful for testing critically the role of a specific repressor protein [23].

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