Effect of 25-hydroxycholesterol and bile acids on the regulation of cholesterol metabolism in Hep G2 cells

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The effect of 25-hydroxycholesterol (25-OH-cholesterol) and chenodeoxycholic (CDC) acid on apoprotein secretion, low-density lipoprotein receptor activity, and [^3H]triacylglycerol secretion in Hep G2 cells was studied. Both 25-OH-cholesterol and CDC acid increased the secretion of apolipoprotein (apo) E by Hep G2 cells. The secretion of apo A-I was slightly lowered (< 10 % decrease). The maximal increase in apo E secretion was observed in culture medium containing 2 μg of 25-OH-cholesterol/ml or 10 μg of CDC acid/ml plus 10 % fetal calf serum. Cholesterol, 7-OH-cholesterol and other bile acids were ineffective in inducing increases in apo E secretion. Another cholesterol synthesis inhibitor, mevinolin, was also ineffective in generating an increase in apoprotein secretion. The data indicated a specific interaction between 25-OH-cholesterol or CDC acid and apo E secretion in Hep G2 cells. Cholesterol synthesis, as measured by the incorporation of [^{14}C]acetic acid into sterols, was repressed in Hep G2 cells in the presence of 25-OH-cholesterol (17 % of control value). CDC acid, on the other hand, increased [^{14}C]acetic acid incorporation (156 % of control value). The number of LDL receptors in Hep G2 cells was decreased after incubation with 25-OH-cholesterol (62 % of control value), but increased significantly after incubation with CDC acid (149 % of control value). The secretion of [^3H]triacylglycerol by Hep G2 cells incubated with 25-OH-cholesterol was greatly increased (248 % of control value). On the contrary, CDC acid did not cause any increase in [^3H]triacylglycerol secretion. The above results suggest that 25-OH-cholesterol and CDC acid have different effects on lipid metabolism in Hep G2 cells. The mRNA levels of apo E increased in cells preincubated with 25-OH-cholesterol and CDC acid, which suggested that the increase in apo E secretion is at least partly due to an increase in synthesis.

INTRODUCTION

The liver is the major organ for synthesizing and secreting plasma lipoproteins that are involved in lipid metabolism. However, the regulation of lipoprotein syntheses by the liver is far from clear. The difficulty in obtaining human liver biopsies hinders any detailed studies. However a human hepatoma cell line (Hep G2) has been developed which has the normal biochemical functions of liver parenchymal cells (Aden *et al.*, 1979; Havekes *et al.* 1983; Dashti *et al.*, 1985; Ellsworth *et al.*, 1986). Furthermore, Hep G2 cells synthesize and secrete the human apolipoproteins A-I, A-II, B, C-II, C-III and E into the medium (Zannis *et al.*, 1981). Therefore they have been used extensively for studying the factors that regulate hepatic lipoprotein secretion (Tam *et al.*, 1985, 1986; Dashti & Wolfbauer, 1987; Havekes *et al.*, 1987). One of the apoproteins that has been of great interest is apo E (Mahley, 1983). Apo E has been shown to be important for both LDL (apo B, E receptor) and chylomicron remnant (E receptor) metabolism (Beisiegel *et al.*, 1988), and in cholesterol-induced high-density lipoprotein (HDL) cholesterol metabolism (Gordon *et al.*, 1983). Furthermore, the three isoforms of apo E in humans were shown to be associated with different types of hyperlipoproteinaemia (Ghiselli *et al.*, 1982). Clearly,

the regulation of apo E synthesis is an important factor in regulating cholesterol metabolism.

Several investigators have shown that the concentration of apo E was greatly increased in animals consuming atherogenic diets. The changes in the apo E levels were highly correlated with the changes in plasma cholesterol concentrations in dogs (Melchior *et al.*, 1984) and in Rhesus monkeys (Melchior *et al.*, 1985). At least part of the increase was due to an increase in apo E mRNA levels in the livers of rats fed on an atherogenic diet (Lin-Lee *et al.*, 1981). Thus it is of interest to study the role of cholesterol in the regulation of apo E synthesis in cell cultures of Hep G2 cells. Due to the low solubility of cholesterol, many investigators have used an oxidized cholesterol, 25-hydroxycholesterol (25-OH-cholesterol), to study the regulation of cholesterol metabolism. 25-OH-cholesterol exerts similar effects as LDL-cholesterol on cholesterol metabolism, i.e. it suppresses both endogenous cholesterol synthesis (Brown & Goldstein, 1974) and the number of LDL receptors (Brown & Goldstein, 1975).

In this study, we discovered unexpectedly that 25-OH-cholesterol also has a specific effect on the secretion of apo E by Hep G2 cells. This effect is not mediated by changes in the endogenous rate of cholesterol synthesis. Furthermore, when other cholesterol metabolites were

Abbreviations used: 25-OH-cholesterol, 25-hydroxycholesterol; CDC acid, chenodeoxycholic acid; LDL, low-density lipoprotein; apo, apolipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; UDC acid, ursodeoxycholic acid; TDC acid, taurodeoxycholic acid; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; LPDS, lipoprotein-deficient serum.

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examined, only one other compound, chenodeoxycholic (CDC) acid, showed a similar effect on apo E synthesis and secretion by Hep G2 cells.

MATERIALS AND METHODS

Materials

Na¹²⁵I (carrier-free) was obtained from Amersham. [³H]Glycerol was obtained from New England Nuclear. [¹⁴C]Acetic acid was purchased from Research Products International. Ursodeoxycholic (UDC) acid was purchased from Sigma. 25-OH-cholesterol, 7-OH-cholesterol, deoxycholic acid and CDC acid were purchased from Steraloids Inc. Cholic acid was purchased from Eastman Kodak, and taurodeoxycholic (TDC) acid was purchased from Calbiochem. All tissue culture supplies were purchased from Grand Island Biological Co. Mevinolin was kindly provided by Merck, Sharp and Dohme. The Hep G2 cell line was generously provided by Dr. Barbara B. Knowles of the Wistar Institute, Philadelphia, PA, U.S.A.

Cell culture

Hep G2 cells were grown in monolayers in medium A [Dulbecco's Modified Eagle's Medium (DMEM)/penicillin (100 units/ml)/streptomycin (100 µg/ml)/Hepes (25 mM, pH 7.4)] plus fetal calf serum (10%). Cells were incubated in a humidified incubator with 5% CO₂ in air at 37 °C. Subcultures for the experiments were obtained by trypsin treatment (37 °C, 3–4 min) of the confluent stock monolayers using a solution of 0.05% trypsin in Dulbecco's phosphate-buffered saline (PBS) containing 0.5 mM EDTA. The cells were seeded in culture dishes (35 mm × 10 mm) at a concentration of 5.0×10^4 cells/dish in 1.5 ml of medium. The cells were grown to subconfluency in 3–4 days, and the medium was changed every 2 days. The cells were washed and incubated in the same medium for 2 more days with the addition of compounds as indicated. 25-OH-cholesterol and cholesterol were dissolved in 95% ethanol at concentrations of 2 mg/ml and 10 mg/ml respectively. CDC acid was dissolved in 50% ethanol (5 mg/ml). The medium was removed after the incubation for further analysis. The monolayers were washed with PBS and dissolved in 0.2 M-NaOH for protein analysis by the method of Lowry *et al.* (1951). 25-OH-cholesterol, CDC acid or ethanol alone did not have any toxic effect on Hep G2 cells throughout the experiments as judged by the cell morphology under an inverted microscope and by the cellular protein content.

Radioimmunoassay

Apo A-I and Apo E were assayed as described (Au *et al.*, 1986a,b). Culture medium without further purification was used for the assays.

Isolation of LDL and lipoprotein-deficient serum

Normal human plasma was obtained from the Mayo Clinic Blood Bank. LDL was isolated by sequential preparative ultracentrifugation as described by Brown & Goldstein (1975). The isolated LDL was washed once and dialysed against 4 litres of buffer containing 0.15 M-NaCl, 10 mM-Tris/HCl (pH 7.4), 1 mM-EDTA and 0.01% NaN₃ with four changes. Lipoprotein-deficient serum (LPDS) was prepared by the method described by Radding & Steinberg (1960).

Radioiodination of LDL

¹²⁵I-labelled LDL was prepared by mixing 100 µg of LDL, 10 µl of 1 M-glycine buffer (pH 10.0) and 10 µl of Na¹²⁵I (1 mCi). The reaction was initiated by adding 10 µl of iodide monochloride (50 µg/ml) and subsequently mixed for 1 min. Unlabelled LDL (1.4 mg) was added to the reaction mixture, and the labelled LDL was separated from unreacted Na¹²⁵I by fractionation on a Sephadex G-25 column. The specific activity of ¹²⁵I-LDL was $(1.5\text{--}2) \times 10^8$ c.p.m./mg of LDL.

LDL binding

The binding of ¹²⁵I-LDL to Hep G2 cells was studied according to the method described by Goldstein & Brown (1974) with slight modifications. On the fourth day of subculture, the cells were incubated with the indicated compounds in medium A containing 10% fetal calf serum, and the incubation was continued for 48 h. At the end of this period, the cells were washed and fresh medium and ¹²⁵I-LDL (10 µg/ml) were added. The incubation was continued for 4 h at 4 °C. The monolayers were washed three times with PBS containing 1% bovine serum albumin and then three additional times with PBS alone. The cells were dissolved in 0.2 M-NaOH, and the radioactivity associated with the cells was measured in a gamma counter.

[¹⁴C]Acetic acid incorporation into sterols

Endogenous cholesterol synthesis by Hep G2 cells was measured on the basis of the incorporation of [¹⁴C]acetic acid into sterols. Hep G2 cells were preincubated in medium A with 5% LPDS with the addition of the indicated compounds for 24 h. [¹⁴C]Acetic acid (1 µCi/1.5 ml) was then added to the medium and the incubation was continued for 8 h at 37 °C. The monolayers were washed four times with PBS, and ¹⁴C-labelled sterols were extracted with 2 ml of hexane/propan-2-ol (3:2, v/v) (Hara & Radin, 1978). The solvent was evaporated and the precipitate was saponified with 1 ml of 1 M-KOH dissolved in methanol/benzene (4:1, v/v) solution at 70 °C for 30 min. The radioactivity incorporated into the unsaponifiable sterols was extracted three times with hexane. These hexane extracts were then combined and back-extracted with 70% ethanol, and radioactivity was measured in a Packard scintillation counter after the addition of 10 ml of scintillation cocktail (AquaSure; Du Pont). More than 80% of the label was incorporated into [¹⁴C]cholesterol as purified by t.l.c.

[³H]Triacylglycerol secretion

Subconfluent Hep G2 cells were grown in medium A with 10% fetal calf serum for 2 days, after which fresh medium A with 5% LPDS plus 25-OH-cholesterol, CDC acid or mevinolin was added. The incubation was continued for 24 h. [³H]Glycerol (2 µCi) was then introduced to the culture medium for 1 h. The cells were washed four times with PBS after labelling and reincubated with fresh medium A containing 5% LPDS (1 ml/dish) for 5 h at 37 °C. At the end of the incubation, half of the medium was extracted with hexane/propan-2-ol (3:2, v/v). The other half of the medium was used for the precipitation of apo B-containing lipoproteins by adding an equal volume of poly(ethylene)glycol 6000 (20% w/v in 0.5 M-glycine buffer, pH 10.0) (Izzo *et al.*, 1981). Human serum (0.1 ml) was added before the addition of poly(ethylene)glycol 6000 as a carrier. The precipitate was collected by

centrifugation at 4 °C and dissolved in 0.2 M-NaOH. Radioactivity in the precipitate was determined using a Packard scintillation counter. The secretion of Hep G2 cells was linear up to 6 h of incubation (results not shown).

mRNA hybridization

The total cellular RNA was extracted from Hep G2 cells by the guanidinium/CsCl method as described by Maniatis *et al.* (1982), except that 4 M-guanidinium isothiocyanate was used. The concentration of RNA was determined spectrophotometrically. Single-stranded apo E RNA probes were prepared from human apo E cDNA clone pE-301 provided by Dr. Jan L. Breslow, Rockefeller University, New York, NY, U.S.A. (Breslow *et al.*, 1982). The largest *Pst*I insert was subcloned into plasmid pSP64 (Melton *et al.*, 1984) in the orientation to produce anti-sense RNA probes. The labelled RNA probe was further purified from a 1% formamide agarose gel (Maniatis *et al.*, 1982) and eluted before use. The specificity of the RNA probe is $(1-2) \times 10^8$ c.p.m./mg. DNA excess equilibrium hybridization was carried out as described by Newman *et al.* (1985) with some modification. After S1 nuclease digestion, the double-stranded RNA-RNA hybrid was precipitated from the solution with 100 µg of carrier salmon sperm DNA and 10% trichloroacetic acid. The precipitates were collected using an Eppendorf microfuge and washed twice with 7.5% trichloroacetic acid, after which the precipitates were dissolved in TE buffer (10 mM-Tris/1 mM-EDTA, pH 8.0) and radioactivity was counted after addition of 10 ml of scintillation cocktail. All the hybridization data were obtained from at least two sets of triplicates. Background counts with no cellular RNA represented less than 10% of experimental values.

RESULTS

Regulation of apo A-I and apo E secretion by a 25-OH-cholesterol and CDC acid in Hep G2 cells

Hep G2 cell cultures were utilized to study the secretion of apo A-I and apo E, the two major apoproteins associated with HDL and very-low-density lipoprotein

(VLDL) fractions respectively. Using the radioimmunoassays developed in this laboratory for apo A-I and apo E, reproducible quantities of apo A-I and apo E can be detected in the culture medium without further purification. 25-OH-cholesterol has been shown to regulate cholesterol metabolism by repressing the synthesis of hydroxymethylglutaryl (HMG)-CoA reductase (Brown & Goldstein, 1974) and LDL receptor activity (Brown & Goldstein, 1975). It was of interest to determine whether or not 25-OH-cholesterol also had an effect on apoprotein synthesis by Hep G2 cells. As shown in Table 1, the concentration of apo A-I secreted by Hep G2 cells was slightly lower (92%) in 25-OH-cholesterol-containing medium. Secretion of apo E, on the other hand, was increased (145%) by 25-OH-cholesterol in the culture medium. In medium containing cholesterol there was only a small increase in both apo A-I and apo E secretion by Hep G2 cells (Table 1). The results indicated that, in addition to regulating cholesterol synthesis and uptake, 25-OH-cholesterol also increases apo E, but not apo A-I, secretion by Hep G2 cells.

To further study the nature of this regulation by 25-OH-cholesterol, another drug commonly used to repress cholesterol synthesis, mevinolin (Tobert *et al.*, 1982), was used under the same conditions as in experiments with 25-OH-cholesterol or cholesterol. The amounts of apo E and apo A-I secreted into the medium in the presence of mevinolin were only slightly changed (104% and 97% of control respectively) (Table 1). The above data suggest that the regulation of apo E secretion by 25-OH cholesterol is specific and therefore probably is not regulated by the levels of endogenous cholesterol synthesis. Several other cholesterol metabolites were also introduced into the culture medium of Hep G2 cells, and their effects on the secretion of apo A-I and apo E were examined (Table 1). Cholic acid, TDC acid, deoxycholic acid and 7-OH-cholesterol did not exert significant effects on the secretion of apo E by Hep G2 cells. However, the amount of apo E secretion was increased (135% of control value) in medium containing CDC acid (Table 1). Surprisingly, the epimer of CDC acid, UDC acid, has no significant effect on apo E secretion by Hep G2 cells. This further demonstrates that the regulation of apo E secretion by

Table 1. Apo A-I and apo E secretion by Hep G2 cells

Hep G2 cells were grown in medium A containing 10% fetal calf serum for 3 days, and the cholesterol metabolites were added. After 2 days of incubation, the medium was withdrawn for radioimmunoassay ($n = 9$). Values are means $\pm$ S.E.M.; * $P < 0.01$. Values in parentheses are percentages of control values (no additions).

Addition	Concentration (µg/ml)	Apo A-I (µg/mg of protein)	Apo E (µg/mg of protein)
None†	—	4.69 $\pm$ 0.13 (100)	1.10 $\pm$ 0.13 (100)
25-OH-cholesterol	2	4.31 $\pm$ 0.28* (93)	1.59 $\pm$ 0.19* (145)
CDC acid	10	4.35 $\pm$ 0.28* (93)	1.48 $\pm$ 0.28* (135)
Cholesterol	10	4.80 $\pm$ 0.38 (102)	1.21 $\pm$ 0.09 (110)
UDC Acid	10	4.39 $\pm$ 0.22* (94)	1.17 $\pm$ 0.13 (106)
Cholic acid	10	4.16 $\pm$ 0.18* (89)	1.10 $\pm$ 0.18 (100)
Deoxycholic acid	10	4.22 $\pm$ 0.33* (90)	1.05 $\pm$ 0.09 (95)
TDC acid	2	4.38 $\pm$ 0.14* (93)	1.18 $\pm$ 0.12 (107)
7-OH-cholesterol	2	4.38 $\pm$ 0.41 (93)	1.25 $\pm$ 0.34 (114)
Mevinolin	1	4.57 $\pm$ 0.48 (97)	1.14 $\pm$ 0.19 (104)

† Ethanol was added to the medium to a final concentration of 1% (v/v).

Table 2 Effect of 25-OH-cholesterol and CDC acid concentrations on apo E synthesis in Hep G2 cells

Different concentrations of 25-OH-cholesterol and CDC acid were added to subconfluent Hep G2 cell cultures, grown for 3 days after passage, in medium A with 10 % fetal calf serum. After 2 days of incubation, the medium was used for analysis ($n = 9$). Values are means $\pm$ S.E.M.; * $P < 0.01$ compared with controls (no additions). Figures in parentheses are percentages of control values.

Addition	Concentration ($\mu\text{g/ml}$)	Apo A-I ($\mu\text{g/mg}$)	Apo E ($\mu\text{g/mg}$)
25-OH-cholesterol	0	4.69 ± 0.13 (100)	1.10 ± 0.13 (100)
	0.5	$4.42 \pm 0.16^*$ (94)	$1.29 \pm 0.08^*$ (117)
	1	$4.35 \pm 0.23^*$ (93)	$1.33 \pm 0.17^*$ (121)
	2	$4.31 \pm 0.20^*$ (92)	$1.59 \pm 0.19^*$ (145)
	3	$3.93 \pm 0.12^*$ (84)	$1.38 \pm 0.19^*$ (125)
CDC acid	0	4.69 ± 0.13 (100)	1.10 ± 0.13 (100)
	2	4.53 ± 0.14 (97)	1.20 ± 0.07 (110)
	5	4.45 ± 0.27 (95)	1.09 ± 0.15 (110)
	10	$4.35 \pm 0.28^*$ (93)	$1.48 \pm 0.28^*$ (135)
	20	$4.17 \pm 0.26^*$ (89)	$1.36 \pm 0.14^*$ (124)

Table 3. Cholesterol synthesis in Hep G2 cells preincubated with 25-OH-cholesterol, CDC acid or mevinolin

[^{14}C]Acetic acid (1 μCi) was added to each culture dish containing 1.5 ml of medium A with 5 % LPDS and 25-OH-cholesterol, CDC acid or mevinolin. The incubation was carried out at 37 °C for 8 h. Each dish was washed extensively with PBS after the incubation. Hexane/propan-2-ol (3:2, v/v) was used to extract [^{14}C]-labelled sterols from the cells as described in the Materials and methods section. The amount of [^{14}C]acetate incorporated into fatty acids is $(2.98\text{--}3.16) \times 10^6$ c.p.m./dish. Values are means $\pm$ S.E.M. for three determinations. Figures in parentheses are percentages of control values.

Medium	Concentration ($\mu\text{g/ml}$)	$10^{-5} \times [^{14}\text{C}]$ -Sterols (c.p.m./mg of cellular protein)
Medium A/5 % LPDS	—	2.35 ± 0.21 (100)
+ 25-OH-cholesterol	2	0.40 ± 0.08 (17)
+ CDC acid	10	3.67 ± 0.77 (156)
+ mevinolin	1	0.43 ± 0.03 (18)

25-OH-cholesterol and CDC acid is very specific. The secretion of apo A-I by Hep G2 cells was slightly decreased in medium containing 25-OH-cholesterol, CDC acid, UDC acid, cholic acid, deoxycholic acid or TDC acid (Table 1). The results were statistically significant ($P < 0.01$) due to the very small variances in the data. However, the biological significance is questionable.

Concentration effects of regulators

To investigate the minimal concentrations of 25-OH-cholesterol and CDC acid needed to maximally increase apo E secretion in Hep G2 cells, various concentrations of 25-OH-cholesterol and CDC acid were added to the culture medium and the incubation continued for 2 days. The results are shown in Table 2. The increase in apo E secretion was highest in medium supplemented with 2 μg of 25-OH-cholesterol/ml (145 %). As for CDC acid,

Table 4. ^{125}I -LDL binding to the LDL receptor in Hep G2 cells preincubated with different cholesterol metabolites

^{125}I -LDL (10 $\mu\text{g/ml}$) was added to each culture dish in medium A for 4 h at 4 °C. After the incubation, the monolayers were washed extensively as described in the Materials and methods section. Total ^{125}I -LDL associated with the cells was dissolved in 0.2 M-NaOH and radioactivity determined using a gamma counter. Values are means $\pm$ S.E.M.

Addition	Concentration (mg/ml)	^{125}I -LDL binding (%)
None	—	100 (0.08*) ($n = 6$)
CDC acid	10	149 ± 1 ($n = 6$)
25-OH-cholesterol	2	62 ± 3 ($n = 6$)
Cholesterol	10	91 ± 3 ($n = 6$)
Cholic acid	10	107 ± 8 ($n = 3$)
Deoxycholic acid	10	111 ± 13 ($n = 3$)
UDC acid	10	88 ± 1 ($n = 3$)
TDC acid	10	101 ± 12 ($n = 3$)

* Expressed as $\mu\text{g/mg}$ of cellular protein.

maximal secretion of apo E by Hep G2 was observed at a concentration of 10 $\mu\text{g/ml}$ in medium containing 10 % fetal calf serum (135 %). All of the following experiments were conducted with the addition of 25-OH-cholesterol at 2 $\mu\text{g/ml}$ or of CDC acid at 10 $\mu\text{g/ml}$ for 48 h.

Regulation of cholesterol synthesis and LDL receptor activity by 25-OH-cholesterol and CDC acid

25-OH-cholesterol was shown to greatly decrease to the amount of HMG-CoA reductase, a key enzyme for cholesterol synthesis in fibroblast cell cultures (Brown & Goldstein, 1974). The effect of CDC acid on cholesterol synthesis was also investigated. The incorporation of [^{14}C]acetic acid into the sterols of Hep G2 was measured (Table 3). Both 25-OH-cholesterol and mevinolin decreased the incorporation of [^{14}C]acetic acid into sterols in Hep G2 cells (17 and 18 % of control value re-

Table 5. [³H]Triacylglycerol secretion by subconfluent Hep G2 cells preincubated with 25-OH-cholesterol, CDC acid or mevinolin

Hep G2 cell cultures were preincubated with medium A containing 5% LPDS and 25-OH-cholesterol, CDC acid or mevinolin for 24 h. [³H]Glycerol (2 μ Ci/dish) was added to the culture and incubated for 1 h, after which monolayers of Hep G2 cells were washed three times with PBS and reincubated with 1 ml of fresh medium A with 5% LPDS for 5 g at 37 °C. At the end of incubation, the medium was withdrawn for further analysis. The cells were washed and dissolved in 0.2 M-NaOH for protein analysis. Half the medium was extracted with hexane/propan-2-ol (3:2, v/v). The other half of medium was mixed with 0.1 ml of human serum and an equal volume of 20% poly(ethylene) glycol 6000 in 0.5 M-glycine buffer, pH 10.0. The apo B-containing lipoproteins were precipitated by centrifugation. Similar results were found in three sets of independent experiments. Values in parentheses are percentages of control values.

Addition	Concentration (μ g/ml)	Total [³ H]-triacylglycerol secreted (c.p.m./mg) (%)	Apo B-containing lipoproteins (c.p.m./mg) (%)
None		9115 (100)	5320 (100)
25-OH-cholesterol	2	22570 (248)	18305 (344)
CDC acid	10	8193 (90)	4992 (94)
Mevinolin	1	5971 (66)	4503 (85)

spectively), as expected. CDC acid, on the other hand, increased the amount of [¹⁴C]sterols in Hep G2 cells (156%). The data suggest that CDC acid and 25-OH-cholesterol have opposite effects on cholesterol synthesis, yet both compounds caused an increase in apo E secretion by Hep G2 cells. Once again, the increase in apo E synthesis probably is not coupled to endogenous cholesterol synthesis in Hep G2 cells.

The activity of the LDL receptor in Hep G2 cells is regulated by the cellular concentration of cholesterol. 25-OH-cholesterol has often been used to repress the amount of LDL receptor activity in fibroblasts (Brown & Goldstein, 1975). The effect of CDC acid on the amount of LDL receptor in Hep G2 cells was also determined. As shown in Table 4, 25-OH-cholesterol decreased the amount of ¹²⁵I-LDL binding to Hep G2 cells (62% of control), while CDC acid increased the binding significantly (149%). The same increase in binding was observed at concentrations of 5, 10, and 20 μ g/ml (results not shown). No significant increase in LDL binding was observed with medium supplemented with other bile acids (Table 4). Once again it is evident that 25-OH-cholesterol and CDC acid do not influence cholesterol metabolism in Hep G2 cells in the same manner.

[³H]Triacylglycerol secretion by Hep G2 cells

Newly synthesized apo E is usually secreted into the culture medium by Hep G2 cells as VLDL. Only a small amount of apo E is associated with apo A-I-containing lipoproteins (Vezina *et al.*, 1988). It was of interest to determine if increased apo E secretion was also associated with increased lipoprotein and VLDL secretion. Hep G2 cells incubated in medium A with the addition of 5% LPDS and 25-OH-cholesterol secreted more [³H]triacylglycerol (248% of control value) and also more apo B-associated [³H]triacylglycerol (344% of control value) (Table 5). However, CDC acid did not induce any significant changes in [³H]triacylglycerol secretion by Hep G2 cells. Mevinolin, which inhibits endogenous cholesterol synthesis, also inhibited [³H]triacylglycerol secretion. In experiments where 1 mM-oleate/bovine serum albumin complex was added to maximize the secretion of triacylglycerol (results not shown), an increase in [³H]triacylglycerol was observed in cells incubated with 25-OH-cholesterol only (170% of

Table 6. mRNA levels in Hep G2 cells incubated with 25-OH-cholesterol and CDC acid

Each hybridization contained 2 μ g or 4 μ g of total cellular RNA and 2×10^5 c.p.m. of apo E RNA probe for experiment I, and 4×10^4 c.p.m. of apo E RNA probe for experiment II. Results were derived from two sets of independent RNA preparations.

Medium	mRNA (% of control)
Experiment I:	
Medium A/fetal calf serum	100 (2.1×10^4 c.p.m./ μ g of total RNA)
+ 25-OH-cholesterol	206 $\pm$ 11
+ CDC acid	146 $\pm$ 22
Experiment II:	
Medium A/LPDS (5%)	100 (1.7×10^3 c.p.m./ μ g of total RNA)
+ 25-OH-cholesterol	306 $\pm$ 172
+ CDC acid	485 $\pm$ 105

control values). No increase was noted in cells incubated with CDC acid. The data suggest that 25-OH-cholesterol induced both apo E and [³H]triacylglycerol (VLDL) secretion by Hep G2 cells. However, the increase in apo E secretion brought about by incubation with CDC acid did not result in an increase in [³H]triacylglycerol secretion.

mRNA levels in Hep G2 cells incubated with 25-OH-cholesterol or CDC acid

The increase in apo E secretion may be due to either increased transport or increased synthesis. The levels of apo E mRNA were measured in Hep G2 cells incubated with 25-OH-cholesterol or CDC acid for 2 days, by hybridization to an apo E RNA probe. As shown in Table 6, the level of apo E mRNA was increased in cells incubated with either 25-OH-cholesterol or CDC acid. The increase is more significant in cells grown in medium A containing 5% LPDS (306% for 25-OH-cholesterol and 485% for CDC acid of control values respectively) than that in cells grown in the presence of 10% fetal calf serum.

DISCUSSION

CDC acid has been used to treat patients with gallstone problems (Alberts *et al.*, 1982). It decreases biliary cholesterol saturation and is effective in the dissolution of cholesterol gallstones, probably by reducing cholesterol secretion into bile, thereby resulting in a decrease in cholesterol saturation. Several studies have demonstrated that bile acids also have some effects on LDL receptor activity in hamsters (Malavolti *et al.*, 1987), probably by altering their cholesterol metabolism. The secretion of VLDL triglycerides by the liver is apparently decreased by CDC acid therapy (Angelin *et al.*, 1978). Therefore bile acids may exert important effects on the overall cholesterol metabolism *in vivo*. The results presented here indicate that CDC acid also has a direct effect on apo E synthesis and secretion, cholesterol synthesis and LDL-receptor activity in Hep G2 cells. Interestingly, UDC acid, the 7 β epimer of CDC acid, had no apparent effect (Tables 1 and 4). Similarly, 7-OH-cholesterol did not increase apo E secretion in Hep G2 cells, although 25-OH-cholesterol did. The data suggest that the regulation by these two compounds of apo E synthesis is rather specific and probably is not due to any changes in endogenous cholesterol synthesis in the cells.

25-OH-cholesterol, an oxidized metabolite of cholesterol (Saucier *et al.*, 1985), has been shown to be a regulator of the amounts of both HMG-CoA reductase and LDL receptors in fibroblast cell culture (Brown & Goldstein, 1975). The present study indicates that 25-OH-cholesterol also regulates apo E synthesis at the molecular level. The promoter/operator region of these three genes may reveal some common sequences necessary for 25-OH-cholesterol regulation. CDC acid, on the other hand, increased the incorporation of [14 C]acetic acid into sterols and the amount of LDL receptors in Hep G2 cells. The different effects on cholesterol metabolism of CDC acid and 25-OH-cholesterol suggest that the regulation of apo E synthesis and secretion by these two compounds is not through a common mediator. CDC acid may be recognized by a different region of the apo E gene sequence. The recently published deletion mapping of the apo E sequence suggests that many regulatory regions may be present (Smith *et al.*, 1988). Increased apo E secretion was apparently associated with increased apo B-containing lipoproteins, presumably in the VLDL and intermediate-density lipoprotein fractions, in cells exposed to 25-OH-cholesterol. However, there was no detectable increase in total lipoprotein or apo B-containing lipoprotein secretion with CDC acid. The results suggest that apo E is secreted either in lipoprotein fractions containing a higher molar ratio of apo E than in normal lipoprotein fractions, or that apo E is secreted into the medium without being associated with any of the lipoprotein classes. Further fractionation of secreted lipoproteins by gel electrophoresis is necessary to clarify this difference. Subconfluent Hep G2 cell cultures were used in these experiments to maximize endogenous cholesterol metabolism. The increase in apo E secretion stimulated by 25-OH-cholesterol or CDC acid with subconfluent Hep G2 cell cultures was similar to that of confluent cultures (results not shown). However, twice the amount of apo E (μ g/mg of cellular protein) was secreted into the medium in 48 h with confluent cultures as compared with subconfluent cultures (results not shown). No differences in apo A-I secretion were

observed. The results suggest that apo E secretion may also be influenced by the metabolic rate of Hep G2 cells and further indicate the multiplicity of the gene regulation of apo E.

The physiological significance of the induction of apo E secretion by 25-OH-cholesterol and CDC acid is not known. However, one can speculate that, when the cells are overloaded with lipoprotein cholesterol, the concentration of 25-OH-cholesterol formed by sterol oxidation may be increased. Subsequently, 25-OH-cholesterol stimulates the secretion of apo E and VLDL by the liver. Preliminary studies suggest that VLDL and LDL both induce an increase in [3 H]triacylglycerol secretion in Hep G2 cells (results not shown). CDC acid, on the other hand, may interfere with the generation of 25-OH-cholesterol from cholesterol, which may in turn increase cholesterol synthesis and LDL receptor activity in the liver. Further studies are needed to elucidate these mechanisms.

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