Cholesterol and bile acid synthesis in Hep G2 cells

Metabolic effects of 26- and 7 α -hydroxycholesterol

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1. Using a human hepatoma (Hep G2) cell line that continually synthesizes 3 β -hydroxy-5-cholenoic acid, lithocholic acid, chenodeoxycholic acid and cholic acid we have determined the metabolism and biological effects of 26-hydroxycholesterol and 7 α -hydroxycholesterol. 2. Addition of 26-hydroxycholesterol to the medium (6 μ M) downregulated cholesterol and chendodeoxycholic acid synthesis. 3. The predominant metabolite of 26-hydroxycholesterol was 3 β -hydroxy-5-cholenoic acid. 4. Cholesterol synthesis was not affected by the addition of 7 α -hydroxycholesterol (6 and 12 μ M). The predominant metabolite of 7 α -hydroxycholesterol was chenodeoxycholic acid. 5. In Hep G2 cells 7 α -hydroxylation of 26-hydroxycholesterol is not well expressed.

INTRODUCTION

The existence of a human liver-cell line that continually produces the same bile acids that are synthesized *in vivo* [1] provides a unique opportunity to determine the biological effects of the naturally occurring intermediates. Both 7 α -hydroxycholesterol and 26-hydroxycholesterol are synthesized in the liver and are metabolized *in vivo* to chenodeoxycholic and cholic acids [2]. Although the major end products are the same, the intermediates are different and their role in regulating hepatic cholesterol synthesis has not been defined.

EXPERIMENTAL

Materials

26-Hydroxycholesterol was synthesized as described previously [3], and 7 α -hydroxycholesterol was purchased from Steraloids, Wilton, NH, U.S.A. Both compounds were chromatographically homogeneous by t.l.c. (silica-gel G) using solvent systems that separated 7 α - and 7 β -hydroxycholesterol (diethyl ether/cyclohexane, 9:1, v/v) and 25- from 26-hydroxycholesterol (hexane/ethyl acetate/propan-2-ol/acetone, 85:15:3:2, by vol.; twice in the same solvent system). $^2\text{H}_2\text{O}$ (99.6%) was purchased from Merck Stable Isotopes, St. Louis, MO, U.S.A. Hep G2 cells were a gift from Dr. Barbara Knowles of the Wistar Institute, Philadelphia, PA, U.S.A. Materials for maintenance of the cell line in our facility have been described previously [4].

Incubation of Hep G2 cells

For studies of cholesterol and bile acid synthesis, subconfluent cultures containing $(3-4) \times 10^7$ cells were maintained in T-75 flasks. Medium containing 10% (v/v) foetal-bovine serum was replaced by 10 ml of serum-free medium, which remained in contact with the cells for 96 h, except in preliminary studies, when the medium was changed at 24 h intervals to establish that bile acid synthesis occurred throughout the entire period. In some studies the medium contained 25% $^2\text{H}_2\text{O}$. Sterols, dissolved in a volume of dimethylformamide no greater than 30 μ l, were added to the serum-free medium

at zero time. Dimethylformamide added in amounts of 5–30 μ l to sterol-free medium did not affect the yield of bile acids at 96 h. In some studies cells were harvested to determine cell number and protein content [4].

Cholesterol and bile acid assays

After preparation of the methyl ester acetates [5], total bile acid in the medium was quantified by g.l.c. (Perkin–Elmer model no. 900 instrument) using a packed glass column containing 3% SP-2550 (Supelco, Bellefonte, PA, U.S.A.) and a flame detector; 10 μ g of 3 α ,7 α -dihydroxy-12-oxo-5 β -cholanoic acid was used as an internal standard. The acetate rather than the trimethylsilyl derivatives were chosen when we found that we could not obtain complete resolution of 3 β -hydroxy-5-cholenoic acid from chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholanoic acid) using conditions similar to those described previously [6]. Also, it should be noted that the most prominent fragment for both these compounds using electron-impact m.s. is $m/z = 370$, notwithstanding the difference in the molecular mass and molecular ion. The major difference in their spectra is the prominent $m/z=249$ fragment that is characteristic of all derivatives of methyl 3 β -hydroxy-5-cholenoate [7]. It was the presence of this fragment that led to the suspicion that 3 β -hydroxy-5-cholenoic acid was present in the medium and to the development of g.l.c. conditions for complete resolution of all the bile acids of interest.

The amount of cholesterol and bile acid synthesized during the 96 h period when the medium contained 25% $^2\text{H}_2\text{O}$ is calculated from the relative intensities of the M_0 and M_{0+5} ions [8]. For Hep G2 cells the M_{0+5} ion represents 15.5% of the total newly synthesized (^2H -labelled) cholesterol [9], and therefore the intensity of M_{0+5} multiplied by 100/15.5 estimates the total amount of newly synthesized cholesterol. The amount of [^3H]cholesterol is determined by the intensity of the M_0 ion multiplied by 100/74, because the natural abundance of ^{13}C yields significant M_{0+1} and M_{0+2} peaks. Thus the amount of newly synthesized cholesterol equals the total amount determined by g.l.c. multiplied by:

$$[M_{0+5} \times (100/15.5)]/[M_{0+5} \times (100/15.5) + M_0 \times (100/74)]$$

Calculation of the amount of newly synthesized 3β -hydroxycholesterol is done in an identical manner. For chenodeoxycholic acid the ' $M_0 \times 100/74$ ' term changes to ' $M_0 \times 100/32$ ' because of the incorporation of ^2H into the A/B ring during the metabolism of cholesterol to bile acid [9].

The medium was harvested and, after addition of the internal standard, the bile acids were extracted by passage through a reverse-phase C_{18} column (Waters, Milford, MA, U.S.A.) using a previously validated method [10]. The methanol eluate, containing more than 90% of the bile acids, including the internal standard, was then solvolysed and the methyl ester acetates were prepared. For the quantification of bile acids, aliquots containing 1–3 μg of internal standard were injected on to the column. In agreement with a previous report [6], we found that the conjugates represented only a small fraction of the total bile acid, and therefore alkaline or enzymic hydrolysis was usually omitted. Neither 3β -hydroxy-5-cholenoic acid nor 3α -hydroxy-5 β -cholanoic acid was detected if the solvolysis step was omitted.

Cholesterol in the medium and the cells was analysed by g.l.c. as described above, using 5β -cholestane- 3β -ol as an internal standard. A 10 μg aliquot of internal standard was added to 1.0 ml of medium. Cholesterol and cholesterol esters were extracted from the cells by overlaying the monolayer with 5 ml of 100% ethanol containing 50 μg of internal standard and leaving the flask at room temperature for 1 h. After extraction with an additional 5 ml of ethanol, a third extraction yielded an amount of cholesterol that was less than 5% of the total cell cholesterol found after saponification using a standard procedure [11].

Isotope-ratio m.s. was performed by using a model 5890 Hewlett-Packard capillary g.l.c. instrument coupled to a model 5970 quadrupole mass detector. A standard CP-19-SIL (Chrompack, Raritan, NJ, U.S.A.) fused-silica column (film thickness 0.20 μm ; column internal diameter 0.25 mm; length 25 m) was used at a helium head pressure of 37.5 kPa. A simultaneous ion-monitoring program was used to quantify both the total area of the molecular ion (m/z – 60 acetate) and the ^2H -enriched species as described previously [9], and a scan program was used for complete analysis of the mass spectrum of the different bile acids.

RESULTS

Bile acid synthesis in Hep G2 cells

The bile acids obtained from the medium after 96 h of contact with Hep G2 cells are shown in Fig. 1(c), and authentic standards are shown in Fig. 2(c). The retention time for each bile acid is identical with that of the standard, and the identity of each bile acid was confirmed by mass-spectral analysis. Chenodeoxycholic acid is the predominant bile acid, and the addition of either 26-hydroxycholesterol or 7 α -hydroxycholesterol affected the rate of its synthesis and that of 3β -hydroxycholenoic acid. Therefore only these bile acids were studied in detail.

Figs. 2(a) and 2(b) show the spectra of chenodeoxycholic and 3β -hydroxy-5-cholenoic acids respectively. Fig. 2 indicates the mass spectrum of these same bile acids when the medium contained 25% $^2\text{H}_2\text{O}$. The series of higher-molecular-mass fragments that follow m/z 370 represent the metabolic processes that occur in

Fig. 1. G.l.c.-m.s. analysis of bile acid obtained from Hep G2-cell medium

(c) Compared with authentic standards (Fig. 2, where the numbered compounds are identified), the predominant bile acid is shown to be chenodeoxycholic (CDCA) acid with lesser amount of lithocholic, 3β -hydroxy-5-cholenoic (HCA) and cholic acids. (a and b) The mass spectra of ^2H -enriched chenodeoxycholic and 3β -hydroxy-5-cholenoic acids are shown and can be compared with authentic standards of the [^1H]compounds (Fig. 2).

Fig. 2. Analysis of bile acid methyl ester acetates

(c) Indicates the retention time of: 1, lithocholic acid; 2, 3β -hydroxy-5-cholenoic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; and 5, cholic acid. (a) and (b) indicate the mass spectra of chenodeoxycholic (CDCA) and 3β -hydroxy-5-cholenoic (HCA) acids respectively.

Table 1. Effect of 26- and 7 α -hydroxycholesterol on the synthesis of cholesterol, 3 β -hydroxy-5-cholenoic acid and chenodeoxycholic acid in Hep G2 cells

Values are means $\pm$ S.D. for three to six independent studies; significance by *t* test: **P* < 0.001; ***P* < 0.05. N.A., not analysed.

Medium	Synthesis (nmol/96 h per dish)		
	Total cell cholesterol	3 β -Hydroxy-5-cholenoic acid	Chenodeoxycholic acid
Control	352 $\pm$ 23	4.3 $\pm$ 0.5	33.4 $\pm$ 1.8
26-Hydroxycholesterol			
1.2 μ M	342 $\pm$ 15	5.6 $\pm$ 1.6	31.6 $\pm$ 2.3
6.0 μ M	311 $\pm$ 4.4**	13.6 $\pm$ 1.9*	18.1 $\pm$ 0.8*
7 α -Hydroxycholesterol			
6.0 μ M	350 $\pm$ 13	2.4 $\pm$ 0.5	43.4 $\pm$ 0.8*
12.0 μ M	358 $\pm$ 19	1.9 $\pm$ 0.5	44.4 $\pm$ 3.1*
26- + 7 α -Hydroxycholesterol			
6.0 μ M + 12 μ M	N.A.	7.8 $\pm$ 0.8**	34.7 $\pm$ 2.0

the transformation of cholesterol into bile acids. The transformation of the sterol ring from a 5-ene-3 β -ol to a 5 β -3 α -ol via the 3-oxo-4-ene intermediate results in the incorporation of three ^2H atoms and thus an increase in the ion intensities of $M_{0+1,2,3}$ [9]. Thus bile acids derived from cholesterol present in the cells before the enrichment of the medium with $^2\text{H}_2\text{O}$ will show an increase in the intensity of m/z 371, 372 and 373. In contrast, bile acids derived from cholesterol that was synthesized after the medium was enriched with $^2\text{H}_2\text{O}$ will contain, in addition, the ^2H that was incorporated into the cholesterol during its synthesis from acetate, the distribution of which is mostly $M_{0+4,5,6,7,8,9}$ [9]. Although some of the ^2H is lost during the oxidation of the side chain of cholesterol to form C_{24} bile acids, the transformation of the sterol ring compensates for this loss because of the acquisition of ^2H , and therefore very little change occurs in the total enrichment of the bile acids derived from the newly synthesized cholesterol. Because there is essentially no change in the ^2H enrichment of the bile acids derived from the newly synthesized cholesterol, it is possible to distinguish the chenodeoxycholic acid derived from cholesterol present in the cells before (m/z 370–373) and after (m/z 374–379) enrichment of the medium with $^2\text{H}_2\text{O}$ [9].

Table 1 summarizes the mean production rate of each bile acid expressed as nmol/96 h per dish, since no

significant differences were found in cell number and in mg of protein/10⁷ cells [2.4 ± 0.4 (S.D.)]. Total cell cholesterol is also given.

Addition of 12 nmol of 26-hydroxycholesterol to the medium had no apparent effect on the total amount of 3 β -hydroxy-5-cholenoic and chenodeoxycholic acids (Table 1). However, addition of 60 nmol to the medium resulted in a significant increase in the amount of 3 β -hydroxy-5-cholenoic acid and a decrease in the amount of chenodeoxycholic acid. A decrease in total cell cholesterol also occurred.

Addition of either 60 or 120 nmol of 7 α -hydroxycholesterol to the medium yielded a significant increase in chenodeoxycholic acid and also augmented synthesis in cultures containing 26-hydroxycholesterol. A decrease in total cell cholesterol did not occur.

Determination of the proportion of the total cell cholesterol that was synthesized during the 96 h period and metabolized to bile acids provides additional insights (Table 2). During the 96 h period, 38%, or 134 nmol (0.38×352), represents newly synthesized cholesterol. In the presence of 6 μM -26-hydroxycholesterol, the amount of newly synthesized cholesterol is decreased to 50 nmol (0.16×311). As a consequence, the amount of chenodeoxycholic derived from the newly synthesized cholesterol also decreases, from 11.7 nmol (0.35×33.4) to 4.7 nmol (0.26×18.1); however, this decrease accounts for only approximately one-half of the apparent total decrease in chenodeoxycholic acid.

For 3 β -hydroxy-5-cholenoic acid the decrease in the amount derived from the newly synthesized cholesterol, from 38 to 13 nmol, does not represent a decrease in the amount derived from newly synthesized cholesterol (0.38×4.3 as compared with 0.13×13.6), but rather reflects the metabolism of some of the [^1H]26-hydroxycholesterol to the [^1H]bile acid, thus accounting for the increase in total bile acid. Similarly, addition of 7 α -hydroxycholesterol to the medium lowers the proportion of M_{0+5} to undetectable levels, indicating that the chenodeoxycholic acid is derived mostly from the [^1H]7 α -hydroxycholesterol added to the medium.

DISCUSSION

The Hep G2 cell line that we obtained [12] did not have the defects in side-chain cleavage noted in a previous

Table 2. Effect of 26- and 7 α -hydroxycholesterol on cholesterol synthesis and the proportion of bile acids derived from newly synthesized (^1H)-cholesterol

Medium	Synthesis (%/96 h)		
	Cholesterol	3 β -Hydroxy-5-cholenoic acid	Chenodeoxycholic acid
Control	38	38	35
26-Hydroxycholesterol			
1.2 μ M	38	30	32
6 μ M	16	13	26
7 α -Hydroxycholesterol			
12 μ M	38	38	0*

* M_{0+5} peak not detected.

report [6], and therefore only C₂₄ bile acids were detected. As was also found previously [6], chenodeoxycholic acid is the predominant bile acid. The small amounts of cholic acid are quite variable and of relatively little quantitative significance. Considering that the Hep G2 cell line was actually from a child with a hepatoblastoma [13], although it is often referred to as a 'a hepatoma', the variable expression of minimal 12 α -hydroxylase activity may reflect the relatively low synthesis of cholic acid in foetal life.

Initially it was thought that bile acids were derived exclusively from cholesterol via initial 7 α -hydroxylation, but studies in rats [14,15], hamsters [16], rabbits [17] and humans [1] indicate that 26-hydroxycholesterol can also yield both chenodeoxycholic and cholic acids and, in addition, that it is the likely source of the 3 β -hydroxy-5-cholenoic acid [18] and 3 β -hydroxy-cholest-5-enoic acid that are found in human plasma [19]. The presence of 26-hydroxycholesterol in normal human plasma [20–22] and its decreased level in cerebrotendinous xanthomatosis [22,23], an inborn error in bile acid synthesis in which a high rate of cholesterol synthesis has been shown to occur [24], also indicates a biological role for this sterol.

The inhibitory effects of many hydroxysterols ('oxysterols'), including 7 α -hydroxycholesterol, on cholesterol synthesis has been reported in L-cells and in other 'in vitro' systems [25]. These data imply that hydroxylated sterol intermediates in bile acid synthesis, regardless of whether they are derived from 7 α -hydroxycholesterol or from 26-hydroxycholesterol, downregulate cholesterol synthesis.

The current findings, however, indicate that 7 α -hydroxycholesterol does not downregulate cholesterol synthesis in hepatocytes. This finding is in keeping with the knowledge that administration of a bile acid sequestrant, such as cholestyramine, is a potent inducer of both hydroxymethylglutaryl-CoA reductase and cholesterol 7 α -hydroxylase [26] and that the latter enzyme is rate-limiting in bile acid synthesis via this metabolic pathway.

Since studies *in vivo* in humans have shown that as much as 40% of administered 26-hydroxycholesterol is metabolized to chenodeoxycholic acid [1], we were surprised to find a significant increase only in 3 β -hydroxy-5-cholenoic acid synthesis.

The decrease in chenodeoxycholic acid synthesis can be attributed to the decrease in cholesterol synthesis. The possibility that a small fraction of the 26-hydroxycholesterol added to the medium was metabolized to ¹H-labelled chenodeoxycholic acid cannot be excluded by the methods used in the present study. However, the finding is different from the known metabolism of 26-hydroxycholesterol *in vivo* and from the metabolism of 7 α -hydroxycholesterol in Hep G2 cells and implies that 7 α -hydroxylation of 26-hydroxycholesterol, which is essential for chenodeoxycholic acid synthesis, is not well-expressed in Hep G2 cells.

It is evident that chenodeoxycholic acid is derived from both the [¹H]cholesterol, present in the Hep G2 cells before the addition of ²H₂O to the medium, and from the [²H]cholesterol that was formed during the 96 h period of incubation. The apparent similarity between the proportions of [²H]cholesterol and of

[²H]chenodeoxycholic acid implies that both the [¹H]- and [²H]cholesterol redistribute intracellularly before their metabolism to bile acid. However, the question of whether newly synthesized cholesterol is preferentially metabolized to bile acid is not addressed by the current experimental design, since only a single value over a 96 h period was obtained. Modifications of the experimental design, such as pulse labelling with ²H₂O for brief intervals, could address this question more precisely.

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REFERENCES

- Anderson, K. E., Kok, E. & Javitt, N. B. (1972) *J. Clin. Invest.* **51**, 112–117
- Gibbons, G. F., Mitropoulos, K. A. & Myant, N. B. (1982) *Biochemistry of Cholesterol*, pp. 195–199, Elsevier Bio-medical Press, Amsterdam, New York and London
- Scheer, I., Thompson, M. J. & Mosettig, E. (1956) *J. Am. Chem. Soc.* **78**, 4733–4736
- Miao, E., Wilson, S. R. & Javitt, N. B. (1988) *Biochem. J.* **255**, 1048–1052
- Ali, S. S. & Javitt, N. B. (1970) *Can. J. Biochem.* **48**, 1054–1057
- Everson, G. T. & Polokoff, M. A. (1986) *J. Biol. Chem.* **261**, 2197–2201
- Sjovall, J., Lawson, A. M. & Setchell, K. D. R. (1985) *Methods Enzymol.* **111**, 87
- Esterman, A., Cohen, B. I. & Javitt, N. B. (1985) *J. Lipid Res.* **26**, 950–954
- Javitt, N. B. & Javitt, J. I. (1989) *Biomed. Environ. Mass Spectrom.* **18**, 640–644
- Setchell, K. D. R. & Worthington, J. (1982) *Clin. Chim. Acta* **125**, 135–144
- Abell, L. L., Levy, B. B., Brodie, B. B. & Kendall, P. E. (1956) *J. Biol. Chem.* **195**, 357–361
- Javitt, N. B., Pfeffer, R., Kok, E., Burstein, S., Cohen, B. I. & Budai, K. (1989) *J. Biol. Chem.* **264**, 10384–10387
- Knowles, B. B., Howe, C. C. & Aden, D. P. (1980) *Science* **209**, 497–499
- Mitropoulos, K. A. & Myant, N. B. (1967) *Biochim. Biophys. Acta* **118**, 551–552
- Javitt, N. B. & Emerman, S. (1970) *Mt Sinai J. Med.* **37**, 477–481
- Wachtel, N., Emerman, S. & Javitt, N. B. (1968) *J. Biol. Chem.* **243**, 5207–5212
- Ayaki, Y., Kok, E. & Javitt, N. B. (1989) *J. Biol. Chem.* **264**, 3818–3821
- Sugiyama, K., Okuyama, S., Imoto, M., Okumura, K., Takagio, K. & Satake, T. (1986) *Gastroenterol. Jpn.* **21**, 608–616
- Alexson, M., Mork, B. & Sjovall, J. (1988) *J. Lipid Res.* **29**, 629–641
- Javitt, N. B., Kok, E., Burstein, S., Cohen, B. & Kutscher, J. (1981) *J. Biol. Chem.* **256**, 12644–12646
- Brooks, C. J. W., McKenna, R. M., Cole, W. J., MacLachlan, J. & Laurie, T. D. C. (1983) *Biochem. Soc. Trans.* **11**, 700–701
- Koopman, B. J., van der Molen, J. C., Wolthers, B. G. & Vanderpas, J. B. (1987) *J. Chromatogr.* **416**, 1–13
- Javitt, N. B., Kok, E., Cohen, B. & Burstein, S. (1982) *J. Lipid Res.* **23**, 627–630
- Salen, G. & Grundy, S. (1973) *J. Clin. Invest.* **52**, 2822–2835
- Kandutsch, A. A. & Chen, H. W. (1973) *J. Biol. Chem.* **248**, 8108–8117
- Cighetti, S., Bosiso, E., Galli, G. & Galli-Kienle, M. (1983) *Life Sci.* **33**, 2483–2488