Bile acid synthesis in HepG2 cells: effect of cyclosporin

Jason Levy, Kornelia Budai, and Norman B. Javitt[1]

Division of Hepatic Diseases, New York University Medical Center, 550 First Avenue, New York, NY 10016

Abstract The hypothesis that cyclosporin specifically affects the pathway of bile acid synthesis that begins with 27-hydroxylation of cholesterol was evaluated in HepG2 cells, which synthesize chenodeoxycholic acid and cholic acid from endogenous 7α-hydroxycholesterol. At a concentration in the medium of 8.3 μM cyclosporin, the proportion of cholic acid increased from 29 ± 7% to 44 ± 6% ($P < 0.001$) with no major change in total bile acid production. Chenodeoxycholic acid synthesis was enhanced by the addition of either 7α-hydroxycholesterol or 5β-cholestane-3α,7α-diol to the medium and cholic acid synthesis was enhanced by the addition of 5β-cholestane-3α,7α,12α-triol to the medium. Cyclosporin significantly inhibited only enhanced chenodeoxycholic acid synthesis, indicating a selective interference in mitochondrial side chain oxidation of less polar intermediates in bile acid synthesis derived from either initial 7α- or initial 27-hydroxylation of cholesterol. The increase in the proportion of cholic acid that occurs in the presence of cyclosporin mimics that occurring in genetically determined sterol 27-hydroxylase deficiency (cerebrotendinous xanthomatosis). Cyclosporin is useful for dissecting the subcellular pathways of bile acid synthesis.—**Levy, J., K. Budai, and N. B. Javitt.** Bile acid synthesis in HepG2 cells: effect of cyclosporin. *J. Lipid Res.* 1994. **35:** 1795–1800.

Supplementary key words cholic acid • chenodeoxycholic acid • cholesterol • 27-hydroxylation • CTX

Because of our interest in the metabolic pathway of bile acid synthesis beginning with 27-hydroxylation of cholesterol (1), a report that cyclosporin selectively inhibits this pathway (2) led us to further evaluate its effects on bile acid synthesis in HepG2 cells.

We showed previously that in this cell line 27-hydroxycholesterol is metabolized almost entirely to 3β-hydroxy-5-cholenoic acid (3) because of markedly low levels of 27-hydroxycholesterol 7α-hydroxylase (4). As a consequence, it can be assumed that virtually all the chenodeoxycholic and cholic acid found in the medium is derived via 7α-hydroxylation of cholesterol. Therefore, a selective effect of cyclosporin on these two pathways should be readily discernible. By using cell culture conditions that result in the endogenous production of both chenodeoxycholic and cholic acid and by supplementing the medium with naturally occurring C_{27} sterol intermediates in bile acid synthesis, we identified a selective effect on chenodeoxycholic acid, independent of initial 7α- or 27-hydroxylation of cholesterol.

METHODS

Cell culture

HepG2 cells were grown to confluence in T-75 flasks (NUNC Intermed, Laboratory Disposable Products, North Haledon, NJ) in 10 ml of Dulbecco's modified Eagle's medium (DMEM, Cat. No. 430-3000EB) containing 10% fetal bovine serum (FBS), both obtained from Gibco Life Technologies, Grand Island, NY. The medium was changed at intervals of 5 days. The monolayer became confluent at approximately 7 days, but the cells were maintained in the confluent state for an additional 20–25 days before use.

After 27–32 days, cells were harvested from the T-75 flasks and were placed together in a single pool. Approximately equal aliquots were plated at high density (approximately 1 × 10^7 cells) into T-25 flasks. Each study was comprised of experimental and control groups of flasks consisting of three to five flasks per group. After seeding, 5 ml of DMEM containing 10% delipidated FBS was added to each flask. In addition, cyclosporin dissolved in ethanol was added to half the flasks. Each study consisted of flasks to which 50 nmol of naturally occurring sterol intermediates in bile acid synthesis dissolved in 10–20 μl of 2-hydroxypropyl-β-cyclodextrin (5) were added and control flasks containing equivalent volumes of the vehicles alone. When it became apparent that cyclosporin had a much greater effect on the metabolism of 5β-cholestane-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; CTX, cerebrotendinous xanthomatosis.

[1]To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 17, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

$3\alpha,7\alpha$-diol than on that of 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol, the study was repeated using the same batch of harvested cells under identical conditions of culture.

Dr. B. I. Cohen provided authentic 5β-cholestane-$3\alpha,7\alpha,12\alpha$-triol and 5β-cholestane-$3\alpha,7\alpha$-diol, and Dr. T. Hoshita provided authentic 7α,27-dihydroxy-cholest-4-ene-3-one.

Deuterated cholesterol was prepared from deuterated tetrahydrodiosgenin using minor modifications of procedures previously described in detail (6–8). After acetylation, deuterated 7α-hydroxycholesterol was prepared by the copper bromide/tertiary butyl perbenzoate reaction (9). The deuterium was present on C_{16} and C_{22} and adjacent carbons (8).

At the time that the cells were seeded into T-25 flasks, duplicate aliquots, referred to as the cell pellet, were also taken for analysis of total cholesterol and total protein. In all studies, the total cholesterol concentration of the duplicate and occasionally triplicate pellets did not vary by more than 5%.

In one study, T-25 flasks were coated with Matrigel (Collaborative Research, Bedford Park, MA) exactly following the directions of the manufacturer. In this study confluent cells were harvested from T-75 flasks at 10 days. After the cells were seeded onto the Matrigel-coated T-25 flasks, DMEM containing 10% FBS (4 days) was alternated with DMEM alone (3 days). The study was continued for a period of 6 weeks and the bile acids secreted into the medium during the 3-day periods when only DMEM was in contact with the cells were identified and quantified by gas–liquid chromatography (GLC).

The cyclosporin A used in this study was a gift from Dr. David Weinstein, Sandoz Research. It was dissolved in ethanol (5 mg/ml) so that the addition of 10 μl to 5.0 ml of medium gave a final concentration of 8.3 μM.

Cholesterol and bile acid analysis by GLC

To determine the total amount of cholesterol produced by the cells during the 72-h period of study, aliquots of the initial cell pellet in duplicate, the harvested cells, and the medium were saponified following the addition of coprostanol (Steraloids, Wilton, NH, Batch G-151) as an internal standard. The internal standard yielded a single peak by GLC analysis after preparation of the acetate. Sterols were then extracted with chloroform and the acetate derivatives were prepared using acetic anhydride/pyridine. Aliquots were injected onto a 60-m fused silica column (i.d. = 0.25 mm) crossbonded with 95% dimethyl polysiloxane and 5% diphenylpolysiloxane (RT_x-5, Restec Corp., Bellefonte, PA). The head pressure was 1.8 kg/cm^2 and the split ratio 1:100. Temperature programming began at 255°C and increased at 1.5°C/min to 285°C, with a total run time of 20 min. Total cholesterol synthesized during the 72-h period was calculated as equal to the sum of the cholesterol in the cells and the medium minus the initial concentration of cholesterol in the cell pellet and was uncorrected for the amount metabolized to bile acids. A Shimadzu Model 14A GLC instrument was used.

Bile acids present in the medium were analyzed as the methyl ester acetates after addition of $3\alpha,7\alpha$-dihydroxy-12-oxo-5β-cholanoic acid as an internal standard, solvolysis, and alkaline hydrolysis (10). Conditions for GLC analysis were the same as for cholesterol acetate with the exception that the initial column temperature was 285°C, increased at 1.5°C/min to 300°C, and then remained isothermal for 10 min (total run time = 20 min).

Analysis of bile acids by HPLC

Because solvolysis, alkaline hydrolysis, and acid extraction can result in large losses of allylic and oxo bile acids, we also prepared the 1-bromoacetylpyrene esters (11) of the bile acids, which also preserves the stereochemistry (12). These esters were analyzed using a gradient high-performance liquid chromatography (HPLC) system (Shimadzu SLC-6B) and fluorescence detector (ex = 360 nm, em = 460 nm; Shimadzu model #530). For these analyses, ursodeoxycholic acid was used as an internal standard and the bile acids in the medium were extracted and eluted from a Sep-Pak cartridge using the procedure described by DeMark and co-workers (13), with the modification that all the bile acids were eluted using 100% methanol.

The 1-bromoacetylpyrene ester derivatives were injected onto a reverse phase C-18 column (3 μm × 15 cm, 4.6 mm i.d., Supelco, PA) using a binary solvent system and a gradient similar to that reported previously (12). The relative retention times (ursodeoxycholic acid 13.4 min, rrt = 1) of the following bile acids as their bromacetylpyrene esters were: glycocholic acid, 0.54; lithocholic acid 3-sulfate, 0.73; glycochenodeoxycholic acid, 0.84; cholic acid, 1.23; glycolithocholic acid, 1.32; 3-oxo,7α-5β-cholanic acid, 1.51; chenodeoxycholic acid, 1.67; $3\alpha,7\alpha$-dihydroxy-chol-5-enoic acid, 1.75; lithocholic acid, 2.0; 3β-hydroxy-5-cholenoic acid, 1.87; and 3β-hydroxy-5-cholestenoic acid, 2.30.

TABLE 1. Bile acid production by HepG2 cells: long-term culture using Matrigel

Week	Bile Acid (µg/3 Day)[a] Chenodeoxycholic	Cholic	Total	Percent Cholic
1	3.3	0.04	3.34	1.2
2	10.0	2.0	12.0	17
3	20.0	10.0	30.0	33
4	26.0	14.6	40.6	36
5	19.0	16.7	35.7	47
6	bacterial contamination			

[a]Medium DMEM + 10% FBS × 4 days alternating with DMEM × 3 days (bile acids harvested from medium); µg/3 day = total bile acid accumulating in medium during 3-day period.

Downloaded from www.jlr.org by guest, on June 17, 2012

TABLE 2. Cholesterol and bile acid synthesis by HepG2 cells

Cell Culture	Cholesterol[a]	Bile Acid: Chenodeoxycholic	Bile Acid: Cholic	Percent Cholic
		nmol/flask/72 h		
Control	99 ± 16[b]	44.7 ± 6.8	21.2 ± 5.5	32 ± 7
+ Cyclo	90 ± 16[b]	31.3 ± 6.9	26.4 ± 6.5	46 ± 6[c]

[a]Calculated as total medium + cell cholesterol − pellet cholesterol uncorrected for the amount metabolized to bile acids.
[b]Mean ± standard deviation base on four studies with five flasks per group (cholesterol) and eight studies with three to five flasks per group (bile acids).
[c]Ranked t test $P < 0.001$.

A standard mixture of unconjugated cholic, chenodeoxycholic, lithocholic, and 3β-hydroxy-5-cholenoic acids analyzed by both GLC and HPLC using 3α,7α-dihydroxy-12-oxo 5β-cholanoic acid and ursodeoxycholic acid as internal standards, respectively, did not vary in amount by more than 10%.

To determine the amounts of chenodeoxycholic and cholic acid present in the medium, the respective sums of the glycine-conjugated and the unconjugated bile acids were used. As previously reported (14), glycine conjugates did not exceed 23% of the total.

GLC-mass spectrometry (MS) analysis

To identify sterols present in the medium, the trimethylsilyl ethers were prepared and were injected onto a column of CP-19 sil (Chrompack, Raritan, NJ) using conditions identical to those previously reported (4).

RESULTS

Table 1 indicates the changes in both total bile acid synthesis and the proportion of cholic acid that occurred when the same flask of HepG2 cells was maintained for 6 weeks using a Matrigel matrix. After week 1, the combined amount of chenodeoxycholic and cholic acid accounted for more than 95% of the total bile acid. Maximum bile acid production occurred at 4 weeks, but the proportion of cholic acid continued to increase. With our routine methods of culture, bacterial contamination occurred frequently at 5–6 weeks. Attempts to recover the cells from the Matrigel after several weeks were not successful and therefore we do not know if the harvested cells would continue to produce the same proportion of cholic acid.

Extrapolation of these findings led us to use HepG2 cells that had been maintained on plastic for periods of up to several weeks. As shown in **Table 2,** the proportion of cholic acid in control cultures containing only the vehicles ranged from 22 to 36% and increased to values that ranged from 38 to 50% ($P < 0.001$) when cyclosporin was present in the medium.

Although preliminary studies showed, as reported previously (2), that a concentration of 10 μM cyclosporin decreased cholesterol synthesis (data not shown), lowering the concentration of cyclosporin to 8.3 μM eliminated major changes in either cholesterol synthesis or total bile acid synthesis that could have accounted for the increase in the proportion of cholic acid (Table 2).

Adding 7α-hydroxycholesterol to the medium to supplement its endogenous production led to a significant increase only in chenodeoxycholic acid (**Table 3**). Although cholic acid production did not increase, using deuterated 7α-hydroxycholesterol made it possible to demonstrate that mixing with the endogenous pool occurred, as the cholic acid that was synthesized was enriched with deuterium (**Fig. 1**).

When cyclosporin was present in the medium, the increment in chenodeoxycholic acid derived from the exogenous 7α-hydroxycholesterol was significantly smaller (Table 3). Analysis of the sterol fractions prepared from the medium indicated the presence of an intermediate

TABLE 3. Effect of cyclosporin on the metabolism of sterol intermediates to bile acids by HepG2 cells

Sterol Intermediate Added to Medium[b]	Increase in Bile Acid Synthesis[a]: Chenodeoxycholic, Control	Chenodeoxycholic, Cyclo	Cholic, Control	Cholic, Cyclo	3β-OH-5-Cholenoic, Control	3β-OH-5-Cholenoic, Cyclo
	nmol/dish/72 h					
7α-Hydroxycholesterol	33 ± 10[c]	12 ± 4[d]	none	none	none	none
5β-Cholestane-3α,7α-diol	37 ± 7	15 ± 4[d]	none	none	none	none
3α,7α,12α-triol	none	none	42 ± 8	37 ± 4	none	none
27-Hydroxycholesterol	none	none	none	none	29 ± 0.6	12 ± 2[d]

[a]Values corrected for endogenous bile acid synthesis from cholesterol.
[b]Fifty nmol of each sterol added to medium.
[c]Values are mean ± SD of two or more studies with a minimum of three dishes per study.
[d]$P < 0.01$.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 17, 2012

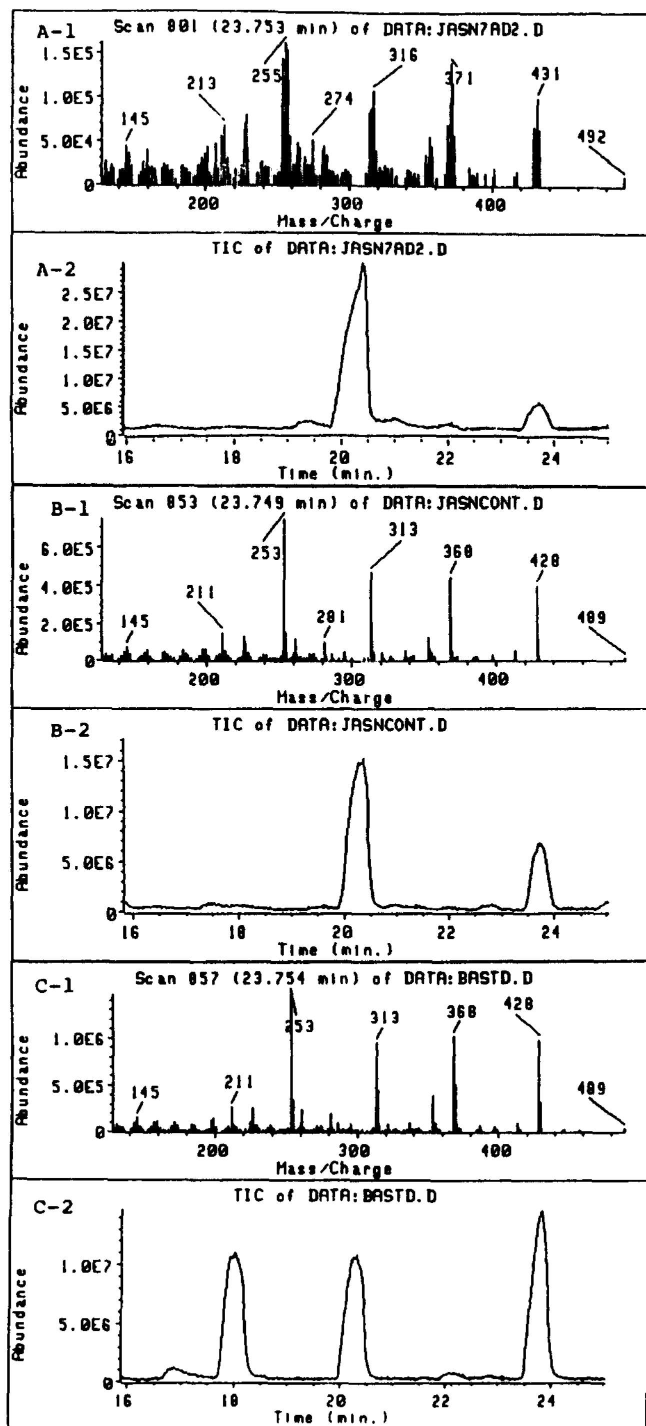

Fig. 1. Metabolism of deuterated 7α-hydroxycholesterol to cholic acid. Panel C-2 (BASTD) indicates the retention times for authentic standards of deoxycholic (10 min), chenodeoxycholic (20.3 min), and cholic acid (23.7 min). The mass spectrum obtained for cholic acid is shown immediately above (panel C-1) (molecular ion = 548 − 60 (acetate) = 488 − 60 = 428). Panel B-2 (JASN-CONT) indicates the retention times of the bile acids recovered from the medium after incubation with HepG2 cells. The mass spectrum of the bile acid at 23.7 min is not significantly different from that obtained for authentic cholic acid. The retention times of bile acids recovered from the medium after incubation of HepG2 cells with medium containing deuterated 7α-hydroxycholesterol is shown in panel A-2 (JASN7AD). The mass spectrum corresponding to cholic acid (23.7 min) contains ion peaks with masses greater than those expected, indicating enrichment with deuterium.

that was detectable only in those media containing added 7α-hydroxycholesterol. GLC-MS analysis, including comparison with an authentic standard, indicated that the metabolite was 7α,27-dihydroxy-cholest-4-ene-3-one. The amount of the metabolite increased from 1.5 nmol to 6.5 nmol in the presence of cyclosporin. HPLC analysis did not indicate the accumulation of either oxo or allylic bile acids in media containing cyclosporin.

Adding 5β-cholestane-3α,7α-diol to the medium also augmented chenodeoxycholic acid production and the increase was again found to be significantly smaller in the presence of cyclosporin (Table 3).

By contrast, cyclosporin had no effect on the increase in cholic acid synthesis that followed the addition of 5β-cholestane-3α,7α,12α-triol (Table 3).

To evaluate the pathway of bile acid synthesis beginning with 27-hydroxylation of cholesterol, the medium was supplemented with 27-hydroxycholesterol. As shown in Table 3, the resulting increase in 3β-hydroxy-5-cholenoic acid was significantly lower in the presence of cyclosporin.

DISCUSSION

Although defects in bile acid synthesis can occur in HepG2 cells, specifically in regard to cholic acid synthesis (14), they appear to be related to cell culture conditions rather than to the deletion of a gene that is essential for the expression of the enzymes needed for bile acid synthesis. The initial report concerning bile acid synthesis in HepG2 cells used cells in their growth phase rather than confluent cells. Defects in cholic acid synthesis were found (14). Our initial study of bile acid composition was done on HepG2 cells that had just become confluent (5–7 days) and no defects in bile acid synthesis were noted (10). Cholic acid accounted for approximately 10% of total bile acid synthesis. After publication of a report that HepG2 cells change their genetic expression as they age in culture (15), we elected to determine whether the expression of the constitutive P-450 12α-hydroxylase, which is a determinant of cholic acid synthesis, also increases with age. Long-term culture using a Matrigel matrix indicated that a progressive increase in cholic acid does occur in cells maintained over a prolonged period. However, the difficulty in recovering these cells from Matrigel led us to extend the length of time that cells were maintained after reaching confluence using standard conditions of culture on a plastic surface.

In the previous study using primary rat and human hepatocytes, it was found that at a concentration of 10 μM cyclosporin caused a 30% decrease in cholesterol synthesis and a 50% decrease in total bile acid synthesis, with an increase in the proportion of cholic acid. Addition of cyclosporin in vitro to enzyme assays for cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase significantly inhibited only the latter assay (2), a finding that led to the

Downloaded from www.jlr.org by guest, on June 17, 2012
ASBMB JOURNAL OF LIPID RESEARCH

ASBMB
JOURNAL OF LIPID RESEARCH

hypothesis of selective inhibition of the 27-hydroxylation pathway. In a more recent report (16), which appeared while our cell culture studies were in progress, it was concluded on the basis of enzyme assays that 27-hydroxylation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol were not significantly inhibited by cyclosporin although some inhibition of hydroxylation of the diol was noted at a 50 μM concentration of cyclosporin. Our finding that the metabolism of 27-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid was also inhibited by cyclosporin indicates a block in side-chain oxidation subsequent to the initial 27-hydroxylation, which would not have been detected by the enzyme assays that were used in the above study and therefore accounts for the difference in the findings and interpretation of the data.

Although it is currently thought that mitochondrial sterol 27-hydroxylase is a polyfunctional enzyme catalyzing all the steps necessary for the formation of the C_{27} acid (17), an additional effect on alcohol dehydrogenases, which could catalyze some of the steps in side-chain oxidation (18), cannot be excluded.

By lowering the concentration of cyclosporin to 8.3 μM we were able to eliminate major effects on both cholesterol synthesis and bile acid production as a determinant of the increase in the proportion of cholic acid. The very low activity of 27-hydroxycholesterol 7α-hydroxylase in HepG2 cells (4) implies that both chenodeoxycholic acid and cholic acid are generated after initial 7α-hydroxylation of cholesterol and that the increase in the proportion of cholic acid indicates 12α-hydroxylation of 7α-hydroxy-cholest-4-ene-3-one, the pivotal endogenous intermediate. Because of the low 27-hydroxycholesterol 7α-hydroxylase activity, no increase in chenodeoxycholic acid synthesis was detected after 27-hydroxycholesterol was added to the medium. However, we consider 3β-hydroxy-5-cholenoic acid as a surrogate for cholest-5-ene-3β,7α,27-triol, the intermediate that would otherwise occur in this pathway leading to chenodeoxycholic acid synthesis. Based on this view, we consider the decrease in 3β-hydroxy-5-cholenoic acid synthesis analogous to inhibition of chenodeoxycholic acid synthesis. Thus, we conclude that cyclosporin inhibits the synthesis of chenodeoxycholic acid regardless of whether it occurs via initial 7α- or initial 27-hydroxylation of cholesterol.

The effect of cyclosporin on bile acid synthesis in HepG2 cells is analogous to that occurring in persons with cerebrotendinous xanthomatosis (CTX). This genetically determined disease is also characterized by a defect in side-chain oxidation attributable to low or absent sterol 27-hydroxylase activity (17) and has been shown to have a selective deficiency in chenodeoxycholic acid synthesis (19). In both HepG2 cells and patients with CTX, virtually all the chenodeoxycholic and cholic acid is derived from the metabolic pathway beginning with 7α-hydroxylation of cholesterol. The proportion of cholic acid in the bile of patients with CTX was found to be 66 ± 19%, compared with 45 ± 5% for a control group of patients (19). An increase in microsomal 12α-hydroxylase was also found (19). However, other studies (20) showed that the 5β-cholestane-3α,7α,12α-triol content of the microsomes in patients with CTX was 46-fold greater than normal, with only a 2-fold increase in the microsomal concentration of 5β-cholestane-3α,7α-diol. The marked increase in the triol was attributed to the increase in residence time of the diol in the endoplasmic reticulum, allowing more time for 12α-hydroxylation to occur (21). According to this view, an increase in 12α-hydroxylation is not essential to change the cholate/chenodeoxycholate ratio.

Although we did not assay 12α-hydroxylase activity, the accumulation of the diol intermediate (7α,27-dihydroxy-cholest-4-ene-3-one) when 7α-hydroxycholesterol was added to the medium of HepG2 cells in the presence of cyclosporin and the lack of any further increase in cholic acid synthesis imply that little change occurred in the activity in this enzyme. Based on this assumption, the effect of cyclosporin on endogenous bile acid production by the HepG2 cells can be attributed to the previously proposed mechanism (21) rather than an induction of 12α-hydroxylase activity.

The selective effect of cyclosporin on side-chain oxidation of some but not all intermediates in bile acid synthesis provides a useful tool for learning more about the intracellular trafficking that occurs during the synthesis of the bile acid molecule. Relatively little is known regarding the determinants of the movements of various intermediates between the subcellular organelles that contain the requisite enzymes for bile acid synthesis.

This work was supported by grant DK32995 from the National Institute of Diabetes, Digestive and Kidney Diseases of the National Institutes of Health. J. L. was supported by a Summer Student Research Program (T35 DK-07421) from the National Institutes of Health.

Manuscript received 5 October 1993, in revised form 18 February 1994, and in re-revised form 13 April 1994.

Downloaded from www.jlr.org by guest, on June 17, 2012

REFERENCES

1. Anderson, K. E., E. Kok, and N. B. Javitt. 1972. Bile acid synthesis in man: metabolism of 7α-hydroxycholesterol-^{14}C and 26-hydroxycholesterol-^{3}H. *J. Clin. Invest.* **51:** 112–117.
2. Princen, H. M. G., P. Meijer, B. G. Wolthers, R. J. Vonk, and F. Kuipers. 1991. Cyclosporin A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis. *Biochem. J.* **275:** 501–505.
3. Javitt, N. B., and K. Budai. 1989. Cholesterol and bile acid synthesis in HepG2 cells. *Biochem. J.* **261:** 989–992.
4. Martin, K. O., K. Budai, and N. B. Javitt. 1993. Cholesterol and 27-hydroxycholesterol 7α-hydroxylation: evidence for two different enzymes. *J. Lipid Res.* **34:** 581–588.
5. De Caprio, J., J. Yun, and N. B. Javitt. 1992. Bile acid and

sterol solubilization in 2-hydroxypropyl-β-cyclodextrin. *J. Lipid Res.* **33:** 441-443.
6. Marker, R. E., and D. L. Turner. 1941. Sterols. CXV. Sapogenins. XLIV. The relation between diosgenin and cholesterol. *J. Am. Chem. Soc.* **63:** 767-771.
7. Arunachalam, T., P. L. MacKoul, N. M. Green, and E. Caspi. 1981. Synthesis of 26-halo-, 26-(phenylseleno)-, and 26-indolylcholesterol analogues. *J. Org. Chem.* **46:** 2966-2968.
8. Javitt, N. B., E. Kok, J. Lloyd, A. Benscath, and F. H. Field. 1982. Cholest-5-ene-3β,26-diol: synthesis and biomedical use of a deuterated compound. *Biomed. Mass Spectrom.* **9:** 61-63.
9. Starka, L. 1962. Reaktion der steroide mit tert.-butylbenzoate. II. Neue synthese des provitamins D_3. *Steroids.* **17:** 126-127.
10. Javitt, N. B., R. Pfeffer, E. Kok, S. Burstein, B. I. Cohen, and K. Budai. 1989. Bile acid synthesis in cell culture. *J. Biol. Chem.* **264:** 10384-10387.
11. Kamada, S., M. Masako, and A. Tsuji. 1983. Fluorescence high-performance liquid chromatographic determination of free and conjugated bile acids in serum and bile using 1-bromoacetylpyrene as a pre-labeling reagent. *J. Chromatogr.* **272:** 29-41.
12. Une, M., N. Izumi, and T. Hoshita. 1993. Stereochemistry of intermediates in the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid to cholic acid by rat liver peroxisomes. *J. Biochem.* **113:** 141-143.
13. DeMark, B. R., G. T. Everson, P. D. Klein, R. B. Showalter, and F. Kern, Jr. 1982. A method for accurate measurement of isotope ratios of chenodeoxycholic and cholic acids in serum. *J. Lipid Res.* **23:** 204-210.
14. Everson, G. T., and M. A. Polokoff. 1986. HepG2. A human hepatoblastoma line exhibiting defects in bile acid synthesis and conjugation. *J. Biol. Chem.* **261:** 2197-2201.
15. Kelly, J. H., and G. J. Darlington. 1989. Modulation of the liver specific phenotype in the human hepatoblastoma line HepG2. *In Vitro Cell. Dev. Biol.* **25:** 217-222.
16. Dahlbäck-Sjöberg, H., I. Björkhem, and H. M. G. Princen. 1993. Selective inhibition of mitochondrial 27-hydroxylation and bile acid intermediates and 25-hydroxylation of vitamin D_3 by cyclosporin A. *Biochem. J.* **293:** 203-206.
17. Cali, J. J., C. Hsieh, U. Franke, and D. W. Russell. 1991. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266:** 7779-7783.
18. Okuda, A., and K. Okuda. 1983. Physiological function and kinetic mechanism of human liver alcohol dehydrogenase as 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase. *J. Biol. Chem.* **258:** 2899-2905.
19. Salen, G., S. Shefer, G. S. Tint, G. Nicolau, B. Dayal, and A. K. Batta. 1985. Biosynthesis of bile acids in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **76:** 744-751.
20. Björkhem, I., H. Oftebro, S. Skrede, and J. I. Pedersen. 1981. Assay of intermediates in bile acid biosynthesis using isotope dilution-mass spectrometry: hepatic levels in the normal state and in cerebrotendinous xanthomatosis. *J. Lipid Res.* **22:** 191-200.
21. Oftebro, H., I. Björkhem, S. Skrede, and A. Schreiner. 1980. Cerebrotendinous xanthomatosis: a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. *J. Clin. Invest.* **65:** 1418-1430.

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 17, 2012