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7α-Hydroxylation of 27-hydroxycholesterol: biologic role in the regulation of cholesterol synthesis

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Abstract The report of a novel cytochrome P450 enzyme in mouse hippocampus (cyp7b) with close homology to cholesterol 7α-hydroxylase led us to determine the substrate specificity with respect to 27-hydroxycholesterol, known to be a potent inhibitor of cholesterol synthesis. Transfection of 293/T cells with pcDNA3.1(+)-mcyp7b was followed by metabolism of 2.5 μM 27-hydroxycholesterol to the 7α-hydroxy intermediate, cholest-5-ene,3β,7α,27-triol, with complete loss of down-regulation of cholesterol synthesis. Addition of 5 μM and 10 μM concentrations of the triol to HepG2 and CHO cells, respectively, also did not reduce cholesterol synthesis. The contrast between the biologic effect on cholesterol synthesis by these two C_{27} hydroxysterols and the wide tissue distribution of both cholesterol 27-hydroxylase and hydroxysterol 7α-hydroxylase implies local regulatory effects prior to their further catabolism in the liver to chenodeoxycholic and cholic acids.—**Martin, K. O., A. B. Reiss, R. Lathe, and N. B. Javitt.** 7α-hydroxylation of 27-hydroxycholesterol:biologic role in the regulation of cholesterol synthesis. *J. Lipid Res.* 1997. **37:** 1053–1058.

Supplementary key words cDNA-mcyp7b • transfection • 293/T cells • CHO cells • cholesterol 7α-hydroxylase • 27-hydroxycholesterol 7α-hydroxylase • cholest-5-ene-3β,7α,27-triol

We previously reported (1) the existence of a novel enzyme in the microsomal fraction of human and hamster liver that catalyzes the 7α-hydroxylation of 27-hydroxycholesterol but not of cholesterol. Further, using enzymological techniques (2), we reported data supporting the view that the same microsomal enzyme catalyzes the 7α-hydroxylation of both the C_{26} and the C_{24} acid intermediates in this pathway, which are then further metabolized to either chenodeoxycholic or cholic acid (3, 4).

Because 27-hydroxycholesterol is a known potent inhibitor of cholesterol synthesis (5, 6), we began a study comparing the biologic effects of the 7α-hydroxy intermediate, cholest-5-ene-3β,7α,27-triol, with those of the parent compound.

During the course of these studies we read a report of a novel P450 enzyme with uncharacterized substrate specificity that has been cloned from rat and mouse hippocampus (mcyp7b) and that has close homology with the cDNA for cholesterol 7α-hydroxylase (7). Studies of 293/T cells transfected with mcyp7b showed that 27-hydroxycholesterol was metabolized to the 7α-hydroxylated intermediate with loss of the potent inhibitory effect of the parent compound on cholesterol synthesis.

MATERIALS AND METHODS

Cell culture and transfection

Chinese hamster ovary (CHO-K1) cells were maintained in F-12 media (Gibco, Grand Island, NY) as described previously (5). For the determination of cholesterol synthesis, the concentrated media were diluted to contain a final concentration of 15% D_2O and 10% delipidated fetal bovine serum (FBS) (8).

HepG2 cells were grown as described previously (9), with the exception that concentrated Dulbecco's modified Eagle's medium (DMEM) was prepared as described above for the determination of cholesterol synthesis.

293/T cells derived from ATCC 293 were grown and maintained in DMEM with 10% FBS. For studies of cholesterol syntesis the medium was changed as described above.

Prior to transfection, a mammalian expression plasmid was constructed from the full-length coding region of mouse cyp7b cDNA (1880bp) that had been inserted between Not I and Eco RI sites in the phagemid pBluescript II KS, kindly supplied by Dr. R. Lathe (7). The

Abbreviations: CHO, Chinese hamster ovary; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GLC, gas–liquid chromatography; MS, mass spectrometry.

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insert (5 μg) was excised with Not I restriction enzyme and the digest underwent electrophoresis in 0.8% agarose gel to obtain the 1880 bp cDNA, which was extracted using QIA quick gel extraction kit according to the manufacturer's protocol.

The insert was then ligated using T4 ligase to pcDNA3.1(+) expression vector (Invitrogen), which had been linearized with Not I and treated with calf intestine alkaline phosphatase.

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These plasmids were then amplified in *E. coli*, INVαF' strain (Invitrogen) and screened for correct orientation using the Bam HI site as a marker. The plasmid that contained the correct orientation of the insert (pcDNA3.1(+)-mcyp7b) was isolated and then further amplified and purified from an overnight culture of *E. coli*, using QIAgen (500) ultrapure plasmid kit.

For transfection, a freshly prepared calcium phosphate precipitate (1 μg/well) of pcDNA3.1((+)-mcyp7b was added to 293/T cells that were 60–70% confluent and was allowed to remain in contact with the cells for 24 h. As a control, parallel transfection of pcDNA3.1(+) without the insert was done.

After 24 h of incubation, the media containing the DNA were removed, the cells were washed with phosphate-buffered saline, fresh media containing 15% D_2O and 10% delipidated FBS were added, and incubation continued for 48 h.

All sterols were dissolved in 45% hydroxypropyl-β-cyclodextrin prior to addition to the medium. The volume added did not exceed 2 μl/ml of medium.

Syntheses

The 7α- and 7β-hydroxy derivatives of 27-hydroxycholesterol were prepared from the diacetate of 27-hydroxycholesterol using copper bromide and tertiary butyl peroxybenzoate as described (10). After removal of the acetate groups with methanolic KOH, each of the isomers was separated and purified by thin-layer chromatography using silica gel G plates and a solvent system of chloroform–acetone 4:1(v/v).

Analyses

Cholesterol, 27-hydroxycholesterol, and its 7α-hydroxy metabolite were measured using coprostanol as an internal standard and a Shimadzu Model GLC 14A instrument with a flame detector as described previously (11). Because the 7α-hydroxy metabolite is an allylic compound and decomposes as the triacetate derivative under the conditions of GLC, the trimethylsilyl derivatives were prepared.

GLC–MS was used to identify the metabolites and to determine the proportion of newly synthesized cholesterol by isotope ratio mass spectrometry. The methods were described in detail previously (8, 11).

The amount of deuterated cholesterol was determined by measuring the enrichment at M_{0+4} (m/z = 372) with a SIM program. Using 15% D_2O, the enrichment at m/z 372 equals 20.8% of the total deuterated species (12). Thus the abundance was multiplied by 4.8 to obtain the total abundance of deuterated species. The abundance of protium cholesterol at m/z = 368 is 71% of the total because of the natural abundance of C_{13} and was therefore multiplied by 1.4 to obtain total abundance of the protium species.

Statistical analysis was performed using the ANOVA program.

RESULTS

As shown in **Table 1,** 27-hydroxycholesterol added to the medium of CHO cells or HepG2 cells in culture has

TABLE 1. Effect of 27-hydroxycholesterol and the 7α-hydroxy metabolite on cholesterol synthesis in cell culture

			Cholesterol		
Cell line	Sterol	Protein	Total	Isotope Ratio 372/368	Synthesis
	μM	μg	μg		%
CHO (3)[a]					
Control		753	43	0.0826	21
27-OH	10	735	34	0.004	1.3[b]
7α,27-OH	10	785	45	0.090	24
HepG2 (3)[a]					
Control		1756	85	0.142	33
27-OH	5	1688	76	0.027	8.4[b]
7α,27-OH	5	1817	79	0.134	31

[a]Mean value of three separate dishes for both control and sterol-added studies; cells were harvested after 72 h.

[b]$P < 0.01$.

TABLE 2. Metabolism of 27-hydroxycholesterol by 293/T cells transfected with mcyp7b cDNA: effect on cholesterol synthesis

Plasmid Transfected	27OH Added to Media	Cell Cholesterol		
		Total	Isotope Ratio 372/368	Synthesis
	μM	μg/mg prot.		%
No transfection (n = 1)	0	28.4	0.111	38.8
No transfection (n = 1)	2.5	21.8	0.023	8.4
Vector alone[a]	0	32.1 ± 0.8[b]	0.117 ± 0.015	41.0 ± 5.2
Vector alone	2.5	24.2 ± 1.6[c]	0.027 ± 0.002[c]	9.5 ± 0.7[c]
Vector-mcyp7b[a]	0	29.8 ± 0.9	0.138 ± 0.023	48.7 ± 8.0
Vector-mcyp7b	2.5	28.9 ± 0.9[d]	0.111 ± 0.012[d]	39.0 ± 4.4[d]

[a]Vector: pcDNA3.1(+), vector-mcyp7b: pcDNA3.1(+) with mcyp7b coding sequence.
[b]Mean ± sd, n = 4.
[c]$P < 0.01$ against no 27OH group.
[d]$P > 0.05$ against no 27OH group, not significantly different.

the known inhibitory effect on cholesterol synthesis. By contrast, cholesterol synthesis is not significantly different from control values when the cells are grown in the presence of the 7α metabolite, cholest-5-ene-3β-7α,27-triol.

Endogenous production of cholest-5-ene-3β-7α,27-triol from 27-hydroxycholesterol added to the medium also abolishes its inhibitory effect on cholesterol synthesis. In the study shown in **Table 2,** addition to the medium of 2.5 μM 27-hydroxycholesterol caused a significant reduction of cholesterol synthesis from 41 to 9.5%, which was prevented completely only in cells transfected with pcDNA3.1(+)-mcyp7b.

Analysis of the medium at the end of each transfection study using 293/T cells indicated that the level of enzyme expression was sufficient to obtain almost complete 7α-hydroxylation. In the study shown in **Table 3,** virtually complete 7α-hydroxylation of 27-hydroxycholesterol added to the medium was obtained within 48 h in medium that initially contained 10 μM 27-hydroxycholesterol. By contrast, transfected CHO cells, not expressing T antigen that facilitates plasmid replication, metabolized less than 10% of added 27-hydroxycholesterol.

GLC–MS analysis of harvested medium indicated the presence of a single 7α-hydroxylated metabolite. As shown in **Fig. 1D,** the 7β-hydroxy derivative of 27-hydroxycholesterol has a much longer retention time (24.3 min) than either 27-hydroxycholesterol (22.6 min) or the 7α-derivative (21.0 min). Analysis of harvested medium (Fig. 1, panel 1B) indicates the presence of a peak with the same retention time as authentic cholest-5-ene-3β,7α,27-triol and comparison of their respective mass spectra (panel 1A and 1C) shows no significant differences.

The medium also contained small peaks of cholesterol (14.3 min) and coprostanol (12.3 min) (Fig. 1, panel C), but 7α-hydroxycholesterol was not detected

TABLE 3. 27-Hydroxycholesterol 7α-hydroxylation after transfection with mcyp7b cDNA

Transfection[a]		27-OH added to Media	Hydroxysterols Found in the Media	
Cells	Plasmid		$7,27(OH)_2$	27OH
		μM	μM	μM
CHO	vector alone[b]	0	[c]	[c]
		10	0.02	10.44
CHO	vector-mcyp7b[d]	0	[c]	[c]
		10	0.88	9.25
293/T	vector alone	0	[c]	[c]
		1	[c]	1.10
293/T	vector-mcyp7b	0	[c]	[c]
		1	0.38	0.01
		10	7.86	0.09
293/T	no plasmid	10	0.02	9.17

[a]Transfections were performed in duplicate; values are the means of two determinations.
[b]Vector: pcDNA3.1(+).
[c]Less than the detectable concentration of 0.01 μM.
[d]Vector-mcyp7b: pcDNA3.1(+) with mcyp7b coding sequence.

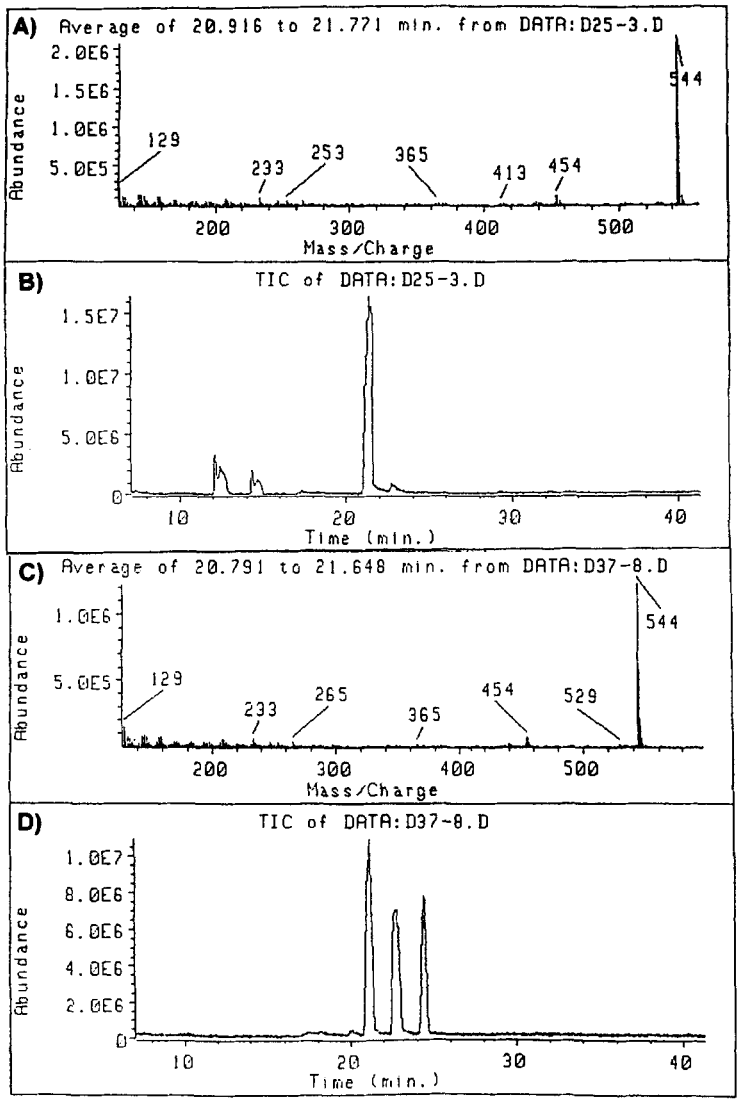

Fig. 1. GLC-MS analysis of 27-hydroxycholesterol and 7α-hydroxy metabolite, cholest-5-ene-3β,7α,27-triol. The bottom panel (D) indicates the retention time for 27-hydroxycholesterol (22.6 min) and both the 7α-hydroxy (21.0 min) and 7β-hydroxy (24.3 min) derivatives. The reduction of the relative retention time for 7α-hydroxy triol derivative compared to its precursor diol, the 7β triol epimer, and to other triols is a known characteristic, useful for identification (22). Analysis of the harvested medium (panel B) indicates a single metabolite with a retention time and mass spectrum (panel A) characteristic of authentic cholest-5-ene-3β,7α,27-triol (panel C) [molecular ion tri TMS ether = m/z 634 (418 + 72 × 3) − 90 (72 + 18) = 544]. Small peaks for coprostanol and cholesterol are also seen (panel B) at 12.3 and 14.3 min, respectively.

in these and other studies in which the medium had been enriched with cholesterol.

DISCUSSION

We (5) and others (6) previously reported the potent inhibitory effect of 27-hydroxycholesterol on cholesterol synthesis and the down-regulation of LDL receptor activity (13). Although the molecular mechanisms that regulate these biologic effects are areas of continuing investigation, considerable evidence has developed to indicate that "oxysterols" interrupt the production of short-chain sterol responsive element binding proteins (SREBPs) that normally enter the nucleus and upregulate transcription of genes governing these processes by binding to specific sequences on their promoter regions (14). Our findings indicate that the formation of a 7α-hydroxylated intermediate completely abolishes this potent biologic effect.

Because the sterol rings of cholesterol and 27-hydroxycholesterol are identical and 7α-hydroxylation occurs at precisely the same site in both compounds, it is reasonable to think that the mRNAs coding for the synthesis of the two enzymes must have close homology. Therefore, when a P450 enzyme from rat and mouse hippocampus was cloned and the cDNA was found to have the closest homology to the known sequence for cholesterol 7α-hydroxylase (7), we evaluated the full-length sequence for expression of an enzyme that might catalyze the 7α-hydroxylation of 27-hydroxycholesterol. In the transfection study shown we found that only cells transfected with mcyp7b metabolized 27-hydroxycholesterol to the 7α-hydroxy metabolite. The absence of 7α-hydroxycholesterol synthesis confirms our previous finding us-

ing the microsomal fraction of hamster liver that the two enzymes are different (1). The absence of 7β-hydroxy or 7-oxo products excludes possible autooxidation.

Thus, it is reasonable to conclude that the enzyme coded for by the cloned cDNA from mouse hippocampus catalyzes the 7α-hydroxylation of 27-hydroxycholesterol. However, although it may be an isoenzyme of that present in the liver, information regarding the homology of the two enzymes awaits further characterization of the liver enzyme.

The wide tissue distribution of both 27-hydroxycholesterol 7α-hydroxylase activity and the mRNA for cholesterol 27-hydroxylase (15) with documented expression of enzyme activity in vascular endothelium (11), macrophages (16), and ovary (17) in addition to liver imply considerable local regulation by C_{27} steroids. Our focus on the regulation of cholesterol synthesis does not preclude other biologic effects that are regulated by the C_{27} steroids generated subsequent to initial C_{27} hydroxylation. Although loss of a down-regulating effect on cholesterol synthesis by 7α-hydroxylation can be considered as part of a catabolic pathway, it may also be interpreted as autocrine regulation by intermediates formed locally in a variety of cells. Thus, the finding that interleukin 1B is a potent inducer of hydroxysterol 7α-hydroxylase activity in the ovary (18) can now be viewed as a local mechanism for increasing the availability of cholesterol for cell growth and/or hormone synthesis. However, until this group of C_{27} hydroxysteroids can be systematically studied and other regulatory effects identified, the presumption that it is a catabolic pathyway ending with the synthesis of bile acids in the liver is consistent with our current knowledge.

Because the activity of HMG-CoA reductase is not always a reliable indicator of cholesterol synthesis in cell culture (19, 20), we developed an isotope ratio technique using D_2O a number of years ago (8, 12). Basic to this technique was the demonstration that the incorporation of deuterium into cholesterol follows a random distribution predictable by the binomial expansion equation. These findings have been fully confirmed recently (21) and should encourage the use of D_2O for determining rates of synthesis for a variety of endogenous products.

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