Suppression of Bile Acid Synthesis, But Not of Hepatic Cholesterol 7α-Hydroxylase Expression, by Obstructive Cholestasis in Humans

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Regulation of bile acid synthesis, a key determinant of cholesterol homeostasis, is still incompletely understood. To elucidate the feedback control exerted on bile acid biosynthesis in humans with obstructive cholestasis, 16 patients with bile duct obstruction were studied. *In vivo* 7α-hydroxylation, reflecting bile acid synthesis, was assayed in 13 of them by tritium release analysis. Serum 27-hydroxycholesterol was determined by gas chromatography-mass spectrometry. In a subgroup, hepatic cholesterol 7α-hydroxylase mRNA was assayed by real-time polymerase chain reaction (PCR), enzyme activity was determined by isotope incorporation, and microsomal cholesterol content was assayed by gas chromatography–mass spectrometry. Age-matched control subjects were studied in parallel. Hydroxylation rates were lower in cholestatic patients (108 ± 33 mg of cholesterol per day, mean ± SEM; controls: 297 ± 40 mg/d; $P < .01$). The reduction was proportional to the severity of cholestasis, and synthetic rates were normalized in 4 subjects restudied after resolution of biliary obstruction. Consistent findings were obtained by analysis of serum 7α-hydroxycholesterol levels. On the other hand, hepatic cholesterol 7α-hydroxylase mRNA, microsomal enzyme activity, and cholesterol content tended to be increased in cholestasis. Finally, serum 27-hydroxycholesterol levels were slightly reduced in cholestatic subjects and were not related with the severity of the disease. Suppression of *in vivo* bile acid synthesis with no corresponding reduction in tissue 7α-hydroxylase expression and activity is consistent with nontranscriptional, posttranslational levels of regulation; these may play a role in the feedback control of bile acid synthesis in particular conditions. Alteration of the alternate biosynthetic pathway seems unlikely according to the present data. (Hepatology 2001;34:234-242.)

Degradation of cholesterol to bile acids is a major mechanism by which cholesterol is eliminated from the organism, and represents a key step in the maintenance of cholesterol homeostasis.[1-3] Hydroxylation at the 7α-position has long been considered the first and rate-limiting step in the metabolic pathway. Such reaction is catalyzed by the P450-dependent microsomal enzyme cholesterol 7α-hydroxylase (EC 1.14.13.17)[4]; the molecular structure of this enzyme has been characterized both in experimental animals and in humans.[1-3]

Regulation of the biosynthetic pathway and of cholesterol 7α-hydroxylase activity is largely dependent on a feedback mechanism exerted by bile acids recirculating to the liver. As clearly shown by different research groups including our own, hydrophobic bile acids are powerful suppressors of cholesterol 7α-hydroxylase expression and bile acid production, as determined in different experimental models[1-3,5-9] and in humans.[10-12] Such regulation is believed to take place mainly at the level of gene transcription; the presence of nuclear receptors for hydrophobic bile acids has recently been highlighted,[13,14] and the role of transcription factors acting on specific sequences of the cholesterol 7α-hydroxylase promoter is now being elucidated.[2,15]

In recent years, attention has also focused on an alternate biosynthetic pathway where side-chain oxidation, catalyzed by mithocondrial sterol 27-hydroxylase (EC 1.14.13.15), precedes 7α-hydroxylation of the sterol nucleus.[16,17] 27-Hydroxylation takes place largely in extrahepatic tissues. This pathway is believed to play a role particularly when the main metabolic pathway is compromised[18,19] or immature, as in neonatal life.[20] The regulatory effects of bile acids on hepatic 27-hydroxylase activity are less defined, in quantitative terms, than those operating on cholesterol 7α-hydroxylase activity[1-3,9]; the alternate pathway and its first committed step appear to be more responsive to cholesterol availability.[21] Regulation of the 7α-hydroxylation occurring on side-chain hydroxylated sterols, catalyzed by oxysterol 7α-hydroxylase[22] and possibly by cholesterol 7α-hydroxylase itself as recently suggested,[23] is also ill-defined.

In this articulated context, cholestasis has long represented an intriguing model in which increasing concentrations of bile acids in the liver did not always induce suppression of bile acid synthesis. Indeed, in the most common model of cholestasis, the bile duct–ligated rat, repeated observations have reported an unexpected increase of *in vitro* hepatic activity of cholesterol 7α-hydroxylase.[24,25] Evidence of the metabolic effects of cholestasis on the 27-hydroxylation pathway is missing; furthermore, studies in humans are extremely scarce, as a result of methodologic problems: the most common tech-

Abbreviations: RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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TABLE 1. Clinical and Biochemical Features of Patients Studied

Patient	Sex	Age	AST/ALT (U/L)	AP (U/L)	γ-GT (U/L)	Bilirubin (mg/dL)	Bile Acids (mmol/L)	Albumin (g/dL)	Score*	Duration of Cholestasis (wk)	Diagnosis
1	M	50	30/147	546	387	1.0	9	4.5	5	5	Chronic pancreatitis
2	M	51	75/128	852	648	8.3	88	3.9	12	5	Common hepatic duct cancer
3	M	56	64/104	480	779	7.0	114	3.4	11	6	Common bile duct stones
4	M	57	67/103	606	611	9.3	130	4.0	13	4	Pancreatic cancer
5	F	60	54/43	410	688	43.6	60	3.6	12	5	Pancreatic cancer
6	M	64	109/170	3563	4218	22.8	199	3.2	18	6	Common hepatic duct cancer
7	M	64	146/154	1759	365	17.9	190	3.5	16	6	Pancreatic pseudocyst
8	M	64	229/352	2486	445	9.3	78	3.8	13	4	Pancreatic cancer
9	M	68	44/156	287	640	7.3	40	3.6	9	3	Common bile duct stones
10	M	70	186/712	952	612	2.7	6	4.2	8	2	Common bile duct stones
11	F	74	88/179	781	416	3.6	175	3.6	10	6	Pancreatic cancer
12	F	77	151/174	468	433	0.8	4	4.3	3	2	Common bile duct stones
13	M	78	103/96	1579	683	6.9	80	3.5	14	6	Vater ampulla cancer
14	F	78	281/407	559	622	4.8	2	3.4	10	3	Common bile duct stones
15	M	79	177/219	2194	693	13.0	126	3.5	17	4	Common hepatic duct cancer
16	F	80	40/80	533	207	1.7	9	3.7	5	3	Common bile duct stones
17	F	82	273/209	1080	561	13.7	56	3.6	16	5	Pancreatic cancer
18	F	51	12/6	143	15	0.7	2	3.7			Colon cancer
19	M	62	14/11	126	33	0.9	3	4.1			Colon cancer
20	M	62	15/21	103	18	1.1	2	3.8			Peptic ulcer
21	M	70	30/28	227	43	1.2	7	3.5			Colon cancer
22	M	78	24/28	167	10	0.7	5	3.2			Gastric cancer
23	M	78	18/11	176	21	0.8	4	3.0			Colon cancer

Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; γ-GT, γ-glutamyl transpeptidase; AP, alkaline phosphatase.
*Score of cholestasis (see Table 2 for details).

niques to quantitate bile acid synthesis and turnover *in vivo* are derived from the isotope-dilution principle,[26] which relies on the assumption of homogeneous redistribution of exogenous bile acids; such a condition may not be fulfilled in conditions of deranged enterohepatic circulation such as cholestasis.

The aim of this study was to investigate the impact of cholestasis on bile acid synthesis *in vivo* and on hepatic expression and activity of cholesterol 7α-hydroxylase in human subjects with bile duct obstruction; the *in vivo* assay was performed using an isotope-release technique, developed by our research group, which does not require exogenous administration of labeled bile acids.

PATIENTS AND METHODS

Patients. Sixteen patients with a clinical and biochemical picture of extrahepatic obstructive cholestasis lasting for 2 to 6 weeks, admitted to the Medical or Surgical Departments of our institution, were investigated. The major clinical and biochemical features of the subjects are shown in Table 1 (patients 1 and 3 to 17). In the attempt to quantitate the severity of cholestasis, an arbitrary scoring system was devised using the biochemical markers that are commonly used as clinical indicators of the disease. This score ranged from 0 (normal) to 3 or 6 (maximal alteration) according to the parameter, as outlined in Table 2. A different weight was given to serum alkaline phosphatase as a sensitive marker and to bilirubin as a more specific indicator of cholestasis. Cholestasis severity could therefore be graded with a score ranging from 0 to 18. The duration of cholestasis was not included in the score both for the difficulty to obtain reliable information about the beginning of the cholestatic picture and in the assumption that it would in any way affect the other biochemical markers.

Physical and laboratory evaluation ruled out intestinal or thyroid disease, and none of the patients had shown clinical signs of liver disease before the onset of cholestasis. Three of them (patients 5, 6, and 8) had radiological or direct (laparotomic) evidence of metastatic liver disease, which was limited in extension and did not appear to be causally related with the onset of cholestasis. They received a standard diet, containing approximately 300 mg of cholesterol per day, adequate to keep their weight constant throughout the study. Patients gave their informed consent to the protocol of the study, which was approved by the local Ethical Committee and conducted according to the Declaration of Helsinki. Four subjects (patients 3, 6, 10, and 13) had the study *in vivo* repeated at least 1 month after resolution of cholestasis (either surgical [patients 6 and 13], endoscopic [patient 3], or spontaneous [patient 10]). Five subjects (patients 4, 5, 6, 8, and 17) undergoing abdominal surgery for the

TABLE 2. Scoring System for the Severity of Cholestasis

Biochemical Parameter		Score
Alkaline phosphatase	<250	0
(U/L)	250-500	2
	500-1,000	4
	>1,000	6
γ-Glutamyl transpeptidase	<50	0
(U/L)	50-500	1
	500-1,000	2
	>1,000	3
Serum bile acids	<10	0
(mmol/L)	10-50	1
	50-100	2
	>100	3
Bilirubin	< 2	0
(mg/dL)	2-4	2
	4-10	4
	>10	6

NOTE. Total score: range 0-18.

management of bile duct obstruction had a specimen of liver tissue taken during operation between 9 and 11 AM, close to the theoretical nadir of bile acid synthesis, for determination of 7α-hydroxylase expression and activity. Biopsy samples were free from metastasis. *In vivo* 7α-hydroxylation rates, determined in 13 of them (all patients except subjects 4, 5, and 8) were compared with those obtained in a control population of 21 subjects spanning the same age range (51-83 years of age), already characterized in detail.[27] The data on *in vitro* mRNA expression and activity of cholesterol 7α-hydroxylase were compared with those of 5 age-matched patients with preserved liver function, undergoing laparotomy for gastrointestinal cancer or complicated peptic disease (Table 1, patients 18, 19, 20, 21, and 23).

***Determination of 7α-Hydroxylation Rates* In Vivo.** The rates of sterol 7α-hydroxylation were assayed by the tritium release assay as described.[11,28] Briefly, trace amounts (200-350 μCi) of [7α-^{3}H]cholesterol (specific activity, 3-10 mCi/mmol) were injected intravenously after an overnight fast. Blood and urine samples were drawn at fixed intervals after tracer administration for 5 to 6 days. The amount of tritium released from the 7α position and joining the body water pool as [^{3}H]water reflects the rate of the 7α-hydroxylation process. This can be quantitated as the ratio between the increment of body water radioactivity, determined by scintillation counting of distilled urine samples, in a fixed time interval, and the mean specific radioactivity (radioactivity/mass ratio) of plasma cholesterol in the same interval (usually 60-72 hours after tracer). Hydroxylation rates were expressed as the amount of cholesterol ultimately undergoing 7α-hydroxylation per day (milligrams per day).[11,28] According to this biochemical approach, the assay quantitates the rate of tritium release from the 7α position regardless of the pathway involved; hydroxylation rates should thus reflect total bile acid synthesis *in vivo* deriving from both classical and alternate metabolic routes.

Analysis of [7α-^{3}H]Cholesterol Enrichment in Liver. This isotope-release technique relies on the assumption of homogeneous mixing of labeled cholesterol with the liver microsome pool.[11,28] To estimate the possible alterations induced by cholestasis on the equilibration process, separate studies were performed in 2 patients (patient 2, cholestatic; and patient 22, noncholestatic) scheduled to undergo surgery, who received tracer infusion 48 hours before laparotomy. Liver samples were obtained and homogenized; the specific radioactivity of free cholesterol in the isolated microsomal fraction and in serum, collected at the same time, were determined as described[11,28] and compared.

Analysis of Bile Acids in Serum. Total bile acids were assayed by standard enzymatic-colorimetric analysis (Enzabile, Nycomed Pharma AS, Oslo, Norway). The composition of the recirculating bile acid pool was determined on fasting serum samples by gas-liquid chromatography with 3α-7α-12-ketocholanoic acid as internal standard, as described,[29] with minor modifications: after thin-layer chromatography, methylated bile acids were extracted with ethyl acetate, acetylated at 0°C in ice by addition of acetic acid and acetic anhydride 7:5 (vol:vol), and acidified with perchloric acid 65% to 70%. After neutralization with NaCl 20%, the acetylated methyl ester derivatives were extracted with ethyl acetate and assayed using Carlo Erba Fractovap 4200 equipment.

Determination of Serum Levels of 27-Hydroxycholesterol and 7α-Hydroxycholesterol. Two hundred fifty nanograms of 19-hydroxycholesterol was added to 0.2 mL serum as internal standard; samples were hydrolyzed and extracted as described.[30] The organic phase was evaporated to dryness under nitrogen and purified by solid-phase extraction. The fraction containing hydroxysterols was eluted with 1.2 mL of toluene-ethyl acetate 2:3 (vol:vol), taken to dryness and treated with trimethylsilyl imidazole:piperidine 1:1 (vol:vol).

Gas-liquid chromatography–mass spectrometry analysis of samples was performed on Hewlett-Packard 5988 equipment in the MID mode monitoring ions at m/z 353 for the detection of 19-hydroxycholesterol and m/z 456 for 27-hydroxycholesterol and 7α-hydroxycholesterol. Calibration curves were prepared, spiking 0.2 mL serum with a fixed amount (250 ng) of internal standard and increasing concentrations (0-50 mg/dL) of the test sterol, and were treated and analyzed as the samples. Concentrations were calculated on the basis of the slope of the standard curve and on the peak area ratio for the tested ions (27-hydroxycholesterol or 7α-hydroxycholesterol/19-hydroxycholesterol) found in the sample. Values obtained in cholestatic patients were compared with the ones obtained in 20 age-matched subjects selected from a wider healthy normolipidemic population.

Assay of Cholesterol 7α-Hydroxylase Activity and of Microsomal Cholesterol Content. Hepatic cholesterol 7α-hydroxylase activity was assayed after isolation of the microsomal fraction by measuring incorporation of [^{14}C]cholesterol into [^{14}C]hydroxycholesterol.[4,31] Microsomal content of cholesterol, 7-oxocholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol were quantitated by gas-liquid chromatography–mass spectrometry with selected ion monitoring using 5α-cholestane as internal standard, as described.[30,32]

Assay of Hepatic Cholesterol 7α-Hydroxylase mRNA by TaqMan Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction. RNA was extracted from liver biopsy using a modification of the guanidinium-cesium chloride centrifugation technique.[33] Reverse-transcriptase (RT) reactions were performed on 3 μg of total RNA using a modification of a described technique.[34] TaqMan real-time quantitative polymerase chain reaction (PCR) assay was performed on a ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA) according to the manufacturer's protocol. Probes and primers used were designed by ABI Primer Express software and obtained from PE Biosystems UK (Warrington, Cheshire, UK). To avoid amplification of DNA sequences, the 7α-hydroxylase forward primer (5′ TGATTGGAAAAAATTTCACTTTGC 3′) was designed on exon 2; the reverse primer (5′ TCAGTGGTATTTCCATCCATCG 3′) was complementary to exon 3; and the 7α-hydroxylase target probe (5′ CATTTGGGCACAGAAGCATTGACCC 3′) was designed on exons 2-3 junction.

Probe was labeled at the 5′ end with the reporter dye molecule, FAM (6-carboxyfluorescein; emission λ 538 nm), and with the quencher, fluor TAMRA (6-carboxytetramethylrodamine; emission λ 582 nm) at the 3′ end. Amplification of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to standardize the amount of target cDNA. Relative quantitation of target cDNA was achieved using ABI PRISM 7700 SDS software according to the manufacturer's protocol.

Briefly, 0.5 μL of 30 μL of cDNAs synthesis mixture was amplified in triplicate for GAPDH in 1× TaqMan Buffer A (ABI TaqMan GAPDH Control Reagents kit). Likewise, 0.5 μL cDNAs was amplified in triplicate for 7α-hydroxylase in 1× TaqMan Buffer A (ABI

TaqMan PCR Core Reagents kit) supplemented with 900 nmol/L 7α-hydroxylase probe, and 900 nmol/L forward and reverse 7α-hydroxylase primers. Each mixture was transferred to a set of thermalcycler tubes. Target and internal controls were incubated at 50°C for 2 minutes and at 75°C for 10 minutes, and then cycled at 95°C for 15 seconds and at 60°C for 1 minute for 40 cycles using an ABI PRISM 7700 sequence detector. The increase in fluorescence (ΔRn) was proportional to the concentration of template in the PCR. PCR cycle number at the threshold line is represented as CT. The relative quantitation of target cDNA was determined as the ΔCT value by subtracting the average GAPDH CT value from the average 7α-hydroxylase CT value. Four validation experiments were performed according to the manufacturer's protocol to test the amplification efficiency and relative quantitation of target and reference. Finally, the relative quantitation of target was determined according to ABI protocols by elaborating and manipulating ΔCTs, allowing determination of the relative amount of 7α-hydroxylase mRNA.

Statistical Evaluation. When appropriate, data were expressed as the mean ± SEM, and the significance of differences was evaluated according to the Student *t* test for independent data. Because 7α-hydroxylation rates were found to decrease with aging,[27] we also aimed to rule out the possible confounding effects of age in the evaluation of differences between groups; therefore, analysis was also performed by ANOVA using age as the covariate, comparing the values obtained in cholestatic patients with those of the control population.[27]

Linear and nonlinear correlation analyses between the investigated parameters were performed by the least-squares method. Statistical analysis was conducted with the aid of the SPSS/PC statistical package on an IBM workstation. Significance was accepted at the $P < .05$ level.

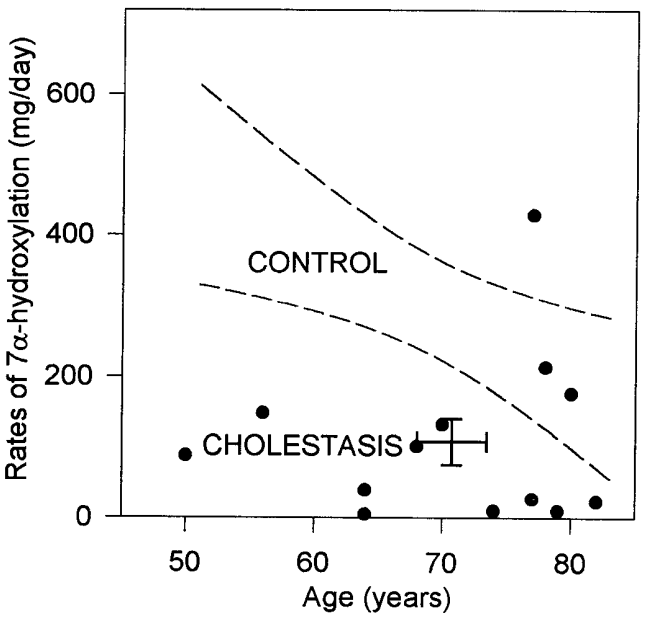

FIG. 1. Effect of obstructive cholestasis on the rates of 7α-hydroxylation. Individual data points of cholestatic patients are plotted vs. age; *error bars* indicate mean ± SEM for age and hydroxylation rates in this group. *Broken lines* indicate the confidence limits of the regression line relating age and 7α-hydroxylation in 21 control subjects[27]; equation of the regression line (mg/day vs. years): $y = 951.5 - 9.41x$ ($r = -.55$). $P < .01$ between control and cholestatic subjects, ANOVA with age as the covariate.

RESULTS

An average 64% reduction in 7α-hydroxylation rates was observed in the group with cholestasis (108 ± 33 mg/d vs. controls, 297 ± 40 mg/d; $P < .01$). In the attempt to minimize the potentially confounding effect of age on 7α-hydroxylation, statistical analysis was also performed using ANOVA with age as the covariate, as illustrated in Fig. 1: a significant ($P < .01$) difference was detected between the two groups, despite a wide data scatter in cholestatic patients.

The values of free cholesterol specific radioactivity in serum and in liver microsomes 48 hours after tracer infusion were as follows: patient 2 (cholestatic), serum: 11,072 dpm/mg; liver (right lobe): 10,509 dpm/mg; liver (left lobe): 12,310 dpm/mg. Patient 22 (noncholestatic), serum: 12,551 dpm/mg; liver: 10,022 dpm/mg. These findings appear to rule out relevant effects of cholestasis on the equilibration within the rapidly exchanging cholesterol pool in our experimental conditions. This is also consistent with preliminary evidence in the bile duct–ligated rat model (Bertolotti M, et al., unpublished data, January 2001).

To characterize the influence of disease severity, we investigated the correlation between serum markers of cholestasis and 7α-hydroxylation. When considered separately, total bile acids were the index showing the strictest correlation with hydroxylation rates ($r = -.63$, $P < .05$), as illustrated in Fig. 2A. A significant inverse correlation was also shown with total bilirubin ($r = -.50$, $P < .05$), whereas no significant relationship was observed with alkaline phosphatase, γ-glutamyl transpeptidase, and transaminases (data not shown). Figure 2B illustrates the correlation between the cholestatic score, calculated as described in Table 2, and the rate of hydroxylation; as expected, an inverse correlation of a higher degree was observed than with either biochemical parameter alone ($r = -.73$, $P < .01$). Similar results were obtained when the cholestasis score was calculated giving the different biochemical parameters the same weight (data not shown). Because the plots illustrated in Fig. 2 also appeared to follow a curvilinear pattern, nonlinear regression analysis was performed between the 7α-hydroxylation rate as the dependent variable, and the other parameters as the independent ones. Exponential and inverse equations were assayed, showing significant correlations (exponential: 7α-hydroxylation vs. bile acids, $r^2 = .60$, $P < .01$; 7α-hydroxylation vs. score, $r^2 = .55$, $P < .01$; inverse: 7α-hydroxylation vs. bile acids, $r^2 = .45$, $P < .05$; 7α-hydroxylation vs. score, $r^2 = .71$, $P < .01$).

Consistent results were obtained with the analysis of serum levels of 7α-hydroxycholesterol, which were significantly decreased in cholestatic subjects (3.2 ± 0.6 μg/dL vs. controls, 6.8 ± 1.0 μg/dL; $P < .01$). As shown in Fig. 3, concentrations of 7α-hydroxycholesterol were inversely correlated with the score of cholestasis and were directly correlated with the rates of *in vivo* 7α-hydroxylation. Serum levels of 27-hydroxycholesterol, the first committed metabolite of the alternate synthetic pathway, were slightly, but significantly, lower in cholestatic subjects (16.1 ± 1.1 μg/dL vs. controls, 22.4 ± 1.1 μg/dL; $P < .01$). As described in patients with cholestasis caused by primary biliary cirrhosis,[30] no correlation was observed between serum 27-hydroxycholesterol and total cholesterol in the cholestatic population ($r = .11$, $P =$ not significant), whereas the correlation was maintained in the control group ($r = .49$, $P < .05$). In cholestatic patients, serum 27-hydroxycholesterol was not correlated with the calculated cholestasis score ($r = .19$, $P =$ not significant) or with any of the biochemical indices of cholestasis considered separately (data not shown).

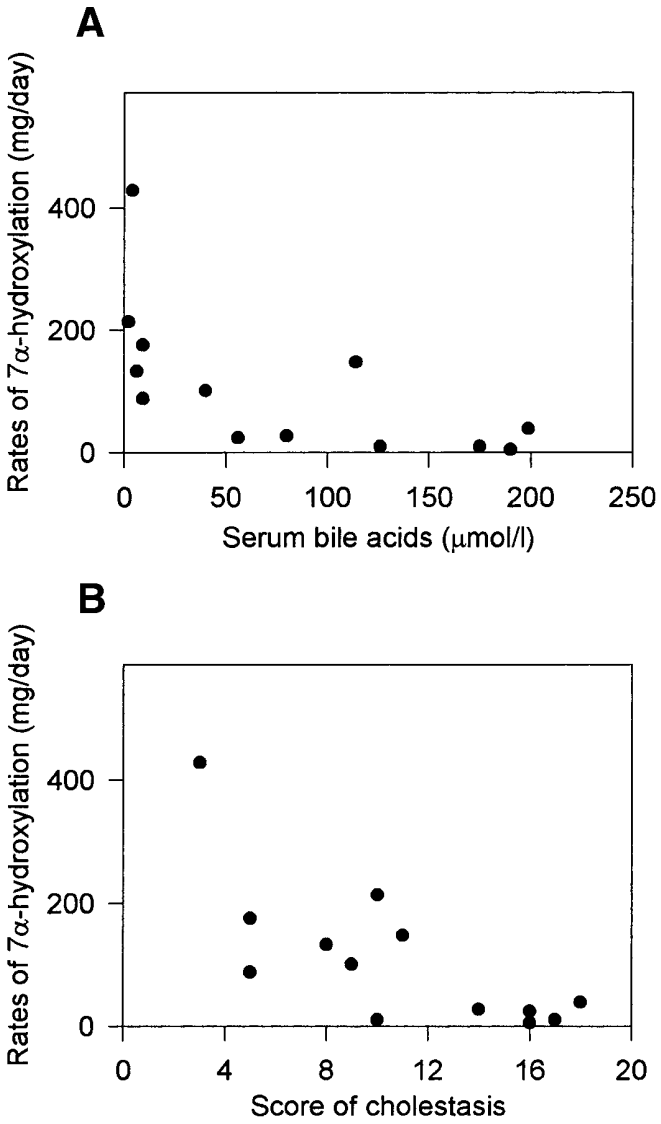

FIG. 2. (A) Individual data points for 7α-hydroxylation rates plotted vs. serum bile acid concentrations in 13 cholestatic patients. $r = -.63$, $P < .05$ by linear regression analysis. (B) Individual data points for 7α-hydroxylation rates vs. cholestasis score in the same subjects. $r = -.73$, $P < .01$ by linear regression analysis.

The ratio of cholic acid to chenodeoxycholic acid content in serum was higher in cholestatic subjects, compared with controls (cholestatic, 1.6 ± 0.9 vs. control, 1.0 ± 0.4; $P < .05$), with a direct correlation of borderline statistical significance with the severity of cholestasis ($r = .53$, $P = .065$), in agreement with previous data in patients with cholestasis of different etiologies.[35] Deoxycholic acid content was lower in cholestatic subjects (7% ± 1% vs. control, 21% ± 1%; $P < .01$).

In 4 patients, the *in vivo* study was repeated after clinical and biochemical resolution of the cholestatic picture. Such resolution was complete in patient 10 after spontaneous gallstone elimination and in patient 3 after endoscopic stone removal, and was only partial in patients 6 and 13 after surgical palliation with a bilio-digestive shunt. Figure 4 compares pre- and posttreatment findings in these subjects. As shown, 7α-hydroxylation rates markedly increase in all patients reinvestigated, reaching the range of noncholestatic subjects. Similarly, serum levels of 7α-hydroxycholesterol increased in 3 of the 4 subjects restudied (patient 3: 5.2 vs. 3.7 μg/dL; patient 6: 3.7 vs. 0.8 μg/dL; patient 13: 3.0 vs. 1.0 μg/dL), and slightly decreased in patient 10 (4.8 vs. 5.1 μg/dL). No change was detected when comparing pretreatment and posttreatment serum 27-hydroxycholesterol levels (data not shown).

In a subgroup of cholestatic subjects undergoing laparotomic surgery, specimens of liver tissues were analyzed for cholesterol 7α-hydroxylase mRNA and activity. Real-time RT-PCR technique was used to assay the former; because cholesterol 7α-hydroxylase mRNA for standard was not available to quantitate its absolute amount, the abundance of 7α-hydroxylase mRNA relative to GAPDH mRNA was used, as shown in Fig. 5A; even if statistical analysis was not per-

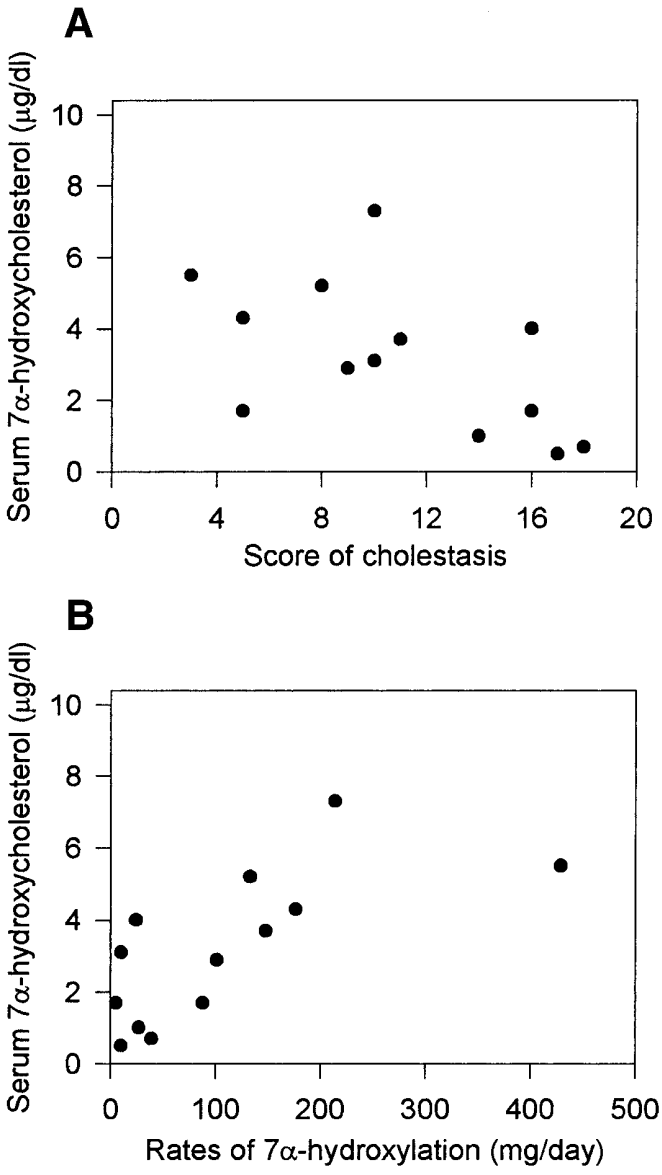

FIG. 3. (A) Individual data points for serum 7α-hydroxycholesterol concentration vs. cholestasis score in 13 cholestatic patients. $r = -.56$, $P < .05$ by linear regression analysis. (B) Individual data points for serum 7α-hydroxycholesterol concentration plotted vs. 7α-hydroxylation rates in the same subjects. $r = .70$, $P < .01$ by linear regression analysis.

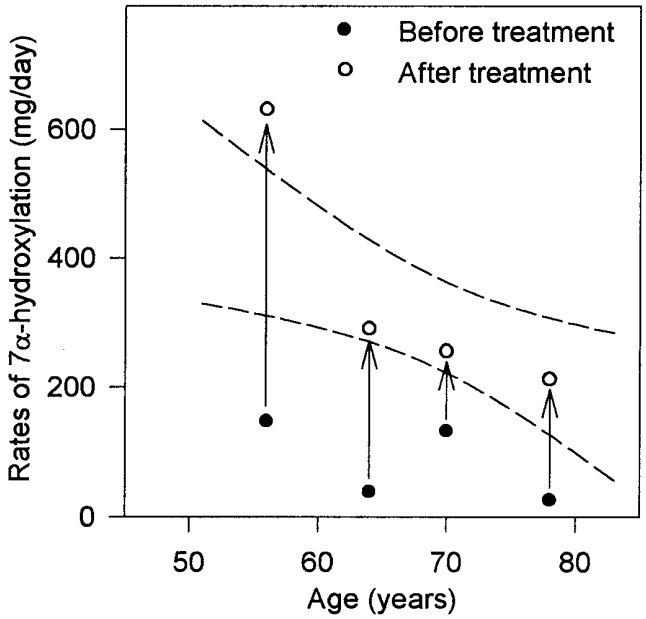

FIG. 4. Effect of resolution of cholestasis on 7α-hydroxylation rates in the 4 subjects reinvestigated. Individual data points for 7α-hydroxylation rates before and after resolution of cholestasis are plotted vs. age and against the confidence limits of the regression line of control subjects (see Fig. 1 for details).

formed because of the relatively small number of observations, mRNA levels tended to be higher in cholestatic subjects. Microsomal cholesterol 7α-hydroxylase activity, assayed by *in vitro* isotope incorporation, was more than doubled in cholestatic subjects, as illustrated in Fig. 5B. Most microsomal preparations were not adequate for determination of cholesterol 7α-hydroxylase activity by gas-liquid chromatography–mass spectrometry[32]; nevertheless, in two specimens from cholestatic subjects in whom this analysis could be performed, enzymatic activity was measurable, whereas in 2 control subjects, no activity was detectable (data not shown) at partial confirmation of the isotope incorporation data. No correlation could be detected between either cholesterol 7α-hydroxylase mRNA or activity and the other parameters of cholestasis, taken separately or as the composite score (mRNA vs. score of cholestasis: $r = .29$, P = not significant; enzyme activity vs. score of cholestasis: $r = .35$, P = not significant). This might be the result of the limited number of observations that were included in the analysis; furthermore, all such patients presented severe disease, and the rather narrow range of cholestasis score (12-18) made it difficult to disclose any correlation trend.

Microsomal cholesterol content was also increased in samples from 4 cholestatic patients (274 $\pm$ 18 nmol/mg protein) compared with 4 control subjects (123 $\pm$ 13 nmol/mg protein). No difference was detected in microsomal content of 7-oxocholesterol and 7β-hydroxycholesterol (data not shown), whereas 25-hydroxycholesterol and 27-hydroxycholesterol were undetectable in all samples.

DISCUSSION

Experimental cholestasis has long represented an intriguing challenge to the classical view of feedback regulation of bile acid synthesis.[24,25] As an attempt to investigate this controversial aspect in humans, we studied a group of patients with bile duct obstruction, a relatively pure model of cholestasis, and no other stigmata of liver dysfunction. The analysis of serum bile acids confirmed a pattern typical of isolated cholestasis, with increased cholic:chenodeoxycholic acid ratio,[35] suggesting normal biosynthetic function. Finally, the increase of hydroxylation rates after resolution of cholestasis is consistent with the absence of permanent liver damage. Bile acid synthesis *in vivo* was assayed by a previously described isotope-release technique,[28] which detects the rate of sterol 7α-hydroxylation deriving from both classical and alternate pathways; this technique involves intravenous administration of [7α-^{3}H]cholesterol, which was shown to quickly equilibrate with the microsomal compartment. The absence of exogenous bile acid administration theoretically makes it an optimal approach in conditions of deranged enterohepatic circulation.

Our experimental data show a significant inhibitory effect of cholestasis on bile acid production *in vivo*, independent of age (Fig. 1) and related to the severity of cholestasis (Fig. 2). Although the number of observations is limited, nonlinear regression analysis of the experimental data shows a curvilin-

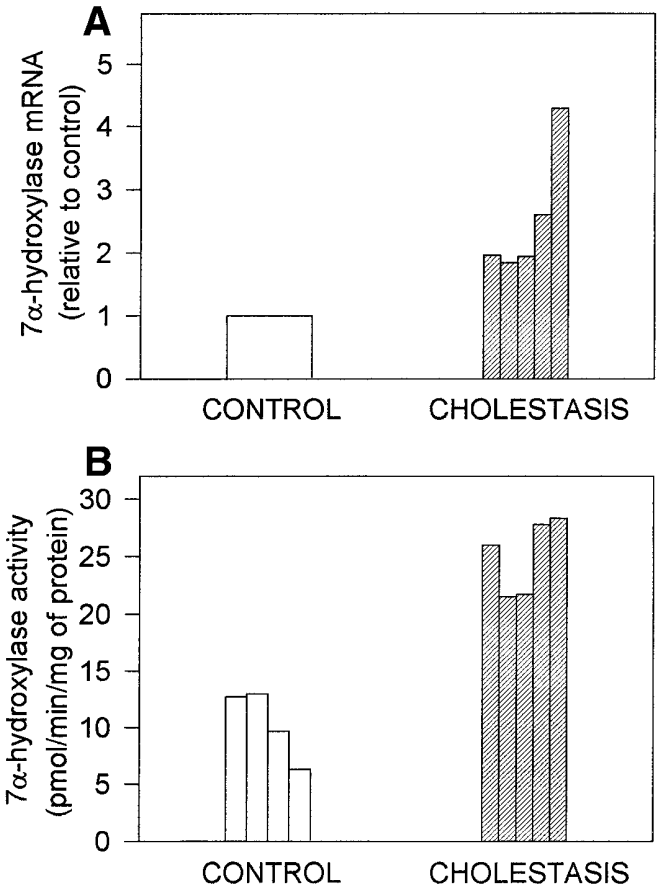

FIG. 5. (A) Relative amount of cholesterol 7α-hydroxylase/GAPDH mRNA content assayed by real-time RT-PCR, expressed as a multiple of the control value. The first column represents the mean (normalized as 1) from 4 values in control subjects (patients 18, 20, 21, and 23). The following *bars* represent individual values from 5 cholestatic subjects. (B) Cholesterol 7α-hydroxylase activity, assayed by isotope incorporation and expressed as picomoles of 7α-hydroxycholesterol formed per minute per milligram of protein. Columns represent individual values from 4 control (patients 18, 19, 20, and 23) and 5 cholestatic subjects.

ear trend. This suggests a threshold effect of cholestasis on 7α-hydroxylation, which might be related to accumulation of bile acids in the liver cell. Serum levels of 7α-hydroxycholesterol, another indicator of bile acid synthesis in humans,[36,37] were also reduced in cholestatic subjects and correlated with disease severity (Fig. 3). Finally, this inhibition seems to be reversible (Fig. 4). Surprisingly, the opposite trend was shown when cholesterol 7α-hydroxylase mRNA and activity were determined in liver samples *in vitro*, as shown in Fig. 5. The literature regarding this is rather scarce and conflicting. Findings in animal models different from the rat are consistent with reduced bile acid synthesis in experimental cholestasis.[38,39] We found direct evidence obtained in human subjects on the effect of obstructive cholestasis on bile acid synthesis *in vivo*; previous reports have investigated indirect markers of bile acid formation, such as serum 7α-hydroxycholesterol[36] and urinary excretion of bile acids and alcohols,[40,41] with results consistent with ours. A report by Salen et al.[42] showed a decrease in the *in vitro* activity of hepatic cholesterol 7α-hydroxylase in patients with bile duct obstruction, in agreement with our findings *in vivo*, but not with our data on enzyme activity. Our findings *in vitro* resemble the ones previously obtained in the rat,[24,25] in which regulation of cholesterol homeostasis is dissimilar from that observed in most species including humans.[43] The bile acid pool of the rat is very hydrophilic, and the hydrophilicity further increases in experimental cholestasis[44]; this might lead, in a situation in which cholesterol synthesis is not repressed,[45] to an increase in bile acid synthesis. The physical-chemical pattern of the recirculating pool in cholestatic patients, with an increased cholic:chenodeoxycholic acid ratio and a reduced content in deoxycholic acid, is also characterized by higher hydrophilicity.[35] This *per se* might be consistent with the observed increase in 7α-hydroxylase expression and activity.

Previous data[46] showed that intravenous infusion of bile acids, in contrast with intraduodenal administration, fails to suppress hepatic cholesterol 7α-hydroxylase in the rat; this suggests an inhibitory role of factor(s) released by the small bowel in the presence of bile acids.[47] Such inhibition could be lost in cholestasis. Finally, the increased 7α-hydroxylase expression might reflect a situation of liver regeneration that is likely to take place in several models of hepatic injury including cholestasis.[48]

At any rate, inhibitory control beyond gene transcription and enzyme translation must take place to account for the findings in our human model. In experimental animals, post-translational regulation of bile acid synthesis was already shown to play an important role.[49] In this context, some reports have shown parallel regulation of 7α-hydroxylase activity and mRNA levels,[6,50] whereas in other experimental models changes in cholesterol 7α-hydroxylase mRNA did not always reflect the corresponding changes in enzyme activity.[7]

These findings, as well as others, raise the possibility that competitive inhibition can also account for changes in the rate of bile acid synthesis. Interestingly, when rats are treated with oxysterols such as 7-oxocholesterol, a competitive inhibitor of cholesterol 7α-hydroxylase, bile acid synthesis is reduced, but *in vitro* 7α-hydroxylase activity and mRNA are up-regulated.[51] We believe that our experimental evidence may be consistent with a similar mechanism; this might be exerted on cholesterol 7α-hydroxylase itself by bile acids and/or other sterol derivatives accumulating in the liver during cholestasis. We could not detect any increase in 7-oxocholesterol and 7β-hydroxycholesterol in our microsomal samples, but we cannot exclude the role of hydrophilic compounds that might be lost during the preparation of microsomes. Up-regulation of 7α-hydroxylase expression and activity could be secondary to reduced production of bile acids or to a direct stimulation by cholesterol or other side-chain–hydroxylated derivatives, which were shown to activate cholesterol 7α-hydroxylase via the liver X receptor α (LXRα) pathway in rodents.[52] The finding of increased microsomal cholesterol content is in line with this view.

Even if this speculation cannot account for all discrepancies encountered in the literature, our data underline the possibility that transcriptional regulation of the cholesterol 7α-hydroxylase gene may not be the only determinant of bile acid synthesis, at least in some conditions. This would also support evidence in humans showing a discrepancy between indices of bile acid synthesis *in vivo* and 7α-hydroxylase expression and activity *in vitro*, as in obesity[53,54] or during deoxycholic acid treatment.[11,12,55] The finding of more pronounced suppression, or lack of increase, of bile acid synthesis *in vivo* compared with the determination of tissue expression of the enzyme is consistent with regulation beyond enzyme transcription and translation.

The control and physiologic roles of the alternate pathway of bile acid synthesis are still a matter of debate. In this report, circulating levels of 27-hydroxycholesterol were slightly decreased in cholestatic patients, but did not show any correlation with the severity of liver disease and did not increase after resolution of cholestasis. It is therefore possible that the reduction in serum 27-hydroxycholesterol may reflect reduced peripheral conversion of cholesterol to its hydroxylated derivative, as postulated for intrahepatic cholestasis,[30] rather than a change in hepatic 27-hydroxylation. The finding is consistent with recent evidence in the rat with estrogen-induced cholestasis.[56] Other reports in the animal also suggest that hepatic 27-hydroxylation is less affected by bile acids, compared with cholesterol 7α-hydroxylation, and is more sensitive to cholesterol availability as a mechanism of regulation.[9,21] Finally, the alternate pathway was shown to account for less than 10% of bile acid production in adult humans.[57] These findings altogether seem to exclude that modifications in the alternate pathway may contribute significantly to the changes in bile acid synthesis observed in our cholestatic patients.

As a final comment, we cannot rule out the presence of an additional biosynthetic pathway that might include a distinct 7α-hydroxylating enzyme, as suggested by recent evidence,[58] or the occurrence of changes in other hydroxylation steps, such as 12α-hydroxylation,[59] which were not analyzed in this study.

Further investigation regarding putative inhibitory compounds of cholesterol 7α-hydroxylase, the regulatory role of factors deriving from the enterohepatic circulation, and the physiologic role of the alternate pathway(s) of bile acid synthesis on hepatic and extrahepatic cholesterol degradation, together with further insight into the molecular control of 7α-hydroxylase, will hopefully help to clarify the regulation of hepatic and whole-body cholesterol homeostasis in humans in the near future.

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