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Isotopomer spectral analysis of intermediates of cholesterol synthesis in patients with cerebrotendinous xanthomatosis

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Abstract

Four patients with cerebrotendinous xanthomatosis (CTX) and 2 healthy controls received a constant proximal intraduodenal infusion of 1-^{13}C-acetate as a stable-isotope–labeled marker of sterol synthesis. One patient was treated with pravastatin (20 mg twice daily) and another patient with chenodeoxycholic acid (250 mg tid). Every hour, venous blood and duodenal samples were obtained. Stable-isotope enrichment of neutral and polar sterols in serum and bile was assessed by gas chromatography/mass spectrometry. Isotopomer spectral analysis was performed on cholesterol, lathosterol, Δ-8-cholestenol, methylsterol, and lanosterol. Stable-isotope labeling of cholestanol, bile acids, and bile alcohols was analyzed by assessing the change over time of the ratio of M + 3 to M + 0. Eleven hours after marker infusion, we found up to 50% newly synthesized lathosterol in serum and up to 80% in bile, with similar results for other cholesterol precursors. In cholesterol, stable-isotope labeling could be demonstrated in all study subjects with a more prominent labeling in bile than in serum. No stable-isotope labeling was detected in cholestanol. Only minor stable-isotope incorporation was detectable in polar sterols in some subjects. Therapy with pravastatin did not have any effect on fractional or absolute synthesis rates or on the concentrations of cholestanol or cholesterol precursors compared to untreated patients with CTX. In contrast, therapy with chenodeoxycholic acid markedly lowered the concentrations of cholestanol and cholesterol precursors, led to a disappearance of bile alcohols, and reduced absolute synthesis rates of lathosterol. Isotopomer spectral analysis proved to be a powerful method to assess the endogenous synthesis of cholesterol precursors in patients with CTX. Higher fractional synthesis in bile than in serum may be due to the size of the pools in bile vs serum. Cholestanol exhibits no marker uptake and is therefore probably synthesized from preformed cholesterol. Biliary cholesterol secretion in patients with CTX is decreased compared to healthy controls.

1. Introduction

Cerebrotendinous xanthomatosis (CTX) is a rare recessively inherited lipid storage disease first described by van Bogaert et al [1]. Clinical characteristics include deposits of cholesterol and cholestanol in tendons, other tissues, and brain in conjunction with progressive neurologic and mental dysfunction. The basic defect has been localized to a mutation on chromosome 2 of the gene encoding sterol-27α-hydroxylase [2], a mitochondrial cytochrome P-450 enzyme that catalyzes the first step of the degradation of the sterol side chain in bile acid biosynthesis. The impaired function of this enzyme leads to a marked reduction of bile acid synthesis [3,4] with a subsequent up-regulation of 7α-cholesterol-hydroxylase, the essential enzyme in bile acid synthesis, followed by a marked increase in endogenous cholesterol biosynthesis. As a consequence, patients with CTX have markedly elevated serum concentrations of bile acid intermediates such as 7α-hydroxy-4-cholesten-3-one [2] and multiple-fold hydroxylated sterol derivatives (bile alcohols), as well as cholesterol precursors and cholestanol [4,5]. It has also been demonstrated that patients with CTX exhibit a high serum concentration of cholesterol precursors

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such as lathosterol [5], which is a marker of endogenous cholesterol synthesis [6-8]. Recently, it has also been shown that urinary excretion of mevalonic acid is markedly elevated in patients with CTX [9].

In 1998, a mouse model with a disrupted gene encoding sterol-27α-hydroxylase (Cyp27) was made available by Rosen et al [10]. As expected, these mice exhibit higher plasma levels of lathosterol and 7α-hydroxycholesterol, and also higher hepatic levels of 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA)-reductase messenger RNA than wild-type mice, indicating an increased endogenous synthesis of cholesterol and an up-regulation of bile acid biosynthesis. Interestingly, $Cyp27^{-/-}$ mice do not develop the clinical features of CTX. Only traces of bile alcohols can be found in their bile and feces, and they do not accumulate cholestanol. It was subsequently demonstrated that microsomal 25- and 26-hydroxylations of 5β-cholestane-3α,7α,12α-triol and microsomal 23R-, 24R-, 24S- and 27-hydroxylations of 5β-cholestane-3α,7α,12α, 25-tetrol are mainly catalyzed by Cyp3A, both in mice and in human beings. In contrast to patients with CTX, $Cyp27^{-/-}$ mice exhibit a marked up-regulation of Cyp3A in response to the accumulation of 5β-cholestane-3α,7α,12α-triol, whereas the up-regulation of sterol-7α-hydroxylase is less pronounced than in patients with CTX. This protects the $Cyp27^{-/-}$ mice from the accumulation of bile acid intermediates and the phenotype seen in patients with CTX [11]. The underlying mechanism seems to be an induction of Cyp3A, which is mediated through the nuclear receptor pregnane X receptor upon activation by 5β-cholestane-3α,7α,12α-triol [12,13]. Unlike in mice, 5β-cholestane-3α,7α,12α-triol is not an effective activator of pregnane X receptor in human beings, thus explaining why patients with CTX do not exhibit a protective up-regulation of Cyp3A and are therefore stricken with the severe clinical manifestations of CTX.

The extent of endogenous synthesis of cholesterol has been quantified in patients with CTX by the fecal balance method [3]. Recently, methods were developed, which allow to assess the kinetics of endogenous synthesis of polymeric compounds such as cholesterol, fatty acids, proteins, or DNA in vivo: mass isotopomer distribution analysis [14] and isotopomer spectral analysis (ISA) [15]. Both methods involve the continuous application of a stable-isotope–labeled monomeric precursor of the compound to be investigated. Stable-isotope enrichment of the polymeric synthesis product is assessed from different compartments (eg, plasma) by gas chromatography/mass spectrometry (GCMS) and compared to a computer-generated model of the expected mass isotopomer pattern in relation to precursor pool enrichment of the labeled precursors. This allows the calculation of fractional precursor enrichments of the respective precursor pool (the ratio of stable-isotope–labeled precursors to all precursors that have access to the endogenous synthesis pathway of the investigated polymeric substance) and fractional synthesis (the ratio of newly synthesized polymeric substance to the total substance pool).

The objective of this study was to investigate the endogenous synthesis of cholesterol and its precursors with ISA in healthy controls and in patients with CTX, either with or without drug treatment. Several cholesterol precursors were investigated for the first time with this method. The extent of stable-isotope labeling of cholesterol precursors, bile acids, and bile alcohols was assessed in this study to clarify the metabolic origin and turnover of these sterols. Furthermore, we determined biliary secretion rates of cholesterol in all subjects by the duodenal perfusion method.

2. Patients and methods

The study was conducted in 4 patients with documented CTX at the university hospital of Siena, Italy. Two healthy male volunteers served as controls and underwent the study at the Department of Clinical Pharmacology at the University of Bonn, Germany. Clinical data of all subjects are given in Table 1. Cerebrotendinous xanthomatosis patient 3 was under treatment with pravastatin 20 mg twice daily, whereas patient 4 with CTX was treated with chenodeoxycholic acid (CDCA) 250 mg tid. Measurements of biliary lipid secretion were performed by the method of Grundy and Metzger [16], as described previously [17,18]. Briefly, in the evening before the study, the subjects were admitted to the hospital and swallowed a triple-lumen tube. The next morning, the tube was positioned by x-ray guidance in the duodenum with the 2 most proximal outlets opposite to the ampulla of Vater and the third 10 cm distally. Gallbladder contraction was performed by intravenous injection of cholecystokinin (0.5 IU/kg body weight), and bile was aspirated from the tube adjacent to the Papilla Vateri. After vigorous shaking, an aliquot of 3 mL was transferred to a vial containing 0.5 mL isopropanol, and the remaining bile was reinfused into the distal lumen. Thereafter, a liquid formula diet (Nutrison, R. Braun, Melsungen, Germany) was infused constantly into the most proximal outlet (1.42 kcal/kg per hour). As a nonabsorbable marker for biliary cholesterol secretion, we added 5 mg of sitostanol to 100 mL of the liquid formula. In addition, 1-^{13}C-acetate (Isotec Inc, Miamisburg, Ohio), as a stable-isotope–labeled monomeric precursor of the polymeric cholesterol molecule, diluted in isotonic saline solution at

Table 1
Clinical profile of patients with CTX and controls

Patients	Age (y)	Sex (m/f)	Body weight (kg)	BMI (kg/m^2)
1	46	f	75	27.5
2	40	f	55	21.0
3	39	m	58	20.1
4	46	m	65	22.5
Controls				
1	26	m	68	21.5
2	27	m	75	22.6

BMI indicates body mass index (body weight/height).

a concentration of 1 mg/mL, was infused through the same proximal site at a constant rate of 1 mg/kg body weight. The infusion period lasted for a total of 11 hours. After starting the infusion of the liquid formula and ^{13}C-labeled acetate (t = 0 hour), constant aspiration of intestinal contents was started from the second distal and third outlet of the tube at a constant rate of ~20 mL/h. Three milliliters of the aspirates were filled into prepared vials containing 0.5 mL of isopropanol. Again, the rest was reinfused into the distal lumen to maintain the enterohepatic circulation. The vials were then closed, shaken vigorously, and stored at −20°C until analysis.

Blood samples were drawn from a venous catheter placed into a forearm before starting the liquid formula infusion and every hour during the study. Blood samples were centrifuged after clotting to obtain the serum that was prepared for storage by adding 2,6-di-*tert*-butyl-4-methyl-phenol (butylated hydroxytoluene) in methanol in a concentration of 100 μg/5 μL. This solution was used to prevent autoxidation of sterols in the samples. Thereafter, serum samples were stored at −20°C until analysis. Sterols from serum samples (100 μL) were extracted in hexane/isopropanol (3:2, vol/vol; 4 mL) after adding 50 μg of 5α-cholestane and 1 μg of epi-coprostanol as internal standards to each sample. After drying under nitrogen and an alkaline hydrolysis (1 N NaOH in 80% ethanol, 1 h at 67°C), neutral sterols were extracted with cyclohexane (twice with 3 mL). After drying under nitrogen, sterols were dissolved in 80 μL of *n*-decane, transferred to GCMS conical glass vials and converted to their trimethylsilyl derivatives (TMSs) by adding 25 μL of a mixture of pyridine (dry), hexanemethyldisilazane, and trichlormethylsilane (9:3:1, vol/vol) (Merck, Darmstadt, Germany). One to two microliters were used for GCMS. For the analysis of cholesterol, the final volume after derivatization was diluted 1/30 with *n*-decane and then another 10 μL of TMS agent was added.

Both neutral and acidic sterols and bile alcohols from duodenal aspirate samples (300 μL) were extracted after adding 50 μg of 5α-cholestane as internal standard for neutral sterols and 50 μg hyodeoxycholic acid as standard for bile acids and bile alcohols. An alkaline hydrolysis was performed (1 N NaOH in 80% ethanol, 1 hour at 67°C) and neutral sterols were extracted with cyclohexane (three times with 3 mL). After drying under nitrogen, neutral sterols were dissolved in 70 mL of *n*-decane, transferred to GCMS conical glass vials and converted to their TMSs by adding 25 μL of the TMS agent.

Bile acids and bile alcohols were extracted by performing a strong alkaline hydrolysis (10 N NaOH, 3 hours at 110°C). After cooling to room temperature, the pH value was brought below 1.5 using 1 to 1.2 mL of concentrated hydrogen chloride. Bile acids and alcohols were extracted with diethylether (twice with 4 mL). After drying under nitrogen, bile acids were methylated by adding methanol (2 mL), dimethoxypropane (1.4 mL), and concentrated hydrogen chloride (10 μL) and stored at room temperature for at least 12 hours. Thereafter, samples were dried under nitrogen and dissolved in 80 μL of *n*-decane. After centrifugating at 2000 rpm for 4 minutes, the clear solution was transferred to a conical GCMS glass vial and 6 μL of TMS agent was added.

Analyses of neutral sterols were performed on a Hewlett-Packard GCMS system (5890 combined with a 5972 mass selective detector; Hewlett-Packard, Wilmington, Del)

Table 2
Sterol concentrations and their ratio to cholesterol in serum in patients with CTX and controls

	Number of samples	Cholesterol	Δ-8-Cholestenol (mg/dL) (mean ± SD)	Lathosterol	Cholestanol
Patients					
1	19	101 ± 14	1.12 ± 0.25	0.50 ± 0.10	1.15 ± 0.17
2	16	131 ± 11	1.76 ± 0.17	0.68 ± 0.06	3.70 ± 0.28
3	19	90 ± 13	0.77 ± 0.26	0.45 ± 0.08	1.40 ± 0.26
4	18	102 ± 30	0.03 ± 0.01	0.06 ± 0.01	0.36 ± 0.08
Controls					
1	14	129 ± 24	0.18 ± 0.11	0.13 ± 0.03	0.35 ± 0.06
2	15	153 ± 10	0.37 ± 0.31	0.12 ± 0.01	0.39 ± 0.21
			Ratio to cholesterol (μg/mg)		
			Δ-8-Cholestenol	Lathosterol	Cholestanol
Patients					
1			11.05 ± 1.66	4.96 ± 0.70	11.34 ± 0.38
2			13.45 ± 0.92	5.23 ± 0.27	28.30 ± 0.69
3			8.63 ± 2.60	5.07 ± 0.77	15.53 ± 1.12
4			0.28 ± 0.10	0.61 ± 0.07	3.57 ± 0.43
Controls					
1			1.43 ± 0.89	0.99 ± 0.09	2.72 ± 0.37
2			2.44 ± 2.04	0.80 ± 0.07	2.54 ± 1.35

Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid).

equipped with a DB-XLB (50 m × 0.25 mm id × 0.25-μm film) for analysis of serum samples of CTX patients (or a DB-XLB [30 m × 0.25 mm id × 0.25 μm film] for analysis of all other samples). Samples were injected at an initial temperature of 150°C for 1 minute, followed by 30°C/min up to 290°C. After the elution of lathosterol, the electron multiplier voltage was raised by 300 for the measurement of lanosterol and methylsterol to increase the sensitivity for these sterols. For the ISA, M + 0 to M + 10 ions were collected for every sterol using a program with equal dwell time for each isotopomer. Single ion monitoring was performed on ions specific for the TMS derivatives of the following sterols: cholesterol (458-468 m/z), cholestanol (460-470 m/z), Δ-8-cholestenol (458-468 m/z), lathosterol (458-468 m/z), methylsterol (472-482 m/z), and lanosterol (498-508 m/z). The internal standard 5α-cholestane was measured at 372 m/z. Peak integration was performed with computer assistance after a manual definition of the peak base line.

Bile acids were measured with identical equipment and temperature settings. Single ion monitoring was performed on TMS-derivative ions specific of the following sterols: chenodeoxycholic (370-380 m/z) and cholic acid (368-378 m/z). Again, peak integration was performed manually. Bile alcohols were measured in the scan modus first to identify peaks by their typical mass spectra [19]. Single ion monitoring was then performed on selected TMS derivative ions specific for the identified sterols.

Cholesterol, Δ-8-cholestenol, lathosterol, methylsterol, and lanosterol were analyzed to calculate fractional precursor enrichment (D) and fractional synthesis at specific times, t, [$g(t)$] as described previously [15,20] using a nonlinear regression method [15]. The fractional precursor enrichment (D) represents the ratio of labeled precursors to the total precursor pool that has access to the endogenous sterol synthesis pathway. The fractional synthesis [$g(t)$] is the ratio of newly synthesized sterol to the total respective sterol pool at specific times (t) after the beginning of stable-isotope marker application. To convert this ratio into a true rate of synthesis, the changes in $g(t)$ values with time were used to solve for the rate constant using the expression $g(t) = G\,(1 - e^{-kt})$. Nonlinear regression, Levenberg-Marquardt algorithm, was used to estimate k from a set of $g(t)$ and t values as described previously [20]. For cholesterol synthesis intermediates such as lathosterol, G was set = 1. Setting G = 1 assumes that all lathosterol appearing in serum was derived from de novo synthesis. To obtain an estimate of the absolute synthesis rate, k is multiplied by the pool size. Here we estimated synthesis per deciliter and thus k was multiplied by the concentration milligrams per deciliter. Because we observe cholesterol intermediates in the plasma, the absolute rate of synthesis here actually refers to the rate of appearance in plasma. These rates were corrected for cholesterol by calculating the ratio to the plasma concentration of cholesterol at the respective time points.

Polar sterols (bile acids and alcohols) and cholestanol were analyzed by calculating the ratio between M + 3 or M + 4 and M + 0 for each measured sterol TMS derivative. Isotopomer spectral analysis was not performed in these sterols because the stable-isotope enrichment turned out to be too small to yield valid results.

Table 3
Ratios of sterols to cholesterol in gallbladder bile in patients with cerebrotendinous xanthomatosis and controls

Ratio to cholesterol (μg/mg)	Patients				Controls	
	1	2	3	4	1	2
Δ-8-Cholestenol	82.47	41.70	38.44	1.03	2.27	1.14
Lathosterol	30.20	17.91	21.78	4.24	7.03	4.61
Cholestanol	49.34	113.40	82.64	16.72	8.52	7.52

Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid).

Table 4
Biliary cholesterol secretion in patients with CTX and controls

	Patients				Controls	
	1	2	3	4	1	2
Cholesterol (mg/h)	42.8 ± 5.6	15.8 ± 10.8	31.5 ± 9.7	34.6 ± 9.8	63.7 ± 10.9	66.6 ± 14.1

Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid).

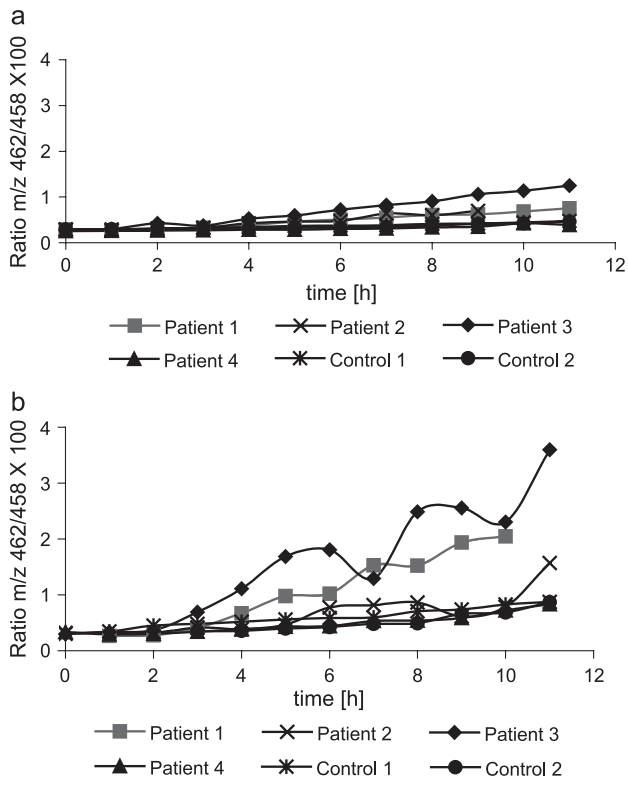

Fig. 1. a, Ratio of m + 4 to m + 0 of cholesterol in serum of patients with CTX and controls. Patient 3 received pravastatin (20 mg twice daily) and patient 4 was treated with CDCA (250 mg tid). b, Ratio of m + 4 to m + 0 of cholesterol in bile of patients with CTX and controls. Patient 3 received pravastatin (20 mg/d twice daily) and patient 4 was treated with CDCA (250 mg tid).

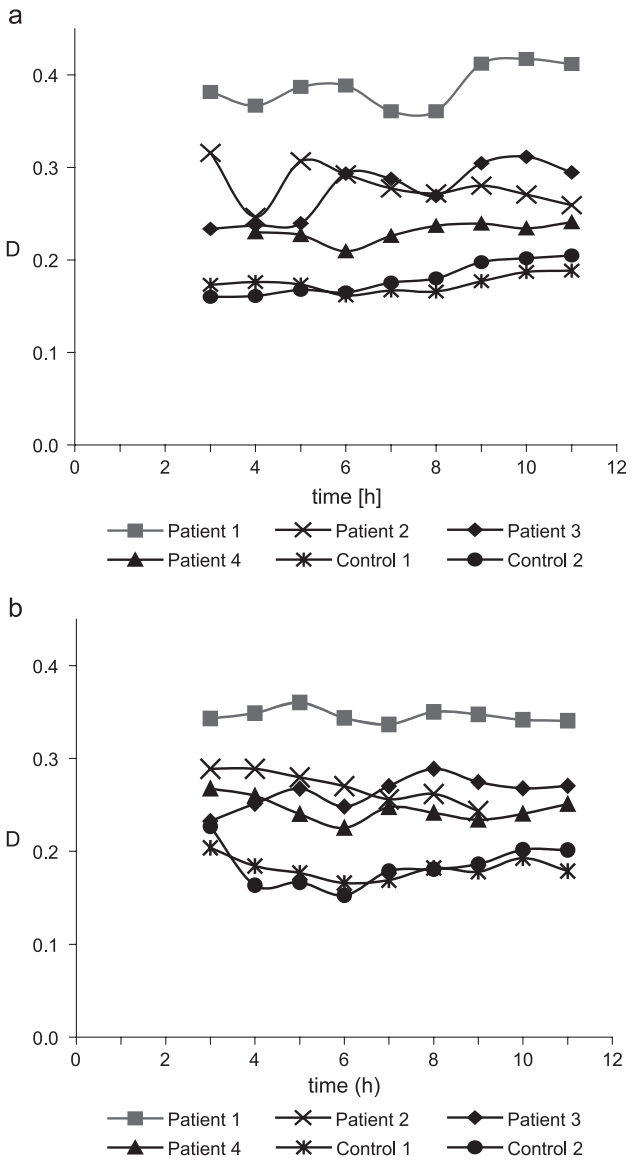

Fig. 2. a, Fractional precursor enrichment (D) of lathosterol in serum of patients with CTX and controls. Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid). b, Fractional precursor enrichment (D) of lathosterol in bile of patients with CTX and controls. Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid).

To quantify the serum and duodenal aspirate concentrations of cholestanol, cholesterol and its neutral precursors, we used a gas chromatograph with flame ionization detector (GC-FID) by Hewlett-Packard (Model 6890) equipped with a Model 7683 automatic sample holder and injector and a DB-XLB (30 m × 0.2 mm id and 0.33-μm film). Peaks were identified by their typical retention times and integration was performed as described above. Concentrations were calculated with the internal standards 5α-cholestane and epi-coprostanol as reference.

Calculation of biliary cholesterol secretion rates was performed as described previously [16]. The duodenal aspirate concentration of cholesterol was divided by sitostanol as determined by GC-FID, and the result was multiplied with the known intraduodenal infusion rate of sitostanol. Finally, the constant infusion rate of cholesterol with the liquid formula was subtracted. The intraduodenal cholesterol infusion rate was calculated from the cholesterol concentration in the liquid formula diet, which is determined by GC-FID as described above. Results are displayed as the average amount of cholesterol secreted into the duodenum per hour for each study subject. Results from the first 4 hours after the start of the marker and liquid formula infusion were not included, because gall bladder

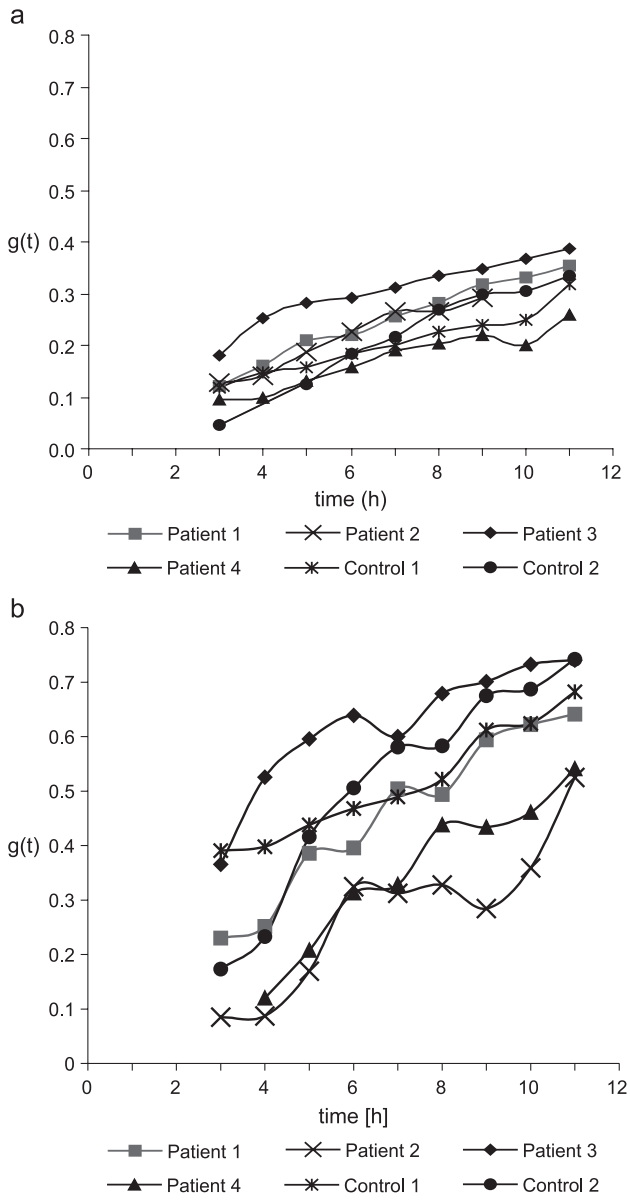

Fig. 3. a, Fractional synthesis $g(t)$ of lathosterol in serum of patients with CTX and controls. Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid). b, Fractional synthesis $g(t)$ of lathosterol in bile of patients with CTX and controls. Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid).

content is still released into the duodenum in the first few hours of the study and no steady state secretion rates were obtained.

3. Results

Table 2 gives the average concentrations of cholesterol, cholestanol, and the 2 cholesterol precursors, lathosterol and Δ-8-cholestenol, in serum and their ratios to cholesterol. In Table 3, the ratios of neutral sterols to cholesterol in gallbladder bile are also provided. The concentrations of Δ-8-cholestenol and lathosterol were higher, both in serum and bile of patients with CTX, compared to controls or in the patient with CTX treated with CDCA. Administration of pravastatin in patient 3 did not seem to have an effect on cholesterol precursors or cholestanol levels compared with untreated patients. Cholesterol concentrations tend to be lower in patients with CTX than in healthy controls in this study. Cholestanol was similarly low in controls and in patient 4 who had been treated with CDCA in contrast to the other patients without CDCA treatment, who presented strikingly higher cholestanol levels.

Patients with CTX secreted less cholesterol into bile than healthy controls (Table 4). Patient 2 showed an extremely low biliary cholesterol secretion rate.

Stable-isotope enrichment in cholesterol was insufficient to yield valid ISA results for many of the early time points during the present study (data not shown). This can probably be attributed to the relatively large size of the cholesterol pool both in patients with CTX and controls, which requires a very long exposure time to stable-isotope marker infusion to achieve notable isotope enrichment. Therefore, we, in addition, investigated the ratio of m + 4 to m + 0 for cholesterol to detect small extents of stable-isotope enrichment. Results for this calculation in serum and bile are presented in Fig. 1a,b, respectively. It can be noted that stable-isotope marker enrichment was detectable in all subjects. Although control subjects and patient 4 with CTX who was treated with CDCA exhibit only small increases in the ratio of m + 4 to m + 0 over time, the other patients with CTX who were not treated with CDCA present markedly higher increases. It was of interest to note that patient 3, who was treated with pravastatin, actually achieved the largest relative isotope enrichment. As a guide to estimate the extent of synthesis, it is noted that ISA results for patient 3 at 11 hours indicated that the fractional synthesis of cholesterol was 2.5% in serum and 11.5% in bile.

In Fig. 2a,b, fractional precursor enrichments of lathosterol are given in serum and bile, respectively, whereas fractional syntheses of lathosterol in serum and bile are presented in Fig. 3a,b. Results obtained within the first 3 hours of the infusion study are not presented here because the small isotope enrichment during the first hours of marker infusion did not allow valid calculations of ISA. It can be seen that fractional precursor enrichment D reaches constant levels both in serum and bile after only 3 to 4 hours of stable-isotope infusion (Fig. 2a,b). In general, patients with CTX tend to achieve a higher precursor enrichment than healthy controls in this study. Fractional syntheses $g(t)$ in this study show a steady increase throughout the marker infusion period in all subjects (Fig. 3a,b). Cerebrotendinous xanthomatosis patients who are not treated with CDCA present a high turnover rate of lathosterol with about 50% newly synthesized lathosterol in serum and about 80% in bile after 11 hours of the study. It is noted that fractional synthesis rates of lathosterol tend to be higher in bile than in serum.

This indicates that the ratio, flux of newly synthesized lathosterol/lathosterol pool size, is greater in bile than in serum.

Fractional synthesis $g(t)$ is the amount of newly synthesized compound divided by the total compound in that pool at time t. To calculate absolute synthesis rates of cholesterol precursors, their fractional synthesis obtained by ISA is used as described in the "Methods" section. The results of this calculation for lathosterol in serum—corrected for cholesterol—are presented in Fig. 4. Here it becomes

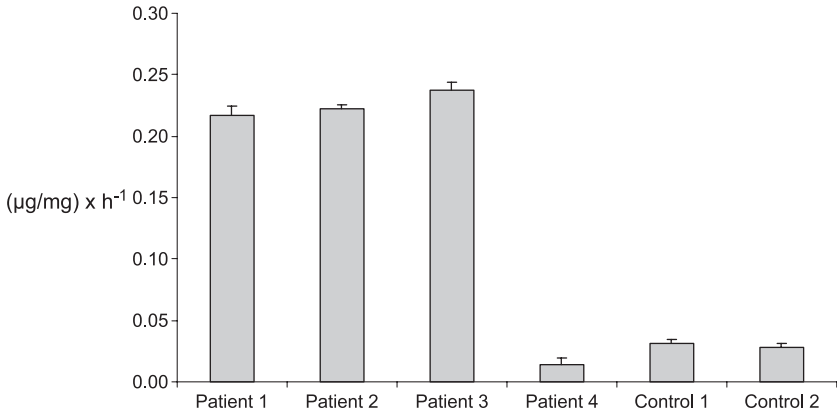

Fig. 4. Rate of appearance of lathosterol in serum of patients with CTX and controls. Patient 3 was on pravastatin (20 mg twice daily) and patient 4 was on CDCA (250 mg tid). Values were calculated, corrected for cholesterol concentration, and multiplied by 1000 to facilitate data interpretation according to the following formula: corrected appearance rate (unit, 1000 h^{-1}) = 1000 × k × [lathosterol]/[cholesterol] (k = rate constant [unit, h^{-1}], [...] = plasma concentration). Data are shown as mean ± standard deviation.

evident that healthy controls and patient 4 with CTX under treatment with CDCA synthesize much less lathosterol than those patients with CTX who are not treated with CDCA. It also becomes evident that treatment with pravastatin in patient 3 does not seem to have any influence on lathosterol synthesis. (To obtain an estimate of the uncorrected rate of appearance of lathosterol in plasma in μg/[dL × h], the numbers presented in Fig. 4 have to be multiplied with their respective cholesterol concentrations as given in Table 2.)

Isotopomer spectral analysis was also performed on Δ-8-cholestenol, methylsterol, and lanosterol. Like with lathosterol, higher fractional synthesis rates of these sterols in bile compared to serum were noticed (data not shown). Linear regression analysis of precursor enrichment and fractional synthesis of lathosterol compared to Δ-8-cholestenol, methylsterol, and lanosterol, respectively, were high, both in serum and in bile (Table 5). In addition, ISA results of these cholesterol precursors were compared between the 2 compartments, serum and bile. Linear correlation factors of fractional precursor enrichment D between serum and bile of lathosterol, Δ-8-cholestenol, methylsterol, and lanosterol were 0.909, 0.732, 0.835, and 0.578, respectively. Fractional synthesis $g(t)$ showed correlation factors of 0.857, 0.732, 0.807, and 0.828 for these sterols, but $g(t)$ was generally higher in bile than in serum. We were not able to detect any remarkable stable-isotope labeling in cholestanol in any of the study subjects in this trial. Therefore, ISA was not performed on cholestanol.

In bile samples, we were able to identify the following acidic sterols by their typical mass spectra and retention times [19]: cholic acid, CDCA, a 3-fold hydroxylated sterol derivative that is most likely 5β-cholestane-3α,7α,12α-triol (here referred to as "triol 1"), 5β-cholest-24-en-3α,7α,12α-triol ("triol 2"), and 5β-cholestane-3α,7α,12α,25-tetrol ("tetrol 1"). We were not able to detect any bile alcohols in healthy controls or in patient 4 with CTX who was under CDCA treatment.

To determine the extent of stable-isotope labeling in polar sterols during the study, we analyzed samples from the beginning (t = 0 hour and t = 1 hour) and the end (t = 10 hours and t = 11 hour) of the marker infusion period. This was done in patients with CTX only. In Table 6, the average ratios of M + 3 to M + 0 of selected polar sterols are compared between the beginning and the end of the marker infusion period in patients with CTX not treated with CDCA. It can be seen that there are small increases in the ratio of M + 3 to M + 0 in triols 1 and 2 in all patients with CTX in whom these bile alcohols were detectable. Cerebrotendinous xanthomatosis patient 1 is the only subject who presents increases of the ratios of M + 3 to M + 0 in all investigated polar sterols. In general, the extent of isotope enrichment in polar sterols is very small—if detectable at all—and cannot be differentiated with certainty from incidental changes in isotopomer ratios. In patient 4 with CTX under therapy with CDCA, bile acids could not be analyzed because their peaks in GCMS chromatograms were overlapping with small unidentified signals that were not found in any of the other study subjects.

Table 5
Correlation of ISA results between lathosterol and other cholesterol precursors in serum and bile, respectively, in patients with CTX

	Correlation with lathosterol in serum	
	D	g(t)
Δ-8-Cholestenol	0.785	0.879
Methylsterol	0.921	0.913
Lanosterol	0.776	0.896
	Correlation with lathosterol in bile	
	D	g(t)
Δ-8-Cholestenol	0.988	0.969
Methylsterol	0.883	0.985
Lanosterol	0.871	0.697

Table 6
Average ratio of M + 3 to M + 0 (in %) of polar sterols at the start and at the end of the marker infusion period in patients with cerebrotendinous xanthomatosis

Patient	1		2		3	
Study period	start	end	start	end	start	end
Cholic acid	1.72	2.85	2.43	2.29	2.74	2.70
CDCA	0.73	0.85	0.66	0.68	0.83	0.80
Triol 1	2.39	3.18	2.48	3.10	2.54	3.21
Triol 2	2.18	2.67	2.22	2.56	2.53	2.77
Tetrol 1	3.81	4.36	4.43	4.43	5.11	4.53

Patient 3 was on pravastatin (20 mg twice daily). In patient 4, treated with CDCA (250 mg tid), no bile alcohols were detected, and GCMS analysis of bile acids was impossible because of overlapping unidentifiable peaks.

4. Discussion

In this study, the application of ISA on cholesterol and cholesterol precursors was investigated in patients with CTX and healthy controls. For the first time, we here report the application of ISA on cholesterol precursors other than lathosterol. In addition, for the first time, biliary secretion rates of cholesterol were determined in patients with CTX. The effect of 2 different drug therapies on sterol concentrations and sterol synthesis rates was assessed, and the turnover of cholesterol precursors, bile acids, and bile alcohols was investigated by analysis of stable-isotope–labeled marker uptake.

It has been well established that serum concentrations of cholesterol precursors can serve as markers of endogenous cholesterol synthesis [6-8]. As expected, in this study, we found higher serum and bile concentrations of the cholesterol precursors lathosterol and Δ-8-cholestenol in patients with CTX not treated with CDCA compared to controls or to the patient with CTX who was under treatment with CDCA. These results correspond well with the differences in lathosterol biosynthesis as determined by ISA in the present study. So the validity of cholesterol precursors as

markers of endogenous cholesterol synthesis has been supported here once again [5].

The cholesterol precursors lathosterol, Δ-8-cholestenol, methylsterol, and lanosterol were investigated successfully with ISA. Precursor pool enrichment reaches a constant level within 3 hours of stable-isotope marker infusion both in serum and bile. Cerebrotendinous xanthomatosis patients presented markedly higher fractional synthesis of lathosterol than healthy controls or the patient with CTX under CDCA treatment. The high turnover rate of cholesterol precursors reflects their rapid conversion to cholesterol. Only little data on the kinetics of endogenous cholesterol synthesis rates have been published before. In a trial with healthy volunteers using mass isotopomer distribution analysis, Empen et al [21] found a fractional synthesis of cholesterol of 4.2% in serum and 5.3% in bile after 15 hours of marker infusion. No cholesterol precursors had been investigated in that trial. Recently, the application of ISA on the cholesterol precursor lathosterol was reported [22]. These authors found de novo synthesis of lathosterol of 50% in serum of healthy volunteers after a marker infusion period of 12 hours. In our trial, fractional synthesis of lathosterol in serum did not reach such high levels in the comparable control group of healthy volunteers. This can only partly be attributed to the shorter infusion time of stable-isotope marker (11 hours in this study vs 12 hours in the trial of Lindenthal et al [22]). Because of the small number of subjects investigated, a stochastic error cannot be excluded in our trial. Differences in lathosterol synthesis among the various study subjects do not appear to be very prominent when looking at the fractional synthesis as determined by ISA only. The concentration of lathosterol in serum and bile is much lower in healthy controls and in the patients with CTX treated with CDCA than in the other patient with CTXs. The absolute synthesis rates obtained by the ISA procedure [20] account for this and use the rate constant to determine the synthesis rate (Fig. 4). This underlines the importance of distinguishing between the $g(t)$ value that is simply a fraction without units and the true synthesis rate that is calculated as amount per time. Both the healthy controls and the patient with CTX who was under treatment with CDCA synthesized much less lathosterol than those patients with CTX who did not receive CDCA. To our knowledge, this is the first time that the tremendous effect of treatment with CDCA on lathosterol synthesis has been demonstrated in patients with CTX.

Precursor pool enrichment of the various cholesterol precursors correlated highly with each other, both in serum and in bile. This result was expected because cholesterol and its precursors are synthesized from the same acetate pool. Especially, the fact that ISA actually produced comparable results of precursor pool enrichment calculations for various cholesterol precursors underlines the validity of the method. Fractional synthesis also showed a high correlation among these cholesterol precursors. This indicates a correlation between the flux of newly synthesized sterol to the plasma and bile and the pool size for the sterols in these compartments. Again, this result was expected, because these cholesterol precursors are all part of the same metabolic pathway. We also found a good correlation of both precursor enrichment and fractional synthesis of individual cholesterol precursors between the 2 compartments, serum and bile. It must be noted, however, that fractional synthesis was generally higher in bile than in serum, indicating a higher flux relative to pool size.

Application of ISA for cholesterol did not produce valid results at early time points, most likely because of the relatively large size of the plasma cholesterol pool. Still, we were able to demonstrate an uptake of stable-isotope marker in cholesterol in all study subjects. As described previously [22], we found a more prominent marker uptake in cholesterol in bile than in serum. The finding of higher turnover rates of cholesterol and its precursors in bile compared to serum reflects the flux relative to pool size.

It has been shown that endogenous cholestanol is synthesized from cholesterol in human beings [23]. Because there was only little stable-isotope marker uptake in cholesterol in this trial, it should be expected that cholestanol is therefore synthesized mainly from unlabeled cholesterol. Indeed, we were not able to detect any stable-isotope labeling in cholestanol in this study. It therefore seems unlikely that there are de novo sources of endogenous cholestanol synthesis. If there was an alternative synthesis pathway from any cholesterol precursor to cholestanol without cholesterol as an intermediate, significant labeling of cholestanol with stable-isotopes could be expected.

In contrast to a previous report [24], treatment of patient 3 with CTX with pravastatin (20 mg twice daily) did not show any effect on the serum concentration of cholestanol compared with untreated patients with CTX, even though the dosage of pravastatin in this trial was 4-fold higher than the dosage applied in the study of Kuriyama et al [24]. This result is in accordance with the report of a single patient with CTX who had been treated with pravastatin for 1 year, which did not change the serum concentration of cholestanol compared to baseline [25]. In the trial reported here, there was also a major difference neither in cholesterol precursor concentrations nor in fractional precursor enrichment or fractional synthesis compared to untreated patients. Because no baseline values before the beginning of treatment were available for this patient, we cannot conclude with certainty that treatment with pravastatin did not produce a benefit for this individual patient. At least, we did not obtain any results that would support the benefit of pravastatin treatment in patients with CTX.

Treatment with CDCA in patient 4 on the other hand produced a marked reduction of cholestanol and cholesterol precursor concentrations both in serum and bile to levels that were comparable with healthy controls. Moreover, treatment with CDCA led to a significant reduction of endogenous cholesterol precursor synthesis to control levels as determined by ISA. In addition, as in healthy controls,

bile alcohols were not detectable in this patient. These findings are in accordance with previously published data by Salen et al [26,27]. We did not investigate the effect of a combined therapy with CDCA and an HMG-CoA-reductase inhibitor, for example, pravastatin or simvastatin. Recently, it was reported that a combination therapy of CDCA (750 mg/d) and simvastatin (40 mg/d) lowers the serum concentration of cholestanol to a larger extent than treatment with CDCA (750 mg/d) alone [28]. The effect of this combination therapy on cholesterol precursor levels and on fractional synthesis rates in patients with CTX still remains to be investigated. However, it has been well established previously that a single long-term treatment with CDCA provides not only a significant reduction of cholestanol and abnormal bile acid levels but also significant clinical benefits for patients with CTX [29]. The mechanism how exogenous CDCA corrects the biochemical abnormalities in patients with CTX seems to be mediated through the nuclear farnesoid X receptor. It was shown recently that CDCA binds to farnesoid X receptor, which then represses the transcription of the gene encoding sterol-7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis [30]. In patients with CTX, this feedback inhibition of bile acid synthesis is impaired because the enzymatic defect at the level of sterol side chain hydroxylation causes a relative lack of bile acids. Substituting exogenous CDCA reinstitutes the inhibition of sterol-7α-hydroxylase, thereby reducing the rapid turnover of cholesterol to 7α-hydroxylated bile acid intermediates, cholestanol and bile alcohols. The reduced turnover of cholesterol eventually leads to a down-regulation of its synthetic pathway, which is reflected in the reduction of cholesterol precursor levels in serum and bile.

Cerebrotendinous xanthomatosis patients demonstrated a lower biliary cholesterol secretion than healthy controls in this trial. We believe that the relatively low biliary cholesterol secretion in patients with CTX is caused by the rapid conversion of cholesterol to 7α-hydroxylated intermediates of bile acid synthesis and bile alcohols. In addition, the serum concentration of total cholesterol is relatively low in patients with CTX in this trial, which is in accordance with previously published data [3], so that there is less cholesterol available for biliary secretion than in healthy controls.

In the analysis of polar sterols, we were able to identify two 3-fold hydroxylated and one 4-fold hydroxylated bile alcohol, apart from cholic acid and CDCA. Bile alcohols were only detectable in patients with CTX who were not treated with CDCA. So treatment with CDCA proves to be very effective in the elimination of bile alcohols in patients with CTX. This is in accordance with previously published data [26]. As expected, both in bile alcohols and in bile acids, there was no or only little uptake of stable-isotope marker. This finding supports the concept that bile acids and bile alcohols are synthesized almost entirely from preformed cholesterol.

In summary, we conclude that ISA is a powerful method to investigate the extent of endogenous synthesis of cholesterol precursors and its response to treatment in patients with CTX. Isotopomer spectral analysis is not helpful in the analysis of cholesterol itself or in the analysis of its metabolic products, a finding that most likely reflects the flux of cholesterol relative to its pool size. To investigate the turnover of cholesterol to cholestanol, bile acids and bile alcohols, we believe that other tracer methods might be more appropriate, for example, the direct application of labeled 7α-cholesterol.

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