Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans. Evidence for involvement of cytochrome P450 3A4

Karl Bodin, Lionel Brétillon, Yacoub Aden, Leif Bertilsson, Ulrika Broomé, Curt Einarsson, Ulf Diczfalusy

► **To cite this version:**

Karl Bodin, Lionel Brétillon, Yacoub Aden, Leif Bertilsson, Ulrika Broomé, et al.. Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans. Evidence for involvement of cytochrome P450 3A4. Journal of Biological Chemistry, 2001, 276 (42), pp.38685-38689. 10.1074/jbc.M105127200 . hal-02681356

HAL Id: hal-02681356
https://hal.inrae.fr/hal-02681356v1

Submitted on 31 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 42, Issue of October 19, pp. 38685–38689, 2001
Printed in U.S.A.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Antiepileptic Drugs Increase Plasma Levels of 4β-Hydroxycholesterol in Humans

EVIDENCE FOR INVOLVEMENT OF CYTOCHROME P450 3A4*

Received for publication, June 5, 2001, and in revised form, August 17, 2001
Published, JBC Papers in Press, August 20, 2001, DOI 10.1074/jbc.M105127200

Karl Bodin‡, Lionel Bretillon§, Yacoub Aden¶, Leif Bertilsson¶, Ulrika Broomé‖, Curt Einarsson‖, and Ulf Diczfalusy‡**

From the Department of Medical Laboratory Sciences and Technology, Divisions of ‡Clinical Chemistry and ¶Clinical Pharmacology and the Department of Medicine, ‖Division of Gastroenterology/Hepatology, Karolinska Institutet, Huddinge University Hospital, SE-141 86 Huddinge, Sweden and §Institut National de la Recherche Agronomique, Unité de Nutrition Lipidique, 21034 Dijon, France

Downloaded from www.jbc.org at INRA Institut National de la Recherche Agronomique, on September 21, 2010

The major cholesterol oxidation products in the human circulation are 27-hydroxycholesterol, 24-hydroxycholesterol, and 7α-hydroxycholesterol. These oxysterols are formed from cholesterol by specific cytochrome P450 enzymes, CYP27, CYP46, and CYP7A, respectively. An additional oxysterol present in concentrations comparable with 7α- and 24-hydroxycholesterol is 4β-hydroxycholesterol. We now report that patients treated with the antiepileptic drugs phenobarbital, carbamazepine, or phenytoin have highly elevated levels of plasma 4β-hydroxycholesterol. When patients with uncomplicated cholesterol gallstone disease were treated with ursodeoxycholic acid, plasma 4β-hydroxycholesterol increased by 45%. Ursodeoxycholic acid, as well as the antiepileptic drugs, are known to induce cytochrome P450 3A. Recombinant CYP3A4 was shown to convert cholesterol to 4β-hydroxycholesterol, whereas no conversion was observed with CYP1A2, CYP2C9, or CYP2B6. The concentration of 4α-hydroxycholesterol in plasma was lower than the concentration of 4β-hydroxycholesterol and not affected by treatment with the antiepileptic drugs or ursodeoxycholic acid. Together, these data suggest that 4β-hydroxycholesterol in human circulation is formed by a cytochrome P450 enzyme.

Cholesterol oxidation products (oxysterols) have recently attracted great interest because of their numerous biological actions. They have been implicated in bile acid biosynthesis, cholesterol transport, and gene regulation (1). In addition, many oxysterols are toxic to cells and induce apoptosis (2–4). These compounds can be formed either by cholesterol autooxidation or by the action of cholesterol-metabolizing enzymes. Several oxysterols can be formed by both mechanisms, *i.e.* 7α-hydroxycholesterol. This oxysterol is a predominant cholesterol auto-oxidation product but is also formed by the hepatic enzyme cholesterol 7α-hydroxylase. Major oxysterols in the human circulation include 27-hydroxycholesterol, 24-hydroxycholesterol, and 7α-hydroxycholesterol (5). One additional oxysterol present in human plasma at a relatively high concentration is 4β-hydroxycholesterol (6). Very little is known about its formation or metabolism. We have shown earlier that small amounts of this oxysterol are formed, together with 4α-hydroxycholesterol, during *in vitro* oxidation of low density lipoprotein, and low levels of the two oxysterols were also found in human atherosclerotic plaques (7). The ratio between 4α- and 4β-hydroxycholesterol was close to one both in oxidized LDL[1] and in plaques, and the amount formed in oxidized LDL was only a small percent of the dominating oxysterol, 7-oxocholesterol. These data suggested that very little 4β-hydroxycholesterol is formed by cholesterol auto-oxidation. Because relatively high levels were reported in human plasma we hypothesized that this compound is formed *in vivo* by an enzymatic reaction. 4α- and 4β-hydroxycholesterol were determined in plasma from volunteers and patients, and it was found that patients treated with certain antiepileptic drugs, known to influence cytochrome P450 enzymes, had 10–20-fold higher plasma levels of 4β-hydroxycholesterol than untreated control subjects. Attempts to identify the cytochrome P450 responsible for the conversion of cholesterol into 4β-hydroxycholesterol were made using recombinant human cytochrome P450 enzymes expressed in insect cells.

EXPERIMENTAL PROCEDURES

Chemicals

Chloroform, ethyl acetate, hexane, and toluene, analytical grade, and methanol (high pressure liquid chromatography grade) were obtained from Merck. 2-Propanol (high pressure liquid chromatography grade) was from Labscan Ltd. (Dublin, Ireland). Butylated hydroxytoluene was obtained from Sigma. EDTA disodium salt and potassium bromide were obtained from Merck. Solid-phase extraction cartridges (100 mg of Isolute® silica) were obtained from Sorbent (Mid Glamorgan, United Kingdom). *tert*-Butyldimethylsilylimidazole-dimethylformamide was obtained from Supelco Inc. (Bellefonte, PA).

Testosterone, diclofenac, and phenacetin were obtained from Sigma. Microsomes (SUPERSOMES®) of recombinant human P450 (CYP1A2, CYP2B6, CYP2C9 (with Arg^{144}), and CYP3A4) expressed in insect cells (BTI-TN-5B1–4), together with NADPH-P450 reductase and cytochrome b_5 (except CYP1A2), were purchased from Gentest (Woburn, MA). All other reagents and chemicals were high purity standard commercial products.

Synthesis of Cholest-5-ene-3β,4β-diol

Cholest-5-ene-3β,4β-diol (4β-hydroxycholesterol) and [26,26,26,27,27,27-$^2H_6$]4β-hydroxycholesterol were synthesized as described previously (6).

* This work was supported by grants from Gunvor och Josef Anérs stiftelse, Swedish Heart Lung Foundation, Stiftelsen för Ålderssjukdomar vid Karolinska Institutet, Stiftelsen Serafimerlasarettet, and Swedish Medical Research Council Project 3143. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Medical Laboratory Sciences and Technology, Division of Clinical Chemistry, Karolinska Institutet, Huddinge University Hospital C1.74, SE-141 86 Huddinge, Sweden. Tel.: 46-8-585-812-53; Fax: 46-8-585-812-60; E-mail: ulf.diczfalusy@chemlab.hs.sll.se.

[1] The abbreviations used are: LDL, low density lipoprotein; GC-MS, gas chromatography-mass spectrometry; LXRα, liver X receptor α.

Healthy Subjects and Sample Collection

Blood was collected from volunteers without any known diseases, after an overnight fast, in evacuated blood collection tubes (Vacutainer, 100 × 16 mm; Becton Dickinson) containing 0.12 ml of 0.34 M EDTA K_3. Blood plasma was prepared by centrifugation at 1400 × *g* for 10 min. Plasma samples were stored at −70 °C until use.

Patients Treated with Antiepileptics

Blood plasma from patients treated with phenobarbital, carbamazepine, phenytoin, and valproate was collected as decoded rest material from therapeutic drug monitoring, at the Department of Clinical Pharmacology, Huddinge University Hospital.

Subjects Treated with Ursodeoxycholic Acid

Patients with uncomplicated cholesterol gallstone disease were treated with a ursodeoxycholic acid (Ursofalk® in 250-mg capsules from Dr. Falk Pharma, Freiburg, Germany) 500-mg bid for 3 weeks prior to laparoscopic cholecystectomy. Blood samples were obtained after an overnight fast before starting the treatment and in the morning prior to operation.

Preparation of Lipoprotein Fractions

Blood, 70 ml, was collected from four healthy volunteers (all non-smokers), two males and two females (age 28, 30, 47, and 47) using the same conditions as described above. Part of the plasma was taken for measurement of total plasma cholesterol and total plasma 4α- and 4β-hydroxycholesterol. Fractionation of plasma into lipoprotein fractions was done according to Havel *et al.* (8). Briefly, lipoprotein fractions were isolated from plasma by ultracentrifugation at 250,000 × *g* at 2 °C. Very low density lipoprotein (0.95–1.006 g/ml) was removed after 24 h; LDL (1.006–1.0063 g/ml) and high density lipoprotein (1.0063–1.21 g/ml) were collected in consecutive spins after adjustment of the density with KBr. The plasma of each volunteer was fractionated separately.

Preparation of Samples from Different Tissues and Organs

Autopsy materials were collected from different organs and tissues from a 72-year-old male patient, 44 h post-mortem, who died after cardiac insufficiency and chronic obstructive lung disease. Autopsy material of a brain was collected from a man who died at the age of 80 by auricular fibrillation and cardiac insufficiency. The post-mortem samples were washed with phosphate-buffered saline and then kept frozen at −20 °C until analysis of cholesterol and 4α- and 4β-hydroxycholesterol.

Incubation with Microsomes of Recombinant Human P450 Expressed in Insect Cells

To investigate whether any of the recombinant enzymes could convert cholesterol to 4β-hydroxycholesterol, 50 pmol of CYP3A4, CYP1A2, CYP2C9, or CYP2B6 in 0.5 ml of incubation buffer (100 mM potassium phosphate, pH 7.4) were pre-incubated with 200 μM cholesterol for 5 min at 37 °C before NADPH (1 mg/ml, final concentration) was added. Cholesterol was added as a hydroxypropyl-β-cyclodextrin solution (9). Microsomes of insect cells without expressed P450 were incubated in the same way. Incubations were terminated after 2 h by adding 50 μl of methanol. After a rapid cooling the samples were subjected to GC-MS analysis as described for plasma samples but without alkaline hydrolysis. Incubations were performed according to the manufacturers recommendations.

The formation of 4β-hydroxycholesterol by CYP3A4 was characterized in more detail by incubating cholesterol at five different concentrations (0, 50, 100, 200, and 400 μM) for 2 h with 25 pmol of enzyme. Incubations were also carried out with different amounts of protein (0, 12.5, 25, 50, and 100 pmol), at 200 μM cholesterol, and for different times (0, 0.5, 1, 2, and 3 h) at 100 μM cholesterol.

Because no conversion of cholesterol to 4β-hydroxycholesterol was observed with CYP1A2, CYP2C9, or CYP2B6, control incubations were carried out with phenacetin, diclofenac, and testosterone as substrates, respectively. All three enzymes converted the control substrates to the expected products, acetamidophenol, 4-hydroxydiclofenac, and 16β-hydroxytestosterone, respectively.

Determination of Cholesterol

Cholesterol in plasma and lipoprotein fractions was determined using a commercial enzymatic method (Roche Diagnostics/Hitachi 917 system). Total cholesterol in tissue homogenate was determined as described earlier (10) by using isotope dilution GC-MS with [26,26,26,27,27,27-$^2H_6$]cholesterol as internal standard.

TABLE I
Plasma concentrations of 4α- and 4β-hydroxycholesterol in healthy volunteers

	4α-Hydroxycholesterol	4β-Hydroxycholesterol	Cholesterol
	ng/ml[a]	*ng/ml*[a]	*mmol/l*[a]
All volunteers (*n* = 125)	6.6 ± 2.8	29 ± 10	4.5 ± 0.8
Males (*n* = 49)	6.8 ± 3.6	26 ± 11	4.3 ± 0.8
Females (*n* = 76)	6.5 ± 2.1	30 ± 10	4.7 ± 0.7

[a] Mean ± S.D.

Determination of 4α- and 4β-Hydroxycholesterol

Alkaline Hydrolysis and Extraction—The procedure for alkaline hydrolysis and extraction of 4β-hydroxycholesterol in tissue homogenate and plasma has been described previously (6). Briefly, to 1 ml of plasma 10 μg of butylated hydroxytoluene and 100 ng of [$^2H_6$]4β-hydroxycholesterol dissolved in 40 μl of toluene was added. Argon was flushed through the vial for 20 min to remove air. Freshly prepared 0.35 M potassium hydroxide in ethanol (10 ml) was added. The alkaline hydrolysis was allowed to proceed for 2 h at room temperature with continuous magnetic stirring. The reaction mixture was transferred to a separatory funnel, and the pH value was adjusted to 7 with phosphoric acid. 18 ml of chloroform and 6 ml of 0.15 M NaCl were added. Thereafter the funnel was vigorously shaken. The organic phase was transferred to a round-bottom flask, and the solvent was evaporated using a rotary evaporator. The residue was dried with ethanol and finally dissolved in 1 ml of toluene. A 100-mg silica solid-phase extraction column (International Sorbent Technology, Mid Glanorgan, UK) was used to separate 4-hydroxycholesterol from cholesterol. The column was conditioned with 2 ml of hexane. The sample (dissolved in toluene) was applied to the column followed by 1 ml of hexane. Cholesterol was eluted with 8 ml of 0.5% 2-propanol in hexane and by adding 5 ml of 30% 2-propanol, 4α- and 4β-hydroxycholesterol were eluted. The solvent was evaporated under a gentle stream of argon, and the residue was derivatized.

Derivatization—4β-Hydroxycholesterol was converted into a *tert*-butyldimethylsilyl ether by treatment with 100 μl of *tert*-butyldimethylsilylimidazole-dimethylformamide (Supelco Inc., Bellefonte, PA) at 22 °C overnight, followed by addition of 1 ml of water and extraction twice with 1 ml of ethyl acetate. After derivatization and removal of solvent under a stream of argon, the samples were dissolved in 100 μl of hexane (6).

Analysis by Gas Chromatography-Mass Spectrometry—Gas chromatography-mass spectrometry was performed on a Hewlett Packard 5890 Series II Plus gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25-μm phase thickness), connected to an HP 5972 mass selective detector and an HP 7673A automatic sample injector. The oven temperature program was as follows: 180 °C for 1 min, 35 °C/min to 270 °C, and then 20 °C/min to 310 °C where the temperature was kept for 17.0 min. Helium was used as a carrier gas with a flow rate of 0.8 ml/min. Samples were splitless injected (1 μl), and the detector temperature was 270 °C. The detector transfer line temperature was set to 280 °C. The mass spectrometer was used in the selected ion monitoring mode, and the following ions (*m/z*) were monitored (retention times in brackets): 573,367 (4β-hydroxycholesterol [16.1 min]; 4α-hydroxycholesterol [17.0 min]) and 579,373 ([$^2H_6$]4β-hydroxycholesterol [16.0 min]). The electron ionization energy was 70 eV.

Ethical Aspects

All studies were approved by the Ethics Committee of Karolinska Institutet at Huddinge University Hospital (Huddinge, Sweden).

RESULTS

Determination of Plasma 4α- and 4β-Hydroxycholesterol in Healthy Volunteers—Plasma concentrations of 4α- and 4β-hydroxycholesterol were determined in 125 healthy volunteers (Table I), and the mean concentrations were found to be 6.6 and 29 ng/ml, respectively. The distribution of plasma 4β-hydroxycholesterol concentrations in the volunteers is shown in Fig. 1.

Downloaded from www.jbc.org at INRA Institut National de la Recherche Agronomique, on September 21, 2010

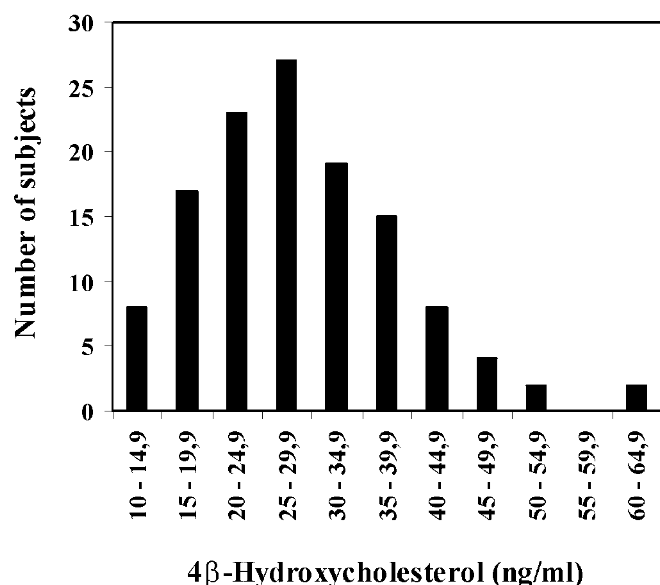

FIG. 1. **Distribution of plasma 4β-hydroxycholesterol in 125 healthy volunteers**.

4β-Hydroxycholesterol was present both in its free form and esterified to long-chain fatty acids. The degree of esterification was determined in plasma from 10 healthy volunteers. 4β-Hydroxycholesterol was present in esterified form to 83% in plasma whereas 4α-hydroxycholesterol was esterified to 70%.

Distribution of 4β-Hydroxycholesterol in Lipoprotein Fractions—Total cholesterol and 4α- and 4β-hydroxycholesterol were determined in plasma collected from four healthy volunteers. The plasma was fractionated into very low density lipoprotein, LDL, and high density lipoprotein fractions, and cholesterol and 4α- and 4β-hydroxycholesterol were determined in the fractions. As shown in Table II, the distribution of 4α- and 4β-hydroxycholesterol paralleled completely the distribution of cholesterol in the lipoprotein fractions with the major part residing in the LDL fraction.

Determination of 4β-Hydroxycholesterol in Human Tissues—The ratio of 4β-hydroxycholesterol to cholesterol was determined in several human tissues from autopsy material. As shown in Table III this ratio did not differ much in the tissues examined except for the brain, which contained 5–6 times higher relative amounts compared with most other tissues. We can not judge how representative these results are, because autopsy material from each tissue was only obtained from one single subject.

Increased Levels of 4β-Hydroxycholesterol in Patients Treated with Certain Antiepileptic Drugs—Plasma concentrations of 4α- and 4β-hydroxycholesterol were determined in patients on monotherapy with different antiepileptic drugs. As shown in Table IV, patients treated with valproate had plasma levels of the two oxysterols very similar to those in healthy subjects (Table I). Patients treated with carbamazepine, phenytoin, and phenobarbital all had significantly ($p < 0.0001$) increased plasma concentrations of 4β-hydroxycholesterol, 7–8-fold higher than those in healthy subjects. Some patients had a 20-fold increase in 4β-hydroxycholesterol. None of the antiepileptic drugs influenced the plasma level of 4α-hydroxycholesterol. Plasma from one patient treated with carbamazepine, with a 4β-hydroxycholesterol concentration of 600 ng/ml, was analyzed for other oxysterols (5). Normal values were found for all oxysterols analyzed, *i.e.* 7α- and 7β-hydroxycholesterol, cholestane-3β,5α,6β-triol, 7-oxocholesterol, and 24-, 25-, and 27-hydroxycholesterol (data not shown). The identity of 4β-hydroxycholesterol was ascertained by full scan GC-MS, and the mass spectrum was found to be identical to what was published previously (7).

TABLE II
Mean distribution of cholesterol, 4α- and 4β-hydroxycholesterol in plasma lipoproteins

VLDL, very low density lipoprotein; HDL, high density lipoprotein.

	VLDL	LDL	HDL
		%	
Cholesterol (n = 4)	4	66	30
4α-OH-cholesterol (n = 3)	3	66	31
4β-OH-cholesterol (n = 4)	2	71	27

TABLE III
Cholesterol related concentrations of 4β-hydroxycholesterol in human tissues

Tissue	4α-Hydroxycholesterol[a]	4β-Hydroxycholesterol[a]
Adrenals	ND	13
Kidney	4	18
Thymus	7	19
Liver	2	21
Lung	ND	24
Smooth muscle	8	25
Adipose tissue	ND	27
Spleen	10	29
Skin	28	30
Tendon	20	33
Duodenum	25	34
Striated muscle	24	37
Bone marrow	17	41
Ileac artery	36	59
Brain	88	165

[a] ng 4-hydroxycholesterol/mg cholesterol. ND, not detected. The brain sample was obtained from one subject, and all other tissue samples were obtained from another subject.

Treatment of Patients with Ursodeoxycholic Acid Leads to Increased Plasma Levels of 4β-Hydroxycholesterol—Four patients, three females and one male, were treated with ursodeoxycholic acid for 3 weeks. Plasma samples were taken before and immediately after treatment. The plasma concentration of 4β-hydroxycholesterol was on average 45% higher after treatment ($p < 0.006$) whereas 4α-hydroxycholesterol was not influenced by the treatment as shown in Table V.

Formation of 4β-Hydroxycholesterol by Recombinant Cytochrome P450 3A4—Insect cell microsomes (SUPERSOMES®) containing recombinant human cytochrome P450 enzymes were incubated with cholesterol. Four different microsomal preparations containing CYP3A4, CYP1A2, CYP2B6, and CYP2C9 were used. Microsomes containing CYP3A4 converted cholesterol into 4β-hydroxycholesterol with a K_m of ~50 μM whereas no conversion could be detected with CYP1A2 and CYP2C9 or CYP2B6. The 4β-hydroxylation of cholesterol by recombinant CYP3A4 was characterized in some detail. Incubations with different amounts of enzyme showed an almost linear response between 25 and 100 pmol CYP3A4/ml (Fig. 2). Substrate saturation was obtained above 100 μM (Fig. 2). Control microsomes without expressed human cytochrome P450 enzymes did not convert cholesterol into 4β-hydroxycholesterol.

DISCUSSION

The major circulating oxysterols in man are 7α-, 24- and 27-hydroxycholesterol. They are all products of enzymatic reactions (11–13). An additional oxysterol present in relatively high concentrations in human plasma is 4β-hydroxycholesterol (6). It has not been known, however, how this oxysterol is formed. *In vitro* oxidation of low density lipoprotein resulted in the formation of small amounts of 4α- and 4β-hydroxycholesterol in a ratio close to one (7) suggesting that the oxysterols were formed by auto-oxidation. The high levels in the human

Downloaded from www.jbc.org at INRA Institut National de la Recherche Agronomique, on September 21, 2010

TABLE IV
Plasma concentrations of cholesterol and 4α- and 4β-hydroxycholesterol in patients treated with different antiepileptics

Antiepileptic drug	Cholesterol	4α-Hydroxycholesterol	4β-Hydroxycholesterol	4β-Hydroxycholesterol
	mmol/l[a]	*ng/ml*[a]	*ng/ml*[a]	*range (ng/ml)*
Valproate (n = 15)	4.5 ± 0.8	5.6 ± 1.1	28 ± 15	13–74
Carbamazepine (n = 15)	5.8 ± 1.5	7.0 ± 2.8	240 ± 142	104–667
Phenytoin (n = 10)	5.1 ± 1.0	5.9 ± 1.6	214 ± 154	102–598
Phenobarbital (n = 5)	4.9 ± 1.1	6.1 ± 2.7	239 ± 226	69–628

[a] Mean ± S.D.

TABLE V
Plasma 4α- and 4β-hydroxycholesterol in patients treated with ursodeoxycholic acid for three weeks

Patient	4α-OH-cholesterol	4β-OH-cholesterol	Increase in 4β-OH-cholesterol
	ng/ml	*ng/ml*	*%*
1 - Before treatment	6.0	17.3	
1 - After treatment	7.4	23.6	36.4
2 - Before treatment	4.5	24.4	
2 - After treatment	4.7	36.0	47.5
3 - Before treatment	6.6	24.4	
3 - After treatment	7.8	36.0	47.5
4 - Before treatment	10.1	25.8	
4 - After treatment	10.3	38.9	50.8

circulation and the high ratio of 4β- to 4α-hydroxycholesterol (usually >3) indicated a potential enzymatic origin of plasma 4β-hydroxycholesterol. Determination of plasma levels of 4β-hydroxycholesterol in 125 healthy volunteers invariably showed higher levels of 4β-hydroxycholesterol compared with 4α-hydroxycholesterol with a mean ratio of 4.7. One subject (not included in the group of healthy volunteers), treated with the antiepileptic drug carbamazepine, had a plasma 4β-hydroxycholesterol concentration over 600 ng/ml compared with 30 ng/ml in the average healthy volunteer (Table I). This led us to investigate the effect of different antiepileptic drugs on the plasma levels of 4α- and 4β-hydroxycholesterol. Patients treated with carbamazepine, phenytoin, or phenobarbital, three drugs known to induce cytochrome P450 enzymes (14, 15), had significantly elevated plasma levels of 4β-hydroxycholesterol compared with patients treated with valproate (Table IV) whereas 4α-hydroxycholesterol levels were similar. Patients on monotherapy with valproate, a drug that does not induce cytochrome P450 enzymes, had normal plasma levels of 4β-hydroxycholesterol. This showed that epilepsy *per se* does not result in elevated plasma levels of 4β-hydroxycholesterol. Because three antiepileptic drugs known to induce cytochrome P450 enzymes elevated plasma 4β-hydroxycholesterol, a number of recombinant human cytochrome P450 enzymes were tested for the ability to 4β-hydroxylate cholesterol. The major human liver P450 enzymes involved in drug metabolism are CYP3A4, CYP1A2, CYP2D6, and the CYP2C subfamily (15). The CYP3A4 enzyme metabolizes the widest range of drugs and endogenous compounds of the different cytochromes (15). Phenytoin, phenobarbital, and carbamazepine induce CYP1A2, CYP2B6, CYP2C9, and CYP3A4 (14–16). Therefore, these cytochromes were tested for cholesterol 4β-hydroxylase activity. The substrates for CYP2D6 are all bases, and their binding to the active site depends on ion-pair interactions (17, 18). Consequently, cholesterol is not expected to be a substrate for CYP2D6.

Although CYP1A2, CYP2C9, and CYP2B6 did not metabolize cholesterol, CYP3A4 converted cholesterol into a polar compound co-chromatographing with 4β-hydroxycholesterol on GC-MS with selected ion monitoring, using an isotope-dilution technique. The identity of the product was verified by full scan GC-MS. Ursodeoxycholic acid has been shown to induce murine CYP3A (19). Plasma concentrations of 4β-hydroxycholesterol in patients before and after treatment with ursodeoxycholic acid was therefore determined. Three weeks of treatment with ur-

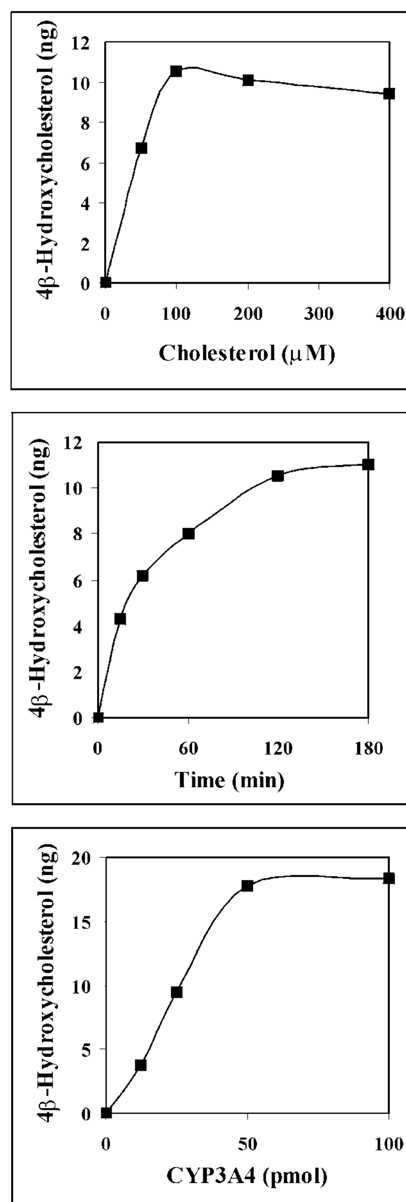

FIG. 2. **Conversion of cholesterol into 4β-hydroxycholesterol by recombinant human CYP3A4.** *Upper panel*, substrate curve. Cholesterol (0, 50, 100, 200, and 400 μM) was incubated with 25 pmol of enzyme for 2 h. *Middle panel*, time curve. Cholesterol (100 μM) was incubated with 25 pmol of enzyme for 0, 0.5, 1, 2, and 3 h. *Lower panel*, protein curve. Cholesterol (200 μM) was incubated with 0, 12.5, 25, 50, and 100 pmol of enzyme for 2 h. The total volume for all incubations was 0.5 ml, and the temperature was 37 °C. The product, 4β-hydroxycholesterol, was determined by gas chromatography-mass spectrometry after derivatization to the *tert*-butyldimethylsilyl ether. [$^2H_6$]4β-Hydroxycholesterol was used as internal standard.

Downloaded from www.jbc.org at INRA Institut National de la Recherche Agronomique, on September 21, 2010

sodeoxycholic acid increased plasma 4β-hydroxycholesterol by 45%. This finding is in accordance with our assumption that CYP3A4 is the enzyme responsible for the conversion of cholesterol into 4β-hydroxycholesterol.

Although recombinant CYP3A4 was shown to convert cholesterol into 4β-hydroxycholesterol *in vitro*, it can not be excluded that another cytochrome is responsible for the normal production of this sterol. Many drugs that induce cytochrome P450 enzymes are relatively nonspecific and may induce several enzymes. Because CYP3A4 is known to be induced by antiepileptics, is abundant in the liver, and was shown to convert cholesterol into 4β-hydroxycholesterol *in vitro* it is a strong candidate enzyme, but it is still possible that another enzyme is responsible for this conversion *in vivo*.

Very little is known about the biological effects of 4β-hydroxycholesterol. It has been reported that 4β-hydroxycholesterol is almost as good an activator for the nuclear receptor liver X receptor α (LXRα) as 24S-hydroxycholesterol, 20S-hydroxycholesterol, and 22R-hydroxycholesterol (20). The most effective activator of LXRα, 24(S),25-epoxycholesterol, binds to the receptor with a K_d of ~200 nM (21). In healthy volunteers the plasma concentration of 4β-hydroxycholesterol is 75 nM, but patients treated with carbamazepine may have 4β-hydroxycholesterol concentrations up to 1500 nM. LXRα is an important transcription factor involved in gene regulation of genes important for cholesterol homeostasis. It is possible that the highly elevated levels of 4β-hydroxycholesterol in patients treated with some antiepileptic drugs may effect transcription of genes responsive to LXRα. One such gene is sterol regulatory element-binding protein-1c, which controls transcription of lipogenic genes (22). A known side effect of treatment with certain antiepileptic drugs is weight gain. Further research will show whether drug-induced formation of 4β-hydroxycholesterol may be related to weight increase seen in patients treated with antiepileptics. 4β-Hydroxylated bile acids have been identified in fetal gallbladder bile where they constituted 5–15% of total biliary bile acids (23, 24).

In summary, we have found evidence for an enzymatic formation of 4β-hydroxycholesterol in man. Patients treated with antiepileptics known to induce CYP3A4 had highly elevated plasma levels of 4β-hydroxycholesterol. Ursodeoxycholic acid, which has also been reported to induce CYP3A, caused an increase in plasma 4β-hydroxycholesterol when given to gallstone patients. Plasma 4α-hydroxycholesterol concentrations were not influenced by treatments that elevated 4β-hydroxycholesterol, indicating that 4α-hydroxycholesterol is formed by cholesterol auto-oxidation or an enzyme not affected by carbamazepine, phenytoin, phenobarbital, or ursodeoxycholic acid.

REFERENCES

1. Russell, D. W. (2000) *Biochim. Biophys. Acta* **1529,** 126–135
2. Schroepfer, G. J., Jr. (2000) *Physiol. Rev.* **80,** 361–554
3. Yin, J., Chaufour, X., McLachlan, C., McGuire, M., White, G., King, N., and Hambly, B. (2000) *Atherosclerosis* **148,** 365–374
4. Nishio, E., and Watanabe, Y. (1996) *Biochem. Biophys. Res. Commun.* **226,** 928–934
5. Dzeletovic, S., Breuer, O., Lund, E., and Diczfalusy, U. (1995) *Anal. Biochem.* **225,** 73–80
6. Breuer, O. (1995) *J. Lipid Res.* **36,** 2275–2281
7. Breuer, O., Dzeletovic, S., Lund, E., and Diczfalusy, U. (1996) *Biochim. Biophys. Acta* **1302,** 145–152
8. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) *J. Clin. Invest.* **34,** 1345–1353
9. Martin, K. O., Budai, K., and Javitt, N. B. (1993) *J. Lipid Res.* **34,** 581–588
10. Björkhem, I., Blomstrand, R., and Svensson, L. (1974) *Clin. Chim. Acta* **54,** 185–193
11. Cohen, J. C., Cali, J. J., Jelenik, D. F., Mehrabian, M., Sparkes, R. S., Lusis, A. J., Russell, D. W., and Hobbs, H. H. (1992) *Genomics* **14,** 153–161
12. Lund, E. G., Guileyardo, J. M., and Russell, D. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7238–7243
13. Cali, J. J., and Russell, D. W. (1991) *J. Biol. Chem.* **266,** 7774–7778
14. Tanaka, E. (1999) *J. Clin. Pharm. Ther.* **24,** 87–92
15. Michalets, E. L. (1998) *Pharmacotherapy* **18,** 84–112
16. Pascussi, J.-M., Gerbal-Chaloin, S., Fabre, J.-M., Maurel, P., and Vilarem, M.-J. (2000) *Mol. Pharmacol.* **58,** 1441–1450
17. Bertilsson, L. (1995) *Clin. Pharmacokinet.* **29,** 192–209
18. Anzenbacher, P., and Anzenbacherová, E. (2001) *Cell. Mol. Life Sci.* **58,** 737–747
19. Paolini, M., Pozzetti, L., Piazza, F., Cantelli-Forti, G., and Roda, A. (1999) *Hepatology* **30,** 730–739
20. Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R., and Mangelsdorf, D. J. (1996) *Nature* **383,** 728–731
21. Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kliewer, S. A., Corey, E. J., and Mangelsdorf, D. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 266–271
22. Schultz, J. R., Tu, H., Luk, A., Repa, J. J., Medina, J. C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D. J., Lustig, K. D., and Shan, B. (2000) *Genes Dev.* **14,** 2831–2838
23. Dumaswala, R., Setchell, K. D. R., Zimmer-Nechemias, L., Iida, T., Goto, J., and Nambara, T. (1989) *J. Lipid Res.* **30,** 847–856
24. Setchell, K. D. R., Dumaswala, R., Colombo, C., and Ronchi, M. (1988) *J. Biol. Chem.* **263,** 16637–16644

Downloaded from www.jbc.org at INRA Institut National de la Recherche Agronomique, on September 21, 2010