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

EVIDENCE FOR INVOLVEMENT OF CYTOCHROME P450 3A4*

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The major cholesterol oxidation products in the human circulation are 27-hydroxycholesterol, 24-hydroxycholesterol, and 7 α -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 7 α - and 24-hydroxycholesterol is 4 β -hydroxycholesterol. We now report that patients treated with the antiepileptic drugs phenobarbital, carbamazepine, or phenytoin have highly elevated levels of plasma 4 β -hydroxycholesterol. When patients with uncomplicated cholesterol gallstone disease were treated with ursodeoxycholic acid, plasma 4 β -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 4 β -hydroxycholesterol, whereas no conversion was observed with CYP1A2, CYP2C9, or CYP2B6. The concentration of 4 α -hydroxycholesterol in plasma was lower than the concentration of 4 β -hydroxycholesterol and not affected by treatment with the antiepileptic drugs or ursodeoxycholic acid. Together, these data suggest that 4 β -hydroxycholesterol in human circulation is formed by a cytochrome P450 enzyme.

cholesterol, and 7 α -hydroxycholesterol (5). One additional oxysterol present in human plasma at a relatively high concentration is 4 β -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 4 α -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 4 α - and 4 β -hydroxycholesterol was close to one both in oxidized LDL¹ 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 4 β -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. 4 α - and 4 β -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 4 β -hydroxycholesterol than untreated control subjects. Attempts to identify the cytochrome P450 responsible for the conversion of cholesterol into 4 β -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 Arg¹⁴⁴), 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-3 β ,4 β -diol

Cholest-5-ene-3 β ,4 β -diol (4 β -hydroxycholesterol) and [26,26,26,27,27,27-²H₆]4 β -hydroxycholesterol were synthesized as described previously (6).

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

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 auto-oxidation or by the action of cholesterol-metabolizing enzymes. Several oxysterols can be formed by both mechanisms, *i.e.* 7 α -hydroxycholesterol. This oxysterol is a predominant cholesterol auto-oxidation product but is also formed by the hepatic enzyme cholesterol 7 α -hydroxylase. Major oxysterols in the human circulation include 27-hydroxycholesterol, 24-hydroxy-

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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 \times 16 mm; Becton Dickinson) containing 0.12 ml of 0.34 M EDTA K₃. Blood plasma was prepared by centrifugation at 1400 \times 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 4 α - and 4 β -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 \times g at 2 $^{\circ}\text{C}$. 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 4 α - and 4 β -hydroxycholesterol.

Incubation with Microsomes of Recombinant Human P450 Expressed in Insect Cells

To investigate whether any of the recombinant enzymes could convert cholesterol to 4 β -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 $^{\circ}\text{C}$ 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 4 β -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 4 β -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 16 β -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

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

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

^a Mean \pm S.D.

described earlier (10) by using isotope dilution GC-MS with [26,26,26,27,27,27-²H₆]cholesterol as internal standard.

Determination of 4 α - and 4 β -Hydroxycholesterol

Alkaline Hydrolysis and Extraction—The procedure for alkaline hydrolysis and extraction of 4 β -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 [²H₆]4 β -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, 4 α - and 4 β -hydroxycholesterol were eluted. The solvent was evaporated under a gentle stream of argon, and the residue was derivatized.

Derivatization—4 β -Hydroxycholesterol was converted into a *tert*-butyldimethylsilyl ether by treatment with 100 μl of *tert*-butyldimethylsilylimidazole-dimethylformamide (Supelco Inc., Bellefonte, PA) at 22 $^{\circ}\text{C}$ 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 \times 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 $^{\circ}\text{C}$ for 1 min, 35 $^{\circ}\text{C}/\text{min}$ to 270 $^{\circ}\text{C}$, and then 20 $^{\circ}\text{C}/\text{min}$ to 310 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$. The detector transfer line temperature was set to 280 $^{\circ}\text{C}$. 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 (4 β -hydroxycholesterol [16.1 min]); 4 α -hydroxycholesterol [17.0 min]) and 579,373 ([²H₆]4 β -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 4 α - and 4 β -Hydroxycholesterol in Healthy Volunteers—Plasma concentrations of 4 α - and 4 β -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 4 β -hydroxycholesterol concentrations in the volunteers is shown in Fig. 1.

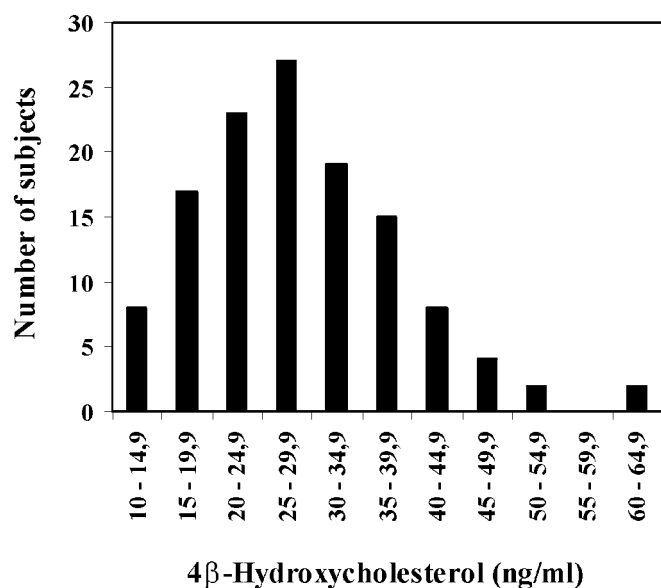


FIG. 1. Distribution of plasma 4 β -hydroxycholesterol in 125 healthy volunteers.

4 β -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. 4 β -Hydroxycholesterol was present in esterified form to 83% in plasma whereas 4 α -hydroxycholesterol was esterified to 70%.

Distribution of 4 β -Hydroxycholesterol in Lipoprotein Fractions—Total cholesterol and 4 α - and 4 β -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 4 α - and 4 β -hydroxycholesterol were determined in the fractions. As shown in Table II, the distribution of 4 α - and 4 β -hydroxycholesterol paralleled completely the distribution of cholesterol in the lipoprotein fractions with the major part residing in the LDL fraction.

Determination of 4 β -Hydroxycholesterol in Human Tissues—The ratio of 4 β -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 4 β -Hydroxycholesterol in Patients Treated with Certain Antiepileptic Drugs—Plasma concentrations of 4 α - and 4 β -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 4 β -hydroxycholesterol, 7–8-fold higher than those in healthy subjects. Some patients had a 20-fold increase in 4 β -hydroxycholesterol. None of the antiepileptic drugs influenced the plasma level of 4 α -hydroxycholesterol. Plasma from one patient treated with carbamazepine, with a 4 β -hydroxycholesterol concentration of 600 ng/ml, was analyzed for other oxysterols (5). Normal values were found for all oxysterols analyzed, *i.e.* 7 α - and 7 β -hydroxycholesterol, cholestane-3 β ,5 α ,6 β -triol, 7-oxocholesterol, and 24-, 25-, and 27-hydroxycholesterol (data not shown). The identity of 4 β -hydroxycholesterol was ascertained by full scan GC-MS,

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

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

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

| Tissue | 4 α -Hydroxycholesterol ^a | 4 β -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.

and the mass spectrum was found to be identical to what was published previously (7).

Treatment of Patients with Ursodeoxycholic Acid Leads to Increased Plasma Levels of 4 β -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 4 β -hydroxycholesterol was on average 45% higher after treatment ($p < 0.006$) whereas 4 α -hydroxycholesterol was not influenced by the treatment as shown in Table V.

Formation of 4 β -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 4 β -hydroxycholesterol with a K_m of $\sim 50 \mu\text{M}$ whereas no conversion could be detected with CYP1A2 and CYP2C9 or CYP2B6. The 4 β -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 4 β -hydroxycholesterol.

DISCUSSION

The major circulating oxysterols in man are 7 α -, 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 4 β -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 4 α - and 4 β -hydroxycholesterol in a ratio close to one (7) suggesting that the oxysterols were formed by auto-oxidation. The high levels in the human

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

| Antiepileptic drug | Cholesterol | 4 α -Hydroxycholesterol | 4 β -Hydroxycholesterol | 4 β -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 4 α - and 4 β -hydroxycholesterol in patients treated with ursodeoxycholic acid for three weeks

| Patient | 4 α -OH-cholesterol | 4 β -OH-cholesterol | Increase in 4 β -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 4 β - to 4 α -hydroxycholesterol (usually >3) indicated a potential enzymatic origin of plasma 4 β -hydroxycholesterol. Determination of plasma levels of 4 β -hydroxycholesterol in 125 healthy volunteers invariably showed higher levels of 4 β -hydroxycholesterol compared with 4 α -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 4 β -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 4 α - and 4 β -hydroxycholesterol. Patients treated with carbamazepine, phenytoin, or phenobarbital, three drugs known to induce cytochrome P450 enzymes (14, 15), had significantly elevated plasma levels of 4 β -hydroxycholesterol compared with patients treated with valproate (Table IV) whereas 4 α -hydroxycholesterol levels were similar. Patients on monotherapy with valproate, a drug that does not induce cytochrome P450 enzymes, had normal plasma levels of 4 β -hydroxycholesterol. This showed that epilepsy *per se* does not result in elevated plasma levels of 4 β -hydroxycholesterol. Because three antiepileptic drugs known to induce cytochrome P450 enzymes elevated plasma 4 β -hydroxycholesterol, a number of recombinant human cytochrome P450 enzymes were tested for the ability to 4 β -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 4 β -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 4 β -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

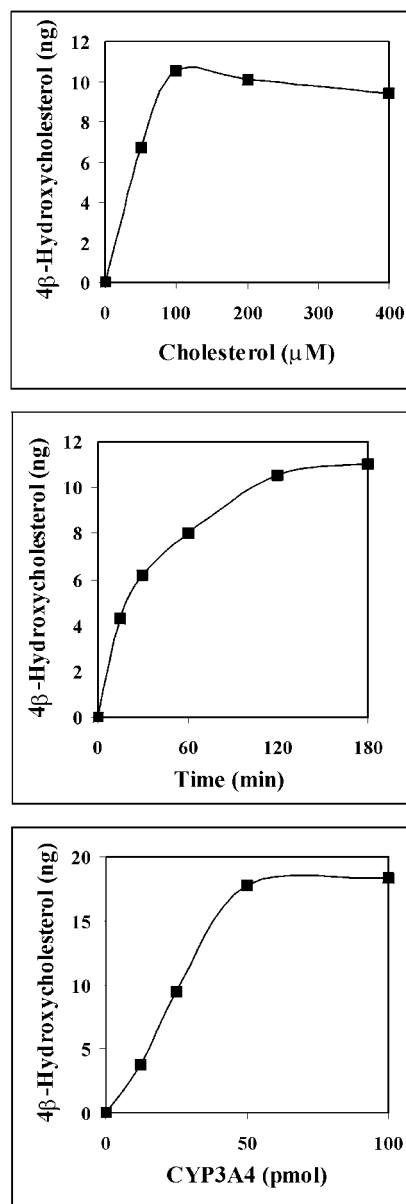


FIG. 2. Conversion of cholesterol into 4 β -hydroxycholesterol by recombinant human CYP3A4. *Upper panel*, substrate curve. Cholesterol (0, 50, 100, 200, and 400 μ M) was incubated with 25 pmol of enzyme for 2 h. *Middle panel*, time curve. Cholesterol (100 μ M) was incubated with 25 pmol of enzyme for 0, 0.5, 1, 2, and 3 h. *Lower panel*, protein curve. Cholesterol (200 μ M) 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, 4 β -hydroxycholesterol, was determined by gas chromatography-mass spectrometry after derivatization to the *tert*-butyldimethylsilyl ether. [²H₆]4 β -Hydroxycholesterol was used as internal standard.

CYP3A (19). Plasma concentrations of 4 β -hydroxycholesterol in patients before and after treatment with ursodeoxycholic acid was therefore determined. Three weeks of treatment with ur-

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

Although recombinant CYP3A4 was shown to convert cholesterol into 4 β -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 4 β -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 4 β -hydroxycholesterol. It has been reported that 4 β -hydroxycholesterol is almost as good an activator for the nuclear receptor liver X receptor α (LXR α) as 24S-hydroxycholesterol, 20S-hydroxycholesterol, and 22R-hydroxycholesterol (20). The most effective activator of LXR α , 24(S),25-epoxycholesterol, binds to the receptor with a K_d of \sim 200 nM (21). In healthy volunteers the plasma concentration of 4 β -hydroxycholesterol is 75 nM, but patients treated with carbamazepine may have 4 β -hydroxycholesterol concentrations up to 1500 nM. LXR α is an important transcription factor involved in gene regulation of genes important for cholesterol homeostasis. It is possible that the highly elevated levels of 4 β -hydroxycholesterol in patients treated with some antiepileptic drugs may effect transcription of genes responsive to LXR α . 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 4 β -hydroxycholesterol may be related to weight increase seen in patients treated with antiepileptics. 4 β -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 4 β -hydroxycholesterol in man. Patients treated with antiepileptics known to induce CYP3A4 had highly elevated plasma levels of 4 β -hydroxycholesterol. Ursodeoxycholic acid,

which has also been reported to induce CYP3A, caused an increase in plasma 4 β -hydroxycholesterol when given to gallstone patients. Plasma 4 α -hydroxycholesterol concentrations were not influenced by treatments that elevated 4 β -hydroxycholesterol, indicating that 4 α -hydroxycholesterol is formed by cholesterol auto-oxidation or an enzyme not affected by carbamazepine, phenytoin, phenobarbital, or ursodeoxycholic acid.

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