The lithocholic acid 6β-hydroxylase cytochrome *P*-450, CYP 3A10, is an active catalyst of steroid-hormone 6β-hydroxylation

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CYP 3A10 is a hamster liver cytochrome *P*-450 (P450) that encodes lithocholic acid 6β-hydroxylase, an enzyme that plays an important role in the detoxification of the cholestatic secondary bile acid lithocholate. Western-blot analysis revealed that the expression of CYP 3A10 protein is male-specific in hamster liver microsomes, a finding that is consistent with earlier analysis of CYP 3A10 mRNA. Since it has not been established whether the specificities of bile acid hydroxylase P450s, such as CYP 3A10, are restricted to their anionic bile acid substrates, we investigated the role of CYP 3A10 in the metabolism of a series of neutral steroid hormones using cDNA directed-expression in COS cells. The steroid hormones examined, testosterone, androstenedione and progesterone, were each metabolized by the expressed CYP 3A10, with 6β-hydroxylation corresponding to a major activity in all three instances. CYP 3A10-dependent steroid hydroxylation was increased substantially when the microsomes were prepared from COS cells co-transfected with NADPH:P450 reductase cDNA. In this case, the expressed P450 actively catalysed the 6β-hydroxylation of testosterone (288 ± 23 pmol of product formed/min per mg of COS-cell microsomal protein), androstenedione (107 ± 19 pmol/min per mg) and progesterone (150 ± 7 pmol/min per mg). Other major CYP 3A10-mediated steroid hydroxylase activities included androstenedione 16α-hydroxylation, progesterone 16α- and 21-hydroxylation, and the formation of several unidentified products. CYP 3A10 exhibited similar $V_{max.}$ values for the 6β-hydroxylation of androstenedione and lithocholic acid (132 and 164 pmol/min per mg respectively), but metabolized the bile acid with a 3-fold lower K_m (25 μM, as against 75 μM for androstenedione). Together, these studies establish that the substrate specificity of the bile acid hydroxylase CYP 3A10 is not restricted to bile acids, and further suggest that CYP 3A10 can play a physiologically important role in the metabolism of two classes of endogenous P450 substrates: steroid hormones and bile acids.

INTRODUCTION

The cytochrome *P*-450 (P450) mono-oxygenases play a central role in the biotransformation of xenobiotics, including drugs, carcinogens and pesticides, as well as endogenous substances, such as steroid hormones, fatty acids and eicosanoids [1,2]. Many individual P450 enzymes have been isolated, purified to apparent homogeneity and characterized at both the protein [3] and the cDNA level [4].

Recently, a hamster liver P450 cDNA with high sequence similarity (≥ 70%) to previously described members of the CYP 3A subfamily was isolated and designated CYP 3A10 [5]. The expression of CYP 3A10 mRNA was found to be highly regulated in hamster liver in response to sex, developmental stage and dietary factors [5]. Thus CYP 3A10 mRNA levels are about 50-fold higher in adult male than in female hamsters, reach a maximum after puberty in males and are elevated in young males fed a cholic acid-supplemented diet. cDNA expression studies revealed that CYP 3A10 is an active lithocholic acid 6β-hydroxylase [5], indicating that this P450 enzyme may play a physiologically important role in bile acid metabolism.

Lithocholic acid is a hydrophobic bile acid that is formed by bacterial 7α-dehydroxylation of chenodeoxycholic acid in the intestine [6]. This cholestatic bile acid is detoxified by rehydroxylation reactions catalysed by liver P450 enzymes [7–9]. In rats and hamsters, lithocholic acid hydroxylation mainly occurs at the 6β position [5,7], whereas in humans the major hydroxylation site is the 6α position [10]. An important question that has not been addressed directly is whether bile acid hydroxylase P450s, such as CYP 3A10, exhibit a narrow specificity that is restricted to the anionic bile acids, or whether they are catalytically competent in the hydroxylation of other endogenous lipophilic compounds, such as steroid hormones. Previous studies that we have carried out in rats suggest that lithocholic acid 6β-hydroxylation is catalysed by liver P450(s) that are distinct from the major catalysts of microsomal steroid hormone 6β-hydroxylation [8]. This finding is consistent with the major differences in polarity and overall shape between bile acids and steroid hormones, and the distinct physiological requirements for regulation of bile acid as compared with steroid-hormone hydroxylation pathways. The recent cloning of CYP 3A10 [5], the first example of a bile acid hydroxylase P450 cDNA characterized to date, provided us with a unique opportunity to address this issue. In the present investigation, we used a COS-cell cDNA expression system to examine the role of CYP 3A10 in the metabolism of three neutral steroid hormones. Our results indicate that CYP 3A10 hydroxylates testosterone, androstenedione and progesterone in a regioselective and stereospecific manner at rates comparable with those observed using lithocholic acid as substrate, and lead us to conclude that this bile acid hydroxylase P450 enzyme is not restricted to the metabolism of bile acids and that it may play a physiologically important role in the metabolism of two distinct classes of endogenous steroidal compounds.

Abbreviations used: P450, cytochrome *P*-450, P450 reductase, NADPH:cytochrome *P*-450 reductase.

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MATERIALS AND METHODS

Materials

[4-^{14}C]Testosterone, [4-^{14}C]androstenedione, [4-^{14}C]progesterone and [4-^{14}C]lithocholic acid (58–60 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Authentic steroid metabolite standards were obtained from sources detailed elsewhere [11]. Murideoxycholic acid and other bile acids were purchased from Steraloids (Wilton, NH, U.S.A.).

Animals and liver microsomes preparation

Golden Syrian hamsters and Fischer 344 rats were supplied by Charles River Laboratories (Wilmington, MA, U.S.A.) and Taconic Inc. (Germantown, NY, U.S.A.) respectively. Hamsters were fed a standard laboratory chow or the same diet supplemented with cholic acid (0.5%, w/w, for 5 days). Rats were maintained on a regular diet and were not treated. Animals were killed and liver microsomes were isolated by methods reported previously [5,12].

Transfection of COS cells

Rat P450 reductase cDNA and hamster CYP 3A10 cDNA were cloned into the expression vector pCMV [13] then transfected into COS-1 cells by the DEAE-dextran method [14]. After a 30 min transfection, the cells were incubated at 37 °C for 48 h and then washed and harvested. COS-cell microsomes were isolated by differential centrifugation, resuspended in 10 mM Tris/acetate (pH 7.4)/1 mM EDTA/0.25 M sucrose, and stored at −80 °C until use.

Steroid-hormone hydroxylase assays

Microsomes isolated from transfected COS cells were assayed for steroid metabolism [11]. Incubation mixtures contained 100 mM Hepes pH 7.4, 0.1 mM EDTA, 100 μg of microsomal protein, 50 μM ^{14}C-labelled steroid substrate (testosterone, androstenedione or progesterone) and 1 mM NADPH in a total volume of 200 μl. Reaction mixtures were incubated for 20 min at 37 °C, then extracted with ethyl acetate and chromatographed on silica-gel t.l.c. plates developed sequentially in multiple solvent systems: AB for testosterone, PQ for androstenedione and NRR for progesterone. The designation solvent system AB, for example, refers to an initial development with solvent system A, after which the t.l.c. plate is air-dried and redeveloped with solvent system B (see Table 1 for a listing of solvent-system compositions). Metabolites were localized by autoradiography and then quantified by liquid-scintillation counting or with a Betagen instrument.

Table 1 Solvent-system compositions

Solvent system	Solvent components	Ratio (v/v or by vol.)
A	Methylene chloride/acetone	4:1
B	Chloroform/ethyl acetate/100% ethanol	40:10:7
H	Chloroform/ethyl acetate	1:2
L	Ethyl acetate/n-hexane/acetic acid	15:4:1
M	Diethyl ether/acetone	4:1
N	Ethyl acetate/n-hexane/acetic acid	16:8:1
O	Benzene/ethyl acetate	3:1
P	Methylene chloride/100% ethanol	97:3
Q	Ethyl acetate/chloroform	1:1
R	Benzene/ethyl acetate/acetone	10:1:1

Identification of steroid metabolites

^{14}C-labelled steroid metabolites formed by CYP 3A10 were initially purified by t.l.c. and subsequently rechromatographed on t.l.c. plates co-spotted with unlabelled, authentic metabolite standards as described previously [11]. Mobilities of the unknown metabolites were then compared with those of the authentic standards by t.l.c. using multiple solvent systems: AA, BB and AB for testosterone metabolites; AA, HH and PQ for androstenedione metabolites; and M, OL and NRR for progesterone metabolites. This approach has been used in our laboratory to identify steroid metabolites formed by both rat and human P450s [15,16].

Other methods

Immunodetection of the expressed P450 was carried out by Western-blot analysis using polyclonal rabbit anti-(rat CYP 3A) antibodies [17]. Lithocholic acid 6β-hydroxylation was assayed by t.l.c. [5]. Microsomal protein was determined by the Bradford method, with BSA as standard.

RESULTS

Immunodetection of P450 expression

Mammalian expression vectors (pCMV-based) containing rat P450 reductase cDNA and/or CYP 3A10 cDNA were constructed and used to transfect COS cells. Western-blot analysis of COS-cell microsomes isolated from CYP 3A10-transfected cells confirmed the expression of CYP 3A10, as revealed using polyclonal rabbit anti-(rat CYP 3A) antibodies (Figure 1, lane 3). Similar levels of CYP 3A10 protein were expressed in cells cotransfected with P450 reductase cDNA (Figure 1, lane 4). As expected, CYP 3A-related proteins were not detected in microsomes prepared from cells transfected with pCMV vector or with P450 reductase in the absence of CYP 3A10 (Figure 1, lanes 1 and 2). Parallel analysis of liver microsomes isolated from adult male hamsters revealed three distinct bands, indicating the presence of multiple CYP 3A-related proteins in hamster liver. The middle band, which was readily apparent in microsomes isolated from 4-week-old (Figure 1, lanes 5 and 6) and 12-week-old male hamsters (lane 9), had the same electrophoretic mobility as cDNA-expressed CYP 3A10 (cf. lanes 3 and 4). This band was absent, or present at a much lower level, in microsomes isolated from female hamsters (lanes 7 and 8), a finding that is consistent with the male-specific expression of CYP 3A10 observed at the RNA level [5]. The microsomal bands of higher and lower mobility than CYP 3A10 respectively correspond to CYP 3A-immunoreactive hamster P450s that have not yet been identified.

Metabolism of steroid hormones by cDNA-expressed CYP 3A10

Expressed CYP 3A10 was evaluated for its capacity to metabolize three neutral steroid hormones: testosterone, androstenedione and progesterone. The steroid metabolites formed by CYP 3A10

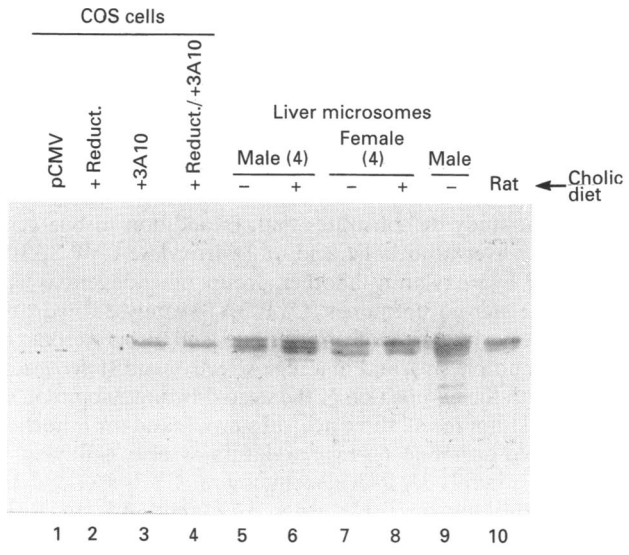

Figure 1 Immunoblot of microsomes isolated from COS cells, hamster liver and rat liver

Shown is a Western blot probed with polyclonal rabbit anti-(rat CYP 3A) antibodies analysing microsomes (15 μg of protein/lane for lanes 1–4 and 5 μg/lane for lanes 5–10) prepared from COS cells transfected with pCMV vector (lane 1), or pCMV containing P450 reductase cDNA (Reduct.; lane 2), CYP 3A10 cDNA (+3A10; lane 3) or from cells transfected with both P450 reductase cDNA and CYP 3A10 cDNA (lane 4). Liver microsomes from 4-week-old male hamsters fed a regular diet (lane 5) or cholic acid-supplemented diet (lane 6) and 12-week-old males fed a regular diet (lane 9) were analysed in parallel and shown to contain three intensely stained bands, with the middle band exhibiting the same electrophoretic mobility as cDNA-expressed CYP 3A10 (cf. lanes 3 and 4). Liver microsomes from 4-week-old female hamsters fed a regular diet (lane 7) or cholic acid-supplemented diet (lane 8) are seen to contain two intensely stained bands, neither one corresponding to the male-specific CYP 3A10 (cf. lanes 3 and 4). Microsomes from adult male rats (lane 10) were included on the blot for comparison.

and the observed rates of hydroxylation are shown in Figure 2 and Table 2 respectively. In microsomes from cells transfected with either pCMV or P450 reductase cDNA (Figures 2a–2c, lanes 1 and 2), little or no steroid metabolism occurred. In contrast, expressed CYP 3A10 metabolized testosterone, androstenedione and progesterone at multiple sites (Figures 2a–2c, lane 3), with the major polar product identified as the 6β-hydroxy derivative in the case of each steroid (see the Materials and methods section and the legend to Fig. 2). The rates of product formation were substantially higher in microsomes prepared from COS cells co-transfected with both CYP 3A10 cDNA and P450 reductase cDNA (Figures 2a–2c, lane 4; Table 2), indicating that the level of endogenous P450 reductase in the COS-cell microsomes is insufficient for optimal rates of P450 catalysis. CYP 3A10, in the presence of cDNA-expressed P450 reductase, actively catalysed the 6β-hydroxylation of testosterone, androstenedione and progesterone (107–288 pmol of product formed/min per mg of microsomal protein). The other major identified sites of metabolism were progesterone 16α- and 21-hydroxylation and androstenedione 16α-hydroxylation. CYP 3A10 also formed several other enzymic products that corresponded to monohydroxysteroids in their t.l.c. mobilities (unknowns 2, 3, 4 and 6) in addition to a less polar metabolite derived from each steroid (unknowns 1, 5 and 7) (Figure 2 and Table 2). None of these products co-migrated with the available collection of authentic steroid metabolite standards [11] and therefore remain unidentified.

Steroid hormones and lithocholic acid as substrates for CYP 3A10

Kinetic analyses were performed to compare directly the efficiency of CYP 3A10-catalysed steroid-hormone versus bile acid 6β-hydroxylation. Apparent K_m and $V_{max.}$ values of 75 μM

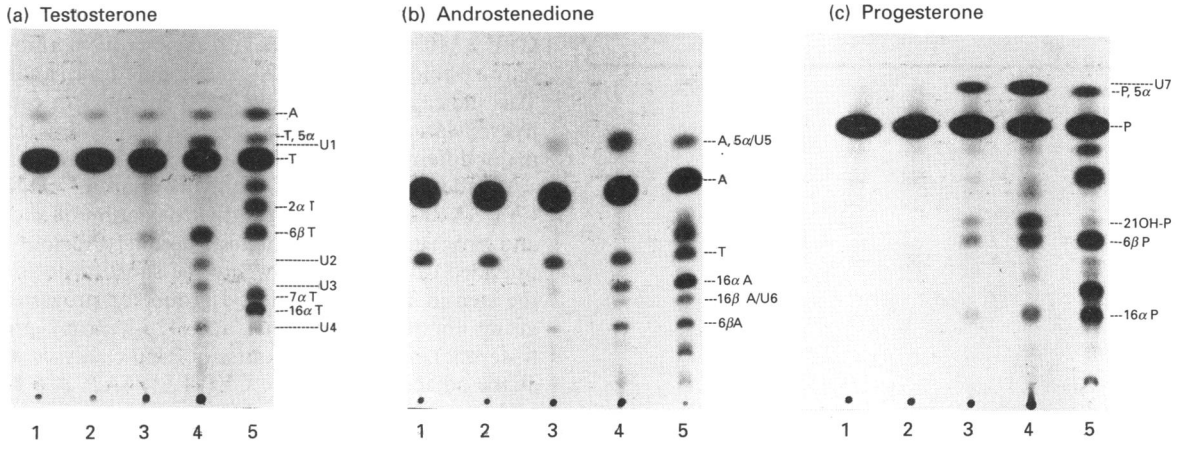

Figure 2 Steroid-hormone metabolism by cDNA-expressed CYP 3A10

Shown are autoradiographs of silica-gel t.l.c. plates resolving testosterone (**a**), androstenedione (**b**), progesterone (**c**) and their respective metabolites formed in incubations with microsomes prepared from COS cells transfected with pCMV vector (lane 1), P450 reductase cDNA (Lane 2), CYP 3A10 cDNA (lane 3) or both P450 reductase cDNA and CYP 3A10 cDNA (lane 4) or with liver microsomes from untreated adult male rats, which serves as a standard for hydroxysteroid metabolites (lane 5). U1–U7 are unknown metabolites. U1 was distinguished from authentic 5-dihydrotestosterone in t.l.c. solvent system AB. U2 co-migrated with authentic 6α-hydroxytestosterone in t.l.c. solvent systems AB and BB, but was distinguished from this compound in system AA. U3 migrated slightly ahead of authentic 11β-hydroxytestosterone and 19-hydroxytestosterone in t.l.c. solvent system BB, but it migrated behind these compounds in systems AB and AA. U4 co-migrated with authentic 15α-hydroxytestosterone in t.l.c. solvent system AB, but it migrated slightly ahead of this compound in system BB and slightly behind in system AA. U5 was distinguished from 5α-dihydroandrostanedione in t.l.c. solvent system PQ. The migration of U6 was similar to [^{14}C]16β-hydroxyandrostenedione formed by purified rat CYP 2B1 [40]. T.l.c. purification and rechromatography in t.l.c. solvent systems PQ, AA and HH resulted in heterogeneity and/or decomposition that is characteristic of the acid-labile 16β-hydroxyandrostenedione [40]. U7 was distinguished from 5α-dihydroprogesterone in t.l.c. solvent system NRR. Abbreviations: T, testosterone; A, androstenedione; P, progesterone; T,5α, 5α-dihydrotestosterone; A,5α, 5α-dihydroandrostenedione; P,5α, 5α-dihydroprogesterone; 6βT, 6β-hydroxytestosterone, etc.

and 132 pmol/min per mg respectively were determined for androstenedione 6β-hydroxylation, and values of 25 μM and 164 pmol/min per mg respectively were determined for lithocholic acid 6β-hydroxylation (Figure 3). CYP 3A10 thus exhibits a 3.7-fold higher efficiency ($V_{max.}/K_m$) for metabolism of lithocholic acid as compared with androstenedione.

Table 2 Steroid hydroxylase activities in microsomes from COS cells transfected with the indicated cDNAs in pCMV expression vectors

Details of metabolite identification and quantification are described in the Materials and methods section. Results are expressed as the means ± S.D. for three independent experiments. Unknowns 1–7 are metabolites that were distinguished from the available collection of authentic monohydroxysteroids [11] (see legend to Figure 2), and thus remain unidentified. Unknowns A and B correspond to the unidentified metabolites of lithocholic acid that migrate just ahead of and just behind lithocholic acid respectively in the t.l.c. assay system of Teixeira and Gil [5]. Activities catalysed by COS cells transfected with the pCMV expression vector without a cDNA insert were comparable with, or less than, those shown for cells transfected with the P450 reductase cDNA. Abbreviation: ND, not determined.

	Activity (pmol/min per mg of protein)		
Metabolite	P450 reductase cDNA	CYP 3A10 cDNA	P450 reductase cDNA + CYP 3A10 cDNA
Testosterone			
6β-Hydroxy	3 ± 2	45 ± 1	288 ± 23
Unknown 1	< 1	35 ± 13	164 ± 28
Unknown 2	< 1	9 ± 5	95 ± 11
Unknown 3	< 1	11 ± 3	71 ± 10
Unknown 4	< 1	3 ± 1	43 ± 8
Androstenedione			
6β-Hydroxy	5 ± 6	23 ± 7	107 ± 19
6α-Hydroxy	7 ± 7	20 ± 4	122 ± 11
Unknown 5	< 1	49 ± 9	406 ± 28
Unknown 6	2 ± 3	10 ± 1	55 ± 14
Progesterone			
16β-Hydroxy	3 ± 2	52 ± 3	150 ± 7
16α-hydroxy	< 1	22 ± 1	68 ± 4
21-Hydroxy	< 1	33 ± 3	145 ± 17
Unknown 7	2 ± 3	101 ± 30	738 ± 77
Lithocholic acid			
6β-Hydroxy	< 1	14	112 ± 22
Unknown A	< 1	ND	121 ± 9
Unknown B	< 1	ND	16 ± 1

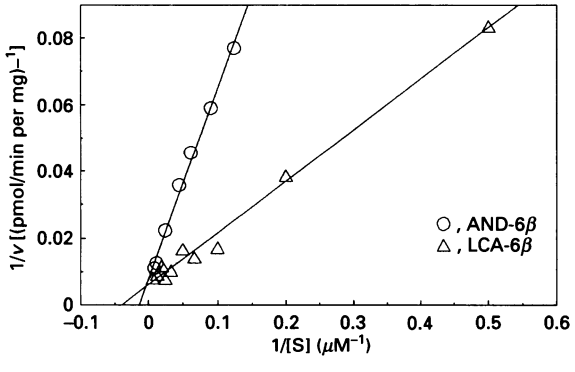

Figure 3 Steady-state kinetic analysis of androstenedione 6β-hydroxylation and lithocholic acid 6β-hydroxylation catalysed by CYP 3A10

Microsomes prepared from COS cells co-transfected with CYP 3A10 and P450 reductase cDNAs in pCMV expression vectors were used for a kinetic analysis of 6β-hydroxylation of lithocholic acid and androstenedione. Experiments were performed as outlined in the Material and methods section. The data are from representative experiments and are shown in the form of Lineweaver–Burk double-reciprocal plots. The K_m and $V_{max.}$ values were determined graphically from these data: androstenedione 6β-(AND-6β) hydroxylation, K_m = 75 μM and $V_{max.}$ = 132 pmol/min per mg; lithocholic acid 6β-hydroxylation (LCA-6β), K_m = 25 μM and $V_{max.}$ = 164 pmol/min per mg.

DISCUSSION

The present study demonstrates that, in addition to bile acids, the hamster liver lithocholic acid 6β-hydroxylase CYP 3A10 is capable of hydroxylating another group of endogenous substances, the steroid hormones. CYP 3A10-catalysed hydroxylation of testosterone, androstenedione and progesterone occurred at multiple sites and in a regioselective and stereospecific manner, with the 6β-position of the steroid B-ring a common site of hydroxylation for all three neutral steroids and for lithocholic acid. Steroid-hormone 6β-hydroxylation is also catalysed by several other family 3A P450s, including rat CYPs 3A1 and 3A2 [18–21], adult human CYPs 3A3, 3A4 and 3A5 [16,22–24], human fetal CYP 3A7 [25] and rabbit CYP 3A6 [26,27]. This suggests that important active-site determinants of steroid hydroxylase regiospecificity are broadly conserved in the CYP 3A gene family.

Several rat and human 3A P450s hydroxylate testosterone primarily at the 6β-position, but also utilize the 2β- and 15β-positions as minor hydroxylation sites. Purified rat CYP 3A1 catalyses formation of 6β-, 2β- and 15β-hydroxytestosterone at ratios of ~ 5:2:1 [20] and cDNA-expressed human CYP 3A4 forms these metabolites at ratios of ~ 24:2:1 [16]. By contrast, no testosterone 2β- or 15β-hydroxylase activity was detected for the hamster testosterone 6β-hydroxylase CYP 3A10 in the present study. CYP 3A10 does metabolize lithocholic acid at several positions in addition to 6β, but these have not been identified [5]. Lithocholic acid differs from the androgens and progesterone by the presence of a carboxy group on its five-carbon D-ring side chain. In addition, the 5β-reduced steroid A–B-ring juncture gives the bile acid a non-planar ring system, which contrasts with the planar ring fusions that characterize the neutral steroid hormones examined in the present study. Nevertheless, lithocholic acid was metabolized at the 6β-position by CYP 3A10 at a rate similar to the steroid hormones and with only a 3-fold difference in K_m as compared with androstenedione. This indicates that the same P450 enzyme can catalyse the 6β-hydroxylation of these two classes of endogenous steroids despite major differences in polarity and overall shape of these molecules.

In addition to steroid 6β-hydroxylation, cDNA expressed CYP 3A10 catalysed the 16α-hydroxylation of androstenedione and progesterone, but not testosterone. Previous studies have provided other examples where the substituent at carbon-17 of the steroid D-ring (17β-acetyl group for progesterone, 17-oxo group for androstenedione and 17β-hydroxy group for testosterone) influences the course of P450-catalysed hydroxylation at the adjacent carbon-16. CYP 1A1, 1A2, 2C6 and 2C13 can each metabolize progesterone to form 16α-hydroxyprogesterone, but the same regioisomer of testosterone or androstenedione is not formed or is formed at much lower rates (CYP 2C13) by these purified P450s [1,3]. Similarly, cDNA-expressed CYP 3A3 and CYP 3A4 are modestly active catalysts of progesterone 16α-hydroxylation, but they have no detectable testosterone or androstenedione 16α-hydroxylase activity [16]. These observations suggest that the 17β-acetyl group of progesterone plays an important role in directing hydroxylation to the adjacent 16α-hydroxy position.

CYP 3A10 metabolized progesterone at the 6β-, 16α- and 21-positions. 21-Hydroxyprogesterone, also known as deoxycorticosterone, has intrinsic hormonal activities, suggesting that CYP

3A10 does not simply catalyse the catabolism of steroid hormones. Of the many liver P450s examined to date, only two others are active progesterone 21-hydroxylases: rabbit CYP 2C5 [28,29] and rat CYP 2C6 [30]. Human liver P450s do not play a significant role in progesterone 21-hydroxylation, since this activity is absent, or present at very low levels, in human liver microsomes [22,31].

The precise function(s) of CYP 3A10 *in vivo* are presently unknown. Since the enzyme's K_m for lithocholic acid 6β-hydroxylation is 3-fold lower than for androstenedione 6β-hydroxylation, the bile acid might serve as the preferred physiological substrate. Whether CYP 3A10 also catalyses 6β-hydroxylation of other bile acids is not known. Many other P450s in the CYP 3A subfamily actively catalyse the oxidation of numerous xenobiotics, including clinically useful drugs [4,32], suggesting that CYP 3A10 might also play a role in drug and xenobiotic metabolism.

The expression of CYP 3A10 mRNA and its associated microsomal lithocholic acid 6β-hydroxylase activity are male-specific in hamster liver [5]. These findings were confirmed at the protein level in the present study and may provide an explanation for the observed sex differences in bile acid metabolism in rodents [33]. The underlying factors governing this sex-dependent expression of CYP 3A10 are not known. However, the expression of other male-specific P450s such as CYP 2A2, 2C11, 3A2 and 4A2 is regulated hormonally, either in a positive or negative manner [17,34–36], and perturbation of hormonal status by drugs [37] and other xenobiotics [38] can lead to major suppression of these hormone-dependent P450s. If CYP 3A10 expression is also influenced by these types of factors, then one consequence could be a decrease in 6β-hydroxylation, leading to an accumulation of lithocholic acid and possibly cholestasis [39]. Preliminary studies demonstrate that in humans, lithocholic acid is also hydroxylated by a member of the CYP 3A subfamily, CYP 3A4 [10], suggesting that the hamster could serve as a useful animal model for studying the role of CYP 3A enzymes in cholestasis.

This work was supported in part by National Institutes of Health grant DK33765 (to D.J.W.) and the American Heart Association grant-In-aid 91010730 (to G.G.). T.C. is the recipient of a Canadian Association of Gastroenterology/Janssen Research Fellowship, J.T. is a March of Dimes Research Foundation Predoctoral Fellow and G.G. is an Established Investigator of the American Heart Association.

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Received 11 August 1992/20 October 1992; accepted 9 November 1992