

CHARACTERIZATION OF TESTOSTERONE 11 β -HYDROXYLATION CATALYZED BY HUMAN LIVER MICROSOMAL CYTOCHROMES P450

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ABSTRACT:

A combination of accelerator mass spectrometry (AMS) and liquid chromatography-tandem mass spectrometry has been used to clarify some new aspects of testosterone metabolism. The main pathway of testosterone oxidative metabolism by human liver microsomes is the formation of 1 β -, 2 α -/ β -, 6 β -, 15 β -, and 16 β -hydroxytestosterones, mainly catalyzed by cytochromes P450 2C9, 2C19, and 3A4. We now report the first determination that 11 β -hydroxytestosterone (11 β -OHT) can also be formed by human liver microsomal fractions. The structures of five hydroxylated metabolites of testosterone (2 β -, 6 β -, 11 β -, 15 β -, and 16 β -OHT) and the C-17 oxidative metabolite androstenedione were determined by liquid chromatography with UV detection at 240 nm and liquid chromatography-tandem mass spectrometry. Corresponding results were obtained by high-performance liquid chromatography-

AMS analysis of incubations of [4-¹⁴C]testosterone with human liver microsomes. 6 β -Hydroxylation was always the dominant metabolic pathway, but 2 β -, 15 β -, and 16 β -OHT, and androstenedione were also formed. The previously undetected hydroxytestosterone, 11 β -OHT, was found to be a minor metabolite formed by human liver microsomal enzymes. It was formed more readily by CYP3A4 than by either CYP2C9 or CYP2C19. 11 β -Hydroxylation was inhibited by ketoconazole (IC₅₀ = 30 nM) at concentrations similar to the IC₅₀ (36 nM) for 6 β -hydroxylation. Therefore, CYP3A4 could be mainly responsible for testosterone 11 β -hydroxylation in the human liver. These findings identify human hepatic biotransformation of testosterone to 11 β -OHT as a previously unrecognized extra-adrenal metabolic pathway.

Cytochrome P450 (P450) enzymes, a superfamily of more than 160 known members, play a major role in the metabolism of numerous physiological substrates, and liver microsomal P450 enzymes are responsible for the biosynthesis or catabolism of steroid hormones. Hydroxylation of active androgens is usually associated with a decrease in biological certainty (Hobkirk, 1979). Endogenous and exogenous testosterone undergoes oxidative metabolism by the human liver cytochrome P450 enzymes (Wood et al., 1988; Yamazaki and Shimada, 1997; Rendic et al., 1999). β -Hydroxylation at either the C6 or C16 position is the major route of testosterone oxidative metabolism, whereas 1 β -, 2 α / β -, and 15 β -hydroxytestosterone are produced as minor metabolites. Human liver enzymes are also found to oxidize testosterone at the C17 position to form androstenedione (Fig. 1).

Various P450 enzymes form hydroxylated steroids highly stereospecifically and regioselectively (Wood et al., 1983). An alteration of the steroid hydroxylation metabolism in liver microsomes therefore indicates altered expression of various P450 enzymes. Liver tumor microsomes, whether from chemically induced or spontaneous tu-

mors, showed a radically modified steroid hydroxylation pattern (Feuer et al., 1986; Lange et al., 1989). The 11 β -hydroxylase is the most thoroughly studied mammalian enzyme involved in steroid hormone biosynthesis. The observation that 11-deoxycorticosterone and 11-deoxycortisol are hydroxylated at the 11 β -carbon position by the enzyme in the mitochondrial fraction of the adrenal suggests that a single reactive site on the enzyme is involved in both cases (Sharma et al., 1962). Biochemical abnormalities are completely linked to the congenital adrenal hyperplasia that causes altered excretion of the tetrahydro-metabolites of these compounds (White et al., 1994; Choi et al., 2002). 11 β -Hydroxylation was confirmed in human adrenal homogenate as the major metabolic pathway of testosterone (Chang et al., 1963) as well as of certain neutral and phenolic steroids (Knuppen and Breuer, 1962; Hudson and Killinger, 1972). On incubating testosterone with a testicular tumor, 11 β -hydroxytestosterone (11 β -OHT) also was observed (Savard et al., 1960). Although the liver is responsible for most of the metabolism of cholesterol and the steroid hormones, to our knowledge, no metabolic study showing the roles of 11 β -hydroxylase on the endogenous steroid metabolism in the human liver has been published.

Whether testosterone may undergo hydroxylation at the C11 position by human liver microsomal P450s has apparently been overlooked, perhaps due in part to techniques that were less specific and less sensitive than those now available. In many studies (van der Hoeven, 1984; Smith et al., 1992; Tachibana and Tanaka, 2001), testosterone metabolites were assayed by HPLC with UV detection

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ABBREVIATIONS: OHT, hydroxytestosterone; LC, liquid chromatography; LC-UV, high-performance liquid chromatography with UV detection; HPLC, high-performance liquid chromatography; AMS, accelerator mass spectrometry; CID, collision-induced dissociation; KTZ, ketoconazole.

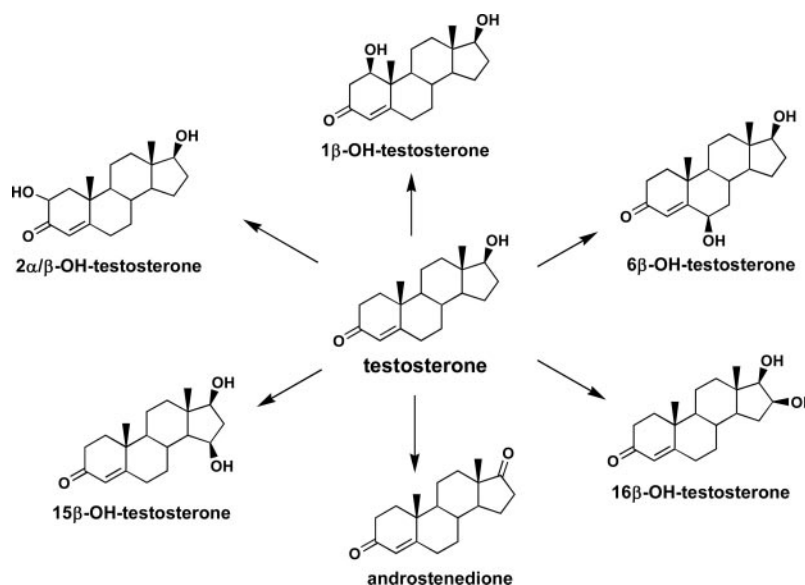


FIG. 1. Oxidative metabolism of testosterone by human liver microsomal cytochrome P450 enzymes.

(LC-UV) and/or gas chromatography-mass spectrometry or LC-mass spectrometry in which 11 β -OHT, which may have been present only at trace levels, might not have been detected. In the present study, we have re-examined hepatic microsomal metabolism of testosterone, using ^{14}C labeling to ensure detection of all metabolites with equal sensitivity and LC-tandem mass spectrometry for structural identification. Individuals with congenital adrenal hyperplasia suffer from hypertension due to a complete deficiency of adrenal 11 β -hydroxylase (Eberlein and Bongiovanni, 1956). In most classic cases of this deficiency, there is overproduction of steroid precursors proximal to the blocked 11 β -hydroxylase, leading to pathological effects (Zachmann et al., 1983). In this paper, we demonstrate that the human liver is capable of generating 11 β -oxygenated steroids from precursors such as testosterone. This could potentially have implications for either diagnosis or therapeutic treatment of adrenal insufficiency diseases.

Materials and Methods

Chemicals and Reagents. Testosterone and its hydroxylated metabolites, as well as androstenedione and 17 α -methyltestosterone, were purchased from Steraloids (Newport, RI). [4- ^{14}C]Testosterone (specific activity, 50 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Pooled human liver microsomes and selectively expressed human cytochrome P450 enzymes (CYP2C9, CYP2C19, and CYP3A4) were purchased from BD Gentest (Woburn, MA). Liver microsome protein content was 20 mg/ml in 250 mM sucrose. Recombinant P450 isoforms were expressed in insect cells selectively transfected with a baculovirus expression system containing the cDNA for human CYP2C9 (total protein concentration, 4 mg/ml; P450 content, 500 pmol/mg), CYP2C19 (3 mg/ml, 360 pmol/mg), and CYP3A4 (5 mg/ml, 400 pmol/mg). The catalytic activities for CYP2C9 (according to diclofenac 4'-hydroxylase), CYP2C19 [(*S*)-mephenytoin 4'-hydroxylase], and CYP3A4 (testosterone 6 β -hydroxylation) were 8000, 900, and 2200 pmol/mg P450/min, respectively.

Single human liver samples were obtained from organ donors or patients undergoing liver resection (Tennessee Donor Service, Nashville, TN). Each liver was tested for pathogenicity using a polymerase chain reaction protocol. Liver samples (0.3 g) were homogenized in 10 ml of ice-cold 10 mM potassium phosphate buffer (pH 7.4) using a Polytron homogenizer. The cell debris, nuclei, and mitochondria were removed by centrifugation at 10,000g for 20 min at 4°C, and then the supernatant was ultracentrifuged at 120,000g for 2 h at 4°C. The pellet was resuspended in ice-cold phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v; Guengerich, 1994). Aliquots

of microsomal fractions were quickly stored at -80°C until used. Protein concentrations of the microsomal fractions were measured in triplicate, with bovine serum albumin as a calibration standard, using commercial protein assay kits (Bio-Rad, Hercules, CA). Cytochrome P450 was estimated by absorbance differences between 450 and 490 nm based on the method of Omura and Sato (1964).

The NADPH-regenerating system used for all NADPH-requiring oxidase assays was obtained from BD Gentest. The system consists of two solutions (solution A, 26.1 mM NADP^+ , 66 mM glucose 6-phosphate, and 66 mM MgCl_2 in H_2O ; solution B, 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium acetate). A stock solution of steroids including 17 α -methyltestosterone was prepared at concentrations of 1 $\mu\text{mol}/\text{ml}$ in methanol. The stock solution was used to prepare a working solution of varying concentrations (1–100 $\mu\text{mol}/\text{ml}$) in methanol.

Microsomal Assays. A 0.3-ml reaction mixture containing 0.33 mg/ml human liver microsomal protein or 120 pmol/ml human CYP2C9, CYP2C19, or CYP3A4, 50 μl of NADPH-regenerating solution A, 20 μl of solution B, and 0.2 μmol of testosterone in 100 mM potassium phosphate (pH 7.4) was incubated at 37°C for 20 min. After incubation, the reaction was terminated by the addition of 0.5 ml of acetonitrile, and 50 μl of methyltestosterone (1 mM) was added. The mixture was then centrifuged (12,000 rpm) for 10 min to precipitate protein. The supernatant, filtered with a VariSep Nylon Syringe Filter (0.2 $\mu\text{m} \times 4$ mm; Varian, Inc., Palo Alto, CA), was dried under a stream of nitrogen and then reconstituted with 50 μl of 50% methanol.

Recovery Test and Calculations. Solutions containing testosterone and its six metabolites at varied concentrations were prepared for method validation. All quantitative calculations were based on the peak area ratios relative to that of the internal standard. To prepare the calibration curve, enzyme-free incubation solutions with increasing concentrations of added analyte (0.1–2 mM testosterone and 0.2–10 μM for all metabolites) and a fixed concentration of added 17 α -methyltestosterone (50 μl ; 1 mM) were analyzed according to the procedure described above. The recovery of the procedure was measured by comparing the responses obtained from the extracted samples to those obtained from the corresponding unextracted reference standards prepared at the same concentrations.

Instrumentation and Chromatography. LC analyses were carried out with an Agilent 1100 binary pumping system (Agilent Technologies, Palo Alto, CA), using a 9725 Rheodyne injector (Rheodyne, Rohnert Park, CA) and a G1314A variable wavelength UV detector set at 240 nm. An aliquot of the sample (2 μl) from each incubation was injected onto a Capcell Pak C18 UG120 column (2.0 \times 150 mm, 5- μm particle size; Shiseido Fine Chemicals, Tokyo, Japan) and eluted at a flow rate of 0.2 ml/min by gradients of solvent A (20 mM ammonium acetate in 10% methanol) and solvent B (90% metha-

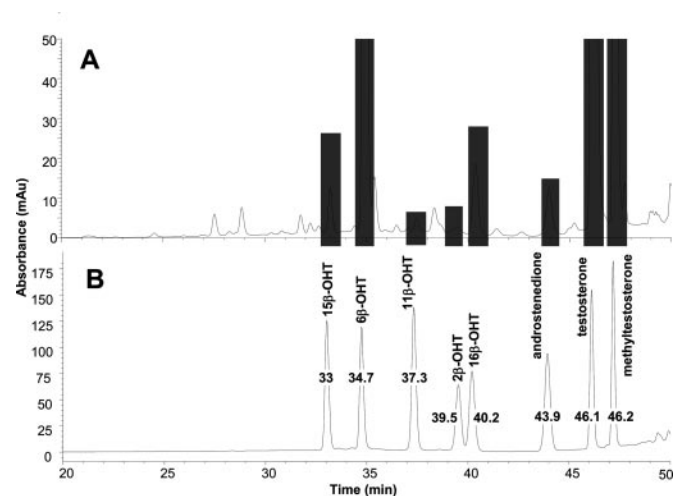


FIG. 2. Representative LC-UV_{240 nm} chromatograms of the testosterone metabolites formed by human liver microsomes (A) and standards (B). The retention times of the standards are overlaid on the corresponding peaks.

no). The typical gradient (solvent B) was as follows: 0 min, 10%; 0 to 10 min, 10%; 10 to 20 min, 30%; 20 to 30 min, 55%; 30 to 38 min, 55%; 38 to 45 min, 100%; 45 to 50 min, 100%. The gradient was then returned to the initial condition (10% B) and held for 8 min before analysis of the next sample.

On-line mass spectrometric analyses were performed using an ion trap (Agilent MSD-Trap XTC) equipped with an electrospray ionization interface. When there was insufficient material for on-line mass spectral analysis, multiple fractions were collected, pooled, and concentrated prior to off-line analysis using flow injection. Collision-induced dissociation (CID) spectra were collected on positive ions using the auto-CID function of the instrument. Nitrogen gas from a liquid-nitrogen dewar boil-off was used as nebulizer (12 psi) and curtain gas (12 l/min). Helium was the collision gas. The heated nebulizer probe temperature and voltage were maintained at 350°C and 5000 V, respectively.

AMS analyses were conducted by the Biological Engineering Accelerator Mass Spectrometry Lab at Massachusetts Institute of Technology. The AMS instrument has previously been described in detail (Lieberman et al., 2004a). The interface used to generate CO₂ from liquid samples has also been described in detail (Lieberman et al., 2004b), and an example of its application to the analysis of HPLC effluent in continuous fashion has been presented (Skipper et al., 2004).

Results

Metabolite Profile/Pooled Liver Microsomes. A representative LC profile for the testosterone metabolite peaks detected after incubation of testosterone with human liver microsomes and NADPH is shown in Fig. 2. The identity of each of the major metabolites was determined through comparison of its LC-UV retention time and its mass spectrum with those of an authentic standard. Using this double match-up method, testosterone, four hydroxylated metabolites (15β-, 6β-, 2β-, and 16β-OHT), and androstenedione were identified. The small peak corresponding to 11β-OHT was tentatively identified based on its LC-UV retention time.

11β-Hydroxylation. To determine whether the peak corresponding to 11β-OHT was produced by P450-catalyzed hydroxylation of testosterone, incubations with pooled human liver microsomes but without added testosterone were conducted. Extracts were analyzed by LC-UV. No peak was detected at the retention time of authentic 11β-OHT. To obtain further evidence that the peak observed at the retention time for 11β-OHT was derived from metabolism of testosterone, we analyzed metabolites produced from ¹⁴C-labeled substrate. ¹⁴C was detected by AMS using a previously described system (Lieberman et al., 2004b; Skipper et al., 2004) that facilitates contin-

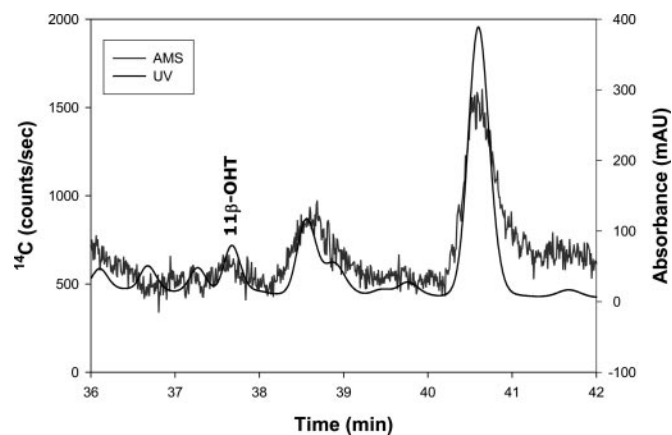


FIG. 3. LC-UV chromatogram and AMS trace of human liver microsomal metabolites in the retention time range of 11β-OHT showing cochromatography for this metabolite. Some of the other UV peaks are not associated with ¹⁴C, suggesting that they are not derived from testosterone.

uous profiling of the isotope concentration in HPLC effluent. Results, shown in Fig. 3, depict the coincident detection of isotope and UV absorbance in a region of the chromatogram that ranges from 11β-OHT to 16β-OHT, and provide a good indication that the peak assumed to be 11β-OHT is derived from testosterone.

Finally, multiple HPLC isolates of the peak corresponding to 11β-OHT (37–38 min) were pooled and concentrated, and the contents were subjected to mass spectrometry. The CID mass spectrum was in good agreement with the mass spectrum of 11β-OHT authentic standard (retention time 37.3 min) (Fig. 4). The other possible isomers, 2α-, 6α-, 7α-, 11α-, and 15α-OHT, were ruled out on the basis of their LC retention times. Liver microsomal formation of 11β-OHT was linear ($r^2 = 0.972$) over the range of 1 to 50 mM substrate concentration.

Metabolism by Recombinant Enzymes. To further characterize the P450 enzyme dependence of 11β-hydroxylation, testosterone was added to the incubation mixture containing recombinant CYP2C9, CYP2C19, or CYP3A4, and the oxidative metabolites of testosterone were analyzed by LC-UV. Five hydroxylated testosterone metabolites and androstenedione were detected in all catalytic enzyme systems. The metabolite profiles were found to be dependent on the nature of the catalytic enzymes. As reported previously (Waxman et al., 1991; Yamazaki and Shimada, 1997), CYP3A4 was a major catalytic enzyme for formation of 15β-, 6β-, and 16β-OHT, whereas it catalyzed formation of 2β-OHT and androstenedione poorly. In contrast, CYP2C9 and CYP2C19 were found to form androstenedione at high rates. In addition, CYP2C9 and CYP2C19 were found in this study to hydroxylate testosterone less efficiently than CYP3A4 at all positions.

In addition, CYP3A4 catalyzed the formation of 11β-OHT at rates higher than those of CYP2C9 and CYP2C19. Catalytic ratios of 11β-OHT versus 6β-OHT in testosterone hydroxylation by recombinant P450 enzymes, including CYP2C9, CYP2C19, and CYP3A4, indicated 6β-OHT as a major product. The ratios of 11β-OHT/6β-OHT were 0.56, 0.40, and 0.11 for CYP2C9, CYP2C19, and CYP3A4, respectively (Fig. 5). The 11β-OHT/6β-OHT ratio in CYP3A4 was 5- and 4-fold lower than those in CYP2C9 and CYP2C19, respectively, whereas the 11β-OHT/testosterone (11β-OHT/T) ratio in CYP3A4 (0.012) was 4 times higher than those in CYP2C9 (0.003) and CYP2C19 (0.003; Fig. 6).

Metabolism by Individual Human Liver Microsomes. All nine human liver microsomal preparations yielded a qualitatively similar metabolite profile. Interindividual variations in levels of each metabolite were evident (data not shown). The formation of 11β-hy-

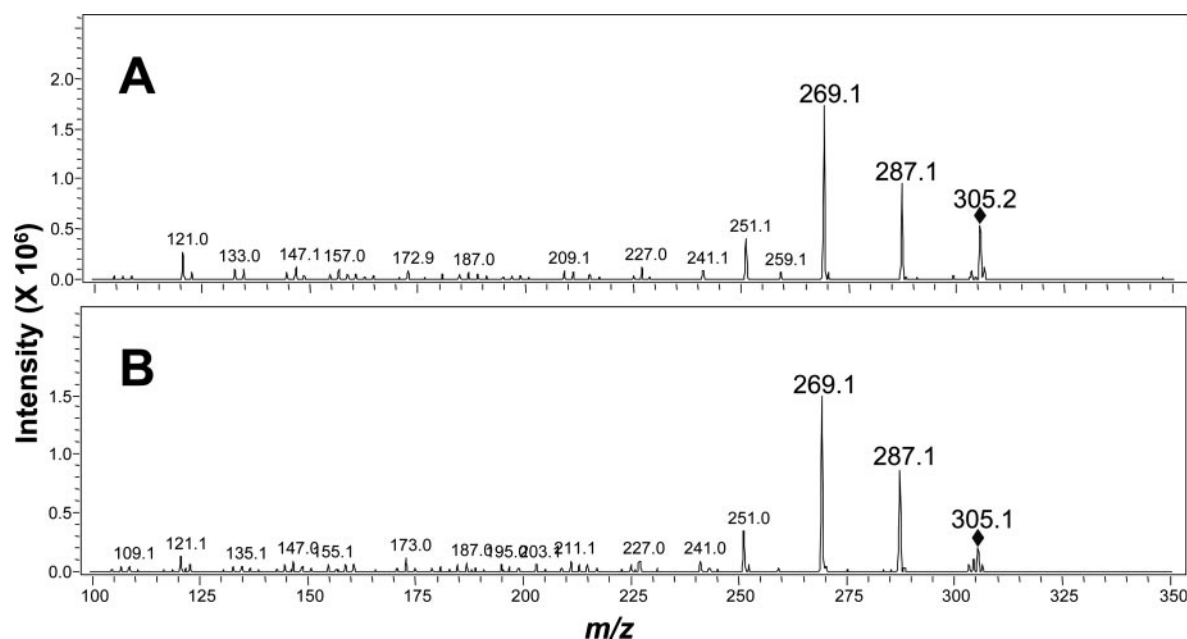


FIG. 4. CID mass spectra of 11 β -OHT as an authentic standard (A) and a metabolite derived from testosterone (B). The CID energy was adjusted so that some of the protonated molecular ion (MH^+ ; m/z 305) was always present in the spectrum.

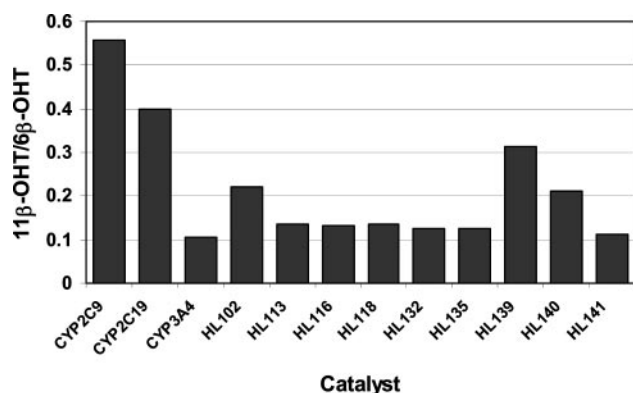


FIG. 5. Ratio of 11 β -OHT to 6 β -OHT produced by recombinant cytochromes P450 2C9, 2C19, and 3A4, and by individual human liver microsomes. Values are the mean of three runs.

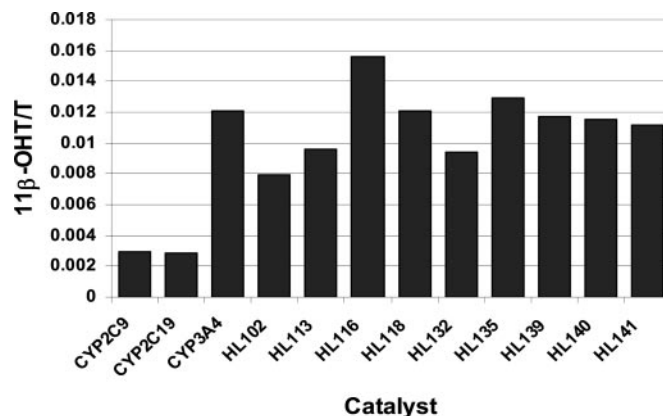


FIG. 6. Ratio of 11 β -OHT to testosterone. Data are from the same samples characterized in Fig. 5.

droxytestosterone was observed in every case. The ratio of 11 β -OHT to 6 β -OHT ranged from 0.11 to 0.31 (Fig. 5). The ratios of 11 β -OHT exhibited somewhat less variation (Fig. 6).

Inhibition of 11 β -Hydroxylation with Ketoconazole. KTZ is a selective inhibitor of CYP3A4 activity in human liver microsomes (IC_{50} = 40 nM), as evaluated by the 6 β -hydroxylation of testosterone (Eagling et al., 1998). To determine whether the CYP3A4 subfamily isoform also plays a predominant role in the 11 β -hydroxylation of testosterone in human liver microsomes, KTZ inhibition studies were conducted. The effects of coinubation with KTZ on testosterone 6 β - and 11 β -hydroxylation are shown in Fig. 7. As predicted, KTZ deactivated testosterone 6 β -hydroxylation, and the formation of 11 β -hydroxytestosterone was also inhibited with similar IC_{50} values, which were 36 nM and 30 nM for 6 β -hydroxylation and 11 β -hydroxylation, respectively.

Extraction Efficiency and Reproducibility. An average of $94.6 \pm 1.7\%$ (mean \pm S.D.) of testosterone and its six oxidative metabolites was recovered when the incubation mixture was extracted and analyzed by LC. Statistical analysis of triplicate determinations of tes-

tosterone and its metabolites showed an average intra-assay variation of <6%.

Discussion

The 11 β -hydroxylation of steroids occurs mainly in the adrenal cortex mitochondria. In the conversion of cholesterol into cortisol in the adrenal cortex, the steroid must move sequentially from the mitochondria (side chain cleavage) to the endoplasmic reticulum (17 α - and 21-hydroxylation) and then back to the mitochondria for 11 β -hydroxylation. Depending on whether the P450 hydroxylase is localized in the mitochondria or in the endoplasmic reticulum, there are two slightly different electron transport chains that function to transfer a pair of electrons from NADPH to the P450 enzyme. The most important component of the electron transport chain is the P450 protein, in that it determines the substrate specificity and dictates the precise site of hydroxylation. In principle, the three-dimensional structure of the substrate-binding domain of a P450 hydroxylase determines which substrates are able to acquire a new hydroxyl group.

A number of studies have shown that P450 enzymes in liver microsomes play an important role in the oxidative metabolism of

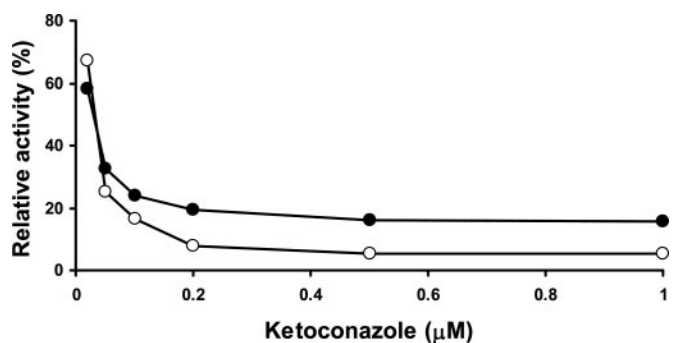


Fig. 7. Inhibition of pooled human liver microsomal activity by ketoconazole. ○, 6 β -hydroxylation; ●, 11 β -hydroxylation. Data are the mean values from three experiments.

steroids at positions other than C-11 (Waxman et al., 1991; Yamazaki and Shimada, 1997). Human liver microsomal CYP3A4 is responsible for the greatest portion of testosterone 6 β - and 16 β -hydroxylation (Kawano et al., 1987; Yamazaki and Shimada, 1997). CYP2C9 and CYP2C19 were also capable of oxidative catalysis of testosterone to form 2 β -, 6 β -, 15 β -, and 16 β -OHT, and androstenedione.

There is evidence for the occurrence of extra-adrenal hydroxylation of steroids at C-11. A virilizing testicular tumor, which exerts its effects by producing androgens, has been shown to form 11 β -OHT (Savard et al., 1960). 11 β -Hydroxylation of osaterone acetate (17 α -acetoxy-6-chloro-2-oxa-4,6-pregnandiene-3,20-dione), an exogenous steroid, by liver microsomal fraction (Minato et al., 1999) has also been demonstrated.

In the present study, hepatic hydroxylation at the 11 β -position of testosterone was observed, and the activity was higher with CYP3A4 than with CYP2C9 and CYP2C19. Chemical inhibition was used to further investigate the role of individual P450s. In many studies, KTZ mainly inhibits CYP3A4 activity at different concentrations (Newton et al., 1995; Yamazaki and Shimada, 1997; Eagling et al., 1998). Our data clearly indicate that KTZ selectively inhibited both testosterone 6 β - and 11 β -hydroxylation by CYP3A4 in human liver microsomes.

Human liver microsomal P450 enzymes are shown to be capable of metabolism of endogenous steroids as well as numerous therapeutic agents, and this metabolism seems to be differentially altered by a number of physiological and pathophysiological conditions. The oxidation of testosterone to 11 β -OHT by 11 β -hydroxylase in the adrenal cortex is well known. Although the physiological significance of the formation of 11 β -OHT by human liver remains unknown at present, our results suggest that this metabolite may participate in the generation of the androgens, which are formed in considerable quantities by the human liver catalyzed by CYP2C9, 2C19, and 3A4, as well as liver-associated, i.e., extra-adrenal, oxidation of testosterone to 11 β -OHT.

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