CHARACTERIZATION OF TESTOSTERONE 11β-HYROXYLATION CATALYZED BY HUMAN LIVER MICROSONAL CYTOCHROMES P450

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ABSTRACT:

A combination of accelerator mass spectrometry (AMS) and liquid chromatography-tandem mass spectrometry (AMS) analysis of incubations of [4-14C]testosterone with human liver microsomes 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 1α-, 2α-/β-, 6α-, 15α-, and 16β-hydroxytestosterones, mainly catalyzed by cytochromes P450 2C9, 2C19, and 3A4. We now report the first determination that 11β-hydroxytestosterone (11β-OHT) can also be formed by human liver microsomal fractions. The structures of five hydroxylated metabolites of testosterone (2β-, 6β-, 11β-, 15β-, and 16β-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. 6β-Hydroxylation was always the dominant metabolic pathway, but 2β-, 15β-, and 16β-OHT, and androstenedione were also formed. The previously undetected hydroxytestosterone, 11β-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. 11β-Hydroxylation was inhibited by ketoconazole (IC50 = 50 nM) at concentrations similar to the IC50 (36 nM) for 6β-hydroxylation Therefore, CYP3A4 could be mainly responsible for testosterone 11β-hydroxylation in the human liver. These findings identify human hepatic biotransformation of testosterone to 11β-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 1α-, 2α-/β-, and 15β-hydroxysteroid 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 tumors, showed a radically modified steroid hydroxylation pattern (Feuer et al., 1986; Lange et al., 1989). The 11β-hydroxyis is the most thoroughly studied mammalian enzyme involved in steroid hormone biosynthesis. The observation that 11-deoxycorticosterone and 11-deoxycortisol are hydroxylated at the 11β-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; Chö et al., 2002). 11β-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, 11β-hydroxytestosterone (11β-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 11β-hydroxylation 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.

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.
(LC-UV) and/or gas chromatography-mass spectrometry or LC-mass spectrometry in which 11β-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 tritium 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 11β-hydroxylase (Eberlein and Bongiovanni, 1956). In most classic cases of this deficiency, there is overproduction of steroid precursors proximal to the blocked 11β-hydroxylase, leading to pathological effects (Zachmann et al., 1983). In this paper, we demonstrate that the human liver is capable of generating 11β-oxygenated steroids from precursors such as testosterone. This could potentially have implications for either diagnosis or therapeutic treatment of adrenal insufficiency.

Materials and Methods

Chemicals and Reagents. Testosterone and its hydroxylated metabolites, as well as androstenedione and 17α-methyltestosterone, were purchased from Steraloids (Newport, RI). [(4,14C)Testosterone (specific activity, 50 mCi/μmol) 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 microsomal protein content was 20 mg/ml in 250 mM succrose. 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 (5α-methylenetoin 4′-hydroxylase), and CYP3A4 (testosterone 6β-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 microsomes (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 100,000 g 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 MgCl2 in H2O; solution B, 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium acetate). A stock solution of steroids including 17α-methyltestosterone was prepared at concentrations of 1 μmol/ml in methanol. The stock solution was used to prepare a working solution of varying concentrations (1–100 μmol/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 0.45-mm filter, was injected onto a Capcell Pak C18 STR 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 17α-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 C18 UG120 column (2.0 x 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-
mass spectrum with those of an authentic standard. Using this double
determined through comparison of its LC-UV retention time and its
shown in Fig. 2. The identity of each of the major metabolites was
bation of testosterone with human liver microsomes and NADPH is
LC profile for the testosterone metabolite peaks detected after incu-

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 analy-

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 (Liberman et al., 2004a). The interface used to generate CO2 from liquid samples has also been de-

Results

Metabolite Profile/Pooled Liver Microsomes. A representative
LC profile for the testosterone metabolite peaks detected after incuba-
tion 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 testo-
sterone, incubations with pooled human liver microsomes but with-
out 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 testos-

terone, we analyzed metabolites produced from 14C-labeled substrate.
14C was detected by AMS using a previously described system
(Liberman et al., 2004b; Skipper et al., 2004) that facilitates contin-
umous 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 stan-
dard (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 concentra-

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 en-
zyme 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 recombi-
nant 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 metab-
olite were evident (data not shown). The formation of 11β-hy-
droxytestosterone was observed in every case. The ratio of 11β-OHT to 6β-OHT ranged from 0.11 to 0.31 (Fig. 5). The ratios of 11β-OHT exhibited somewhat less variation (Fig. 6).

Inhibition of 11β-Hydroxylation with Ketoconazole. KTZ is a selective inhibitor of CYP3A4 activity in human liver microsomes (IC₅₀ = 40 nM), as evaluated by the 6β-hydroxylation of testosterone (Eaglin et al., 1998). To determine whether the CYP3A4 subfamily isoform also plays a predominant role in the 11β-hydroxylation of testosterone in human liver microsomes, KTZ inhibition studies were conducted. The effects of coincubation with KTZ on testosterone 6β- and 11β-hydroxylation are shown in Fig. 7. As predicted, KTZ deactivated testosterone 6β-hydroxylation, and the formation of 11β-hydroxysteroid was also inhibited with similar IC₅₀ values, which were 36 nM and 30 nM for 6β-hydroxylation and 11β-hydroxylation, respectively.

Extraction Efficiency and Reproducibility. An average of 94.6 ± 1.7% (mean ± 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 testosterone and its metabolites showed an average intra-assay variation of <6%.

Discussion

The 11β-hydroxylation of steroids occurs mainly in the adrenal cortex mitochondria. In the conversion of cholesterol to cortisol in the adrenal cortex, the steroid must move sequentially from the mitochondria (side chain cleavage) to the endoplasmic reticulum (17α- and 21-hydroxylation) and then back to the mitochondria for 11β-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...
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 6β- and 16β-hydroxylation (Kawano et al., 1987; Yamazaki and Shimada, 1997). CYP2C9 and CYP2C19 were also capable of oxidative catalysis of testosterone to form 2β-, 6β-, 15β-, and 16β-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 11β-OHT (Savard et al., 1960). 11β-Hydroxylation of osaterone acetate (17α-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 11β-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 testosterone was observed, and the activity was higher with CYP3A4.


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