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Purification and Characterization of Constituent Androstenedione 15α-Hydroxylase (Cytochrome $P450_{15\alpha AD}$) from Mouse Liver

SEX- AND TISSUE-DEPENDENT EXPRESSION

*Mahesh C. Sharma, Meena R. Sharma, Seong-Joo Jeong and Bernard H. Shapiro**

LABORATORIES OF BIOCHEMISTRY, UNIVERSITY OF PENNSYLVANIA SCHOOL OF VETERINARY MEDICINE, PHILADELPHIA, PA 19104-6048, U.S.A.

ABSTRACT. Hepatic microsomal androstenedione 15α-hydroxylase (i.e. cytochrome $P450_{15\alpha AD}$) was purified from female CD-1 mice. Protein purification was monitored in eluates from Fractogel, DEAE-Sephacel, and hydroxylapatite columns at heme absorbing 417 nm, by cytochrome P450 content, reactivity to monoclonal antibody against female-specific rat cytochrome P450 2C12, and androstenedione 15α-hydroxylase activity. The catalytic activity for androgens of the purified cytochrome $P450_{15\alpha AD}$, exhibiting a high degree of regioselectivity and stereospecificity, was restricted to the 7α- and 15α-hydroxylation of androstenedione, representing, respectively, >5% and >93% of the total metabolites. Polyclonal antibodies against cytochrome $P450_{15\alpha AD}$ exhibited a concentration-dependent and very selective inhibition of hepatic microsomal androstenedione 7α- and 15α-hydroxylation and a 60% inhibition of benzphetamine demethylation, the latter drug appearing to be a much more effective substrate than androgens. Cytochrome $P450_{15\alpha AD}$ accounted for about 3% of the total P450 in female mouse liver microsomes. The apparent subunit molecular weight of $P450_{15\alpha AD}$ was 53,000, and the protein appeared as a single band on sodium dodecyl sulfate-polyacrylamide gels. The isoform was intensely expressed in both liver and lung of CD-1 female mice and was female-predominant in the livers of five or eight strains examined; it was sex-independent in the remaining three strains. Amino-terminal sequence analysis indicates that cytochrome $P450_{15\alpha AD}$ is a member of the murine cytochrome P450 2c subfamily. BIOCHEM PHARMACOL 52;6:901–910, 1996.

KEY WORDS. androstenedione 15α-hydroxylase, murine; cytochrome $P450_{15\alpha AD}$, murine hepatic; cytochrome P450, murine isoform; cytochrome P450, tissue specificity, murine; drug metabolism, murine; sexual dimorphism, cytochrome P450, murine; murine P450 2c

The P450† enzymes, referred to as mixed-function oxidases or monooxygenases, are a superfamily of enzymes responsible for the metabolism of such endogenous compounds as steroids, fatty acids, thyroid hormone, prostanoids, bile acids, and biogenic amines. In addition, many of the P450 enzymes originally identified as drug-metabolizing enzymes metabolize a wide range of exogenous chemicals, e.g. xenobiotics, ethanol, insecticides, herbicides, food additives, and environmental pollutants. Whereas these enzymes are regarded generally as deactivating or detoxifying agents, they may also function to enhance the biological activity of endogenous compounds (e.g. glucocorticoids, leukotrienes) as well as increase the toxicity of some environmental chemicals (e.g. carcinogens). To date, more than 200 P450 genes have been characterized in such diverse organisms as bacteria, snails, flies, fishes, birds, and mammals in this ancient gene superfamily that has existed for over 3.5 billion years [1–3]. Within the past decade or so an enormous amount of information has been published regarding the identification and characterization of scores of forms of P450 and their genes. In a vast number of these studies, the rat has been the model of interest [3, 4]. Indeed, we know more about the regulation, expression, and catalytic activities of rat P450 than of any other species.

In contrast to the rat where some two dozen or so constituent isoforms of hepatic P450 have been characterized extensively at a molecular and regulatory level [2, 3, 5], far fewer constituent forms of P450, with diverse and overlapping catalytic activities, and representing a small fraction of the total P450 pool, have been identified in the mouse liver [3]. Testosterone 15α-hydroxylase appears to be specific for

* Corresponding author: Dr. Bernard H. Shapiro, Laboratories of Biochemistry, Department of Animal Biology, University of Pennsylvania School of Veterinary Medicine, 3800 Spruce St., Philadelphia, PA 19104-6048. Tel. (215) 898-1772; FAX (215) 898-9923.

† *Abbreviations:* P450, cytochrome P450; PVDF, polyvinylidene defluoride; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; and anti-P450, antibody against particular cytochromes P450, i.e. 2C7, 2C11, and 2C12.

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the female-predominant*, constituent murine P450 2a-4 [6], whereas testosterone 16α-hydroxylase may be representative of the constituent, male-specific P450 2d-9 [7] and testosterone 2α-hydroxylase is indicative of a presently unassigned constituent member of the P450 2d subfamily [8]. In addition, testosterone is also hydroxylated at the 16α-position by at least one or more female-specific, inducible forms of P450 in the 2b subfamily that are actually expressed constituently in some mouse strains [3, 9, 10]. Murine P450 2a-5, sharing >96% nucleotide sequence similarity with P450 2a-4 [11], selectively catalyzes 7-hydroxylation of coumarin [12]. Recently, several new murine isoforms have been cloned and expressed at the cDNA level, e.g. Cyp3a-16 [13], 2c-29 [14], and 2e-1 [15]. Since many of these newly identified murine P450 cDNAs have not been characterized at a protein and/or catalytic level, there remain numerous "orphan" constituent monooxygenase reactions in murine liver for which no dependent forms of P450 have been identified [16]. Since hepatic drug-metabolizing enzyme activities are expressed at higher levels in female than in male mice [17], we have used a monoclonal antibody prepared against the major female-specific rat P450 2C12 [18] to probe the hepatic microsomes of female mice for previously unidentified forms of P450.

MATERIALS AND METHODS

Materials

[4-^{14}C]Testosterone (57.3 mCi/mmol), [4-^{14}C]androstenedione (53.9 mCi/mmol), and [2-^{14}C]hexobarbital (8.59 mCi/mmol) were purchased from DuPont NEN Research Products (Boston, MA). Fractogel EMD TMAE-650 (M), chloride form matrix (tentacle ion exchange), was a gift from EM Separation Technology (Gibbstown, NJ). DEAE-Sephacel was purchased from Pharmacia (Uppsala, Sweden), and Biogel-HTP, PVDF membranes for sequencing, Biobeads SM-2 (20–50 mesh), and materials used for protein electrophoresis were from Bio-Rad Laboratories (Hercules, CA). NitroPure transfer membranes were purchased from Micron Separations (Westboro, MA) and ECL western blotting detection reagents were obtained from Amersham (Arlington Heights, IL). Lubrol PX was obtained from ICN Biochemicals (Costa Mesa, CA). NADPH-cytochrome P450-reductase was purified from untreated murine hepatic microsomes as we previously described [19]. All other chemicals were of the highest available purity.

Microsomal Preparation and Solubilization

Animals were housed in the University of Pennsylvania Laboratory Animal Resources facility, under the supervision of certified Laboratory Animal Medicine veterinarians, and were treated according to a research protocol approved by the University's Institutional Animal Care and Use Committee. Untreated 14- to 16-week-old female CD-1 mice (Charles River Laboratories, Wilmington, MA) were housed for 2 weeks on hardwood bedding in plastic cages provided with acidified water (as a prophylactic against the pathogen *Pseudomonas aeuringosa*) and mouse diet, *ad lib.* Four hundred mice were quickly decapitated, their livers were perfused *in situ* with chilled saline and homogenized (10%, w/v) in a Tris-sucrose buffer, pH 7.4, containing protease inhibitors leupeptin (4.2 μM), antipain (3.3 μM), and PMSF (0.57 mM). Microsomes were prepared according to our standard method [20]. The resultant microsomal pellets were suspended in homogenizing buffer, pooled (a fraction of which was stored at 0–4° for measuring steroid hydroxylations and drug metabolism in intact microsomes), and solubilized by the dropwise addition of sodium cholate (2.5 mg/mg protein) in a 100 mM potassium phosphate buffer, pH 7.4, containing 100 μM DTT, 100 μM EDTA, and 20% glycerol (buffer A) with continuous stirring for 60 min at 4°. The solubilized microsomes were centrifuged at 105,000 *g* for 60 min, and the supernatant was dialyzed overnight against buffer A containing 0.25% sodium cholate (buffer B)..

Protein Purification

All purification steps were carried out at 4° unless otherwise stated. The dialysate derived from the solubilized microsomes was applied to a Fractogel column (2.5 × 30 cm) that was equilibrated with 600 mL of buffer B. After sample application (7.8 μmol of P450 in 100 mL), the column was first washed with 600 mL of buffer B and P450s were eluted with buffer B containing 0.1% Lubrol. The 10-mL fractions were monitored for heme absorbance at 417 nm, and the absorbance peaks were assayed for P450 content and for reactivity to a monoclonal antibody against the predominant female-specific rat P450 2C12 [18].

Fractions eluted with 0.1% Lubrol and containing measurable P450 and exhibiting maximal absorbance at 417 nm and strong reactivity to anti-P450 2C12 were pooled, concentrated in a Centriprep-30, and dialyzed overnight against 2 L of a 10 mM potassium phosphate buffer, pH 7.9, containing 100 μM DDT, and 100 μM EDTA, 20% glycerol, 0.1% Lubrol, and 0.25% sodium cholate (buffer C). The dialyzed fraction was applied to a DEAE-Sephacel column (2.5 × 20 cm), pre-equilibrated with 300 mL of buffer C, and washed until the initial fractions absorbing at 417 nm (exhibiting no reactivity against anti-P450 2C12) passed through the column. The putative $P450_{15\alpha AD}$, monitored at 417 nm and with anti-P450 2C12, was eluted in fractions 20 through 28 with a linear gradient of KCl (0 to 0.3 M) in buffer C. Each fraction contained 5 mL of eluate.

Fractions enriched for reactivity to anti-P450 2C12 from the DEAE-Sephacel column were pooled, concentrated

* The terms sex-dependent, sex-predominant or -dominant and sex-specific are often used indiscriminantly. We use sex- or gender-dependent to imply that expression levels are dependent upon the existence of gender; sex- or gender-predominant indicates that expression levels, regardless of magnitude, are consistently greater in one gender; and sex- or gender-specific implies that expression is basically restricted to only one gender.

with a Centriprep-30, dialyzed against a 5 mM potassium phosphate buffer, pH 7.25, containing 100 μM DTT, 100 μM EDTA, 20% glycerol, and 0.1% Lubrol, (buffer D), and applied to a hydroxylapatite column (1.5 × 10 cm) pre-equilibrated with buffer D. The column was washed with buffer D until initial fractions absorbing at 417 nm were eluted. Fractions containing the $P450_{15\alpha AD}$ eluted with a linear potassium phosphate gradient and found to be electrophoretically homogeneous were pooled, concentrated with a Centricon-30, and dialyzed overnight with 5 mM potassium phosphate buffer, pH 7.4, containing 100 μM DTT, 100 μM EDTA, and 20% glycerol; the buffer was replaced two to three times. To monitor enzymatic activity, the detergent had to be removed by first washing the dialysate on a hydroxylapatite column (1.5 × 10 cm) with detergent-free buffer D. Then the putative $P450_{15\alpha AD}$ was eluted from the column with 200 mM buffer D containing no detergent. Fractions reactive to anti-P450 2C12 were pooled, concentrated with a Centricon-30, and dialyzed overnight with detergent-free buffer D; any remaining detergent residue was removed by incubating the dialysate with SM-2 Biobeads for 90 min at 4°, and the final protein product was divided into aliquots and stored at −80°.

Enzyme Reconstitution

The catalytic activities were studied in the isolated protein and reconstituted as previously described [21], with minor modifications. That is, the enzymatic reactions (500 μL) were determined, under conditions of linear kinetics, in a 50 mM phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$, 1.5 mM NADPH, 50 μg dilauroylphosphatidylcholine, 500 units murine NADPH-cytochrome P450-reductase, and 100–500 pmol of the putative $P450_{15\alpha AD}$. The reaction mixture was preincubated for 5 min at room temperature. Next, 50 μmol of either testosterone or androstenedione dissolved in 10 μL of methanol and spiked with 200,000 dpm of the respective [^{14}C]androgen was added. The reaction was started by the addition of the NADPH-electron generating system consisting of 0.50 μmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, and 5 μmol $MgCl_2$. After incubating for 20 min at 37° in a shaking water bath, the reaction was terminated by the addition of 2 mL of ethyl acetate and centrifuged. The organic phase was dried under a gentle stream of nitrogen, redissolved in 50 μL of methanol, and resolved on a one-dimensional thin-layer chromatography system capable of distinguishing 37 metabolites of androstenedione and testosterone [22]. In control incubations, the reaction mixture contained all components except that the protein was added after termination of the reaction with ethyl acetate. After chromatography, plates were exposed to X-ray films with intensifying screens and quantified by scintillation counting of the scraped spots. Steroid metabolites were identified by comparing their mobilities to standard steroids run in our laboratory and found to be indistinguishable from those of previous reports [22]. Benzphetamine [23] and cocaine [24] demethylase activities were measured spectrophotometrically, and hexobarbital hydroxylase was determined by a radioenzymatic assay [20]. P450 content was measured as previously reported [25].

Electrophoresis

SDS–PAGE was performed in 7.5% acrylamide slab gels (20–30 mamp/gel) according to Laemmli [26], and proteins were visualized by staining with Coomassie brilliant blue. The standard protein molecular weight markers were phosphorylase *b* (95,500), glutamate dehydrogenase (55,000), ovalbumin (43,000), lactate dehydrogenase (36,000), and carbonic anhydrase (29,000).

Protein concentrations were estimated by the method of Bradford [27] using bovine serum albumin as the standard protein.

N-*terminal Sequence Analysis*

Approximately 100 pmol of our purified, putative $P450_{15\alpha AD}$, electrophoretically transferred to a PVDF membrane, was sequenced by the phenylthiohydantoin method [28, 29]. *N*-terminal amino acid sequencing was performed by the Wistar Protein Microchemistry Laboratory (Philadelphia, PA). The amino acid yield/cycle was comparable to that previously reported for the amino-terminal sequence analyses of other P450s [2, 8], and indicated the presence of only a single protein.

Antibody Preparation

Polyclonal antibodies raised against our putative $P450_{15\alpha AD}$ were produced in female New Zealand white rabbits by Dr. J. K. Noel (HRP Inc., Denver, PA). Approximately 140 μg of our purified protein mixed with Freund's complete adjuvant was injected nodal and intradermally. Two weeks later, 100 μg of the protein mixed in Freund's incomplete adjuvant was administered intradermally. Four weeks after the first immunization, a booster dose of 50 μg protein mixed with Freund's incomplete adjuvant was injected intradermally, and the rabbit was exsanguinated 2 weeks later. Antibody titer was determined by ELISA precoated with solubilized antigen.

Western Blotting

Microsomes were prepared from liver of male and female AKR/J, C57BL/6J, BALB/cJ, 129/J, A/J, C3H/HeJ, DBA/2J (Jackson Laboratory, Bar Harbor, ME), and CD-1 (Charles River Laboratories) mice, as well as from lung, kidney and total brain of CD-1 females, and assayed by western blotting [30] for the presence of the putative $P450_{15\alpha AD}$ protein. Briefly, 10 μg of microsomal protein was electrophoresed on 0.75 mm SDS–polyacrylamide (7.5%) gels and electroblotted onto nitrocellulose filters. The blots were probed by a 1:15,000 titer of polyclonal anti-mouse $P450_{15\alpha AD}$ rabbit

immunoglobulin G and detected by an enhanced chemiluminescence procedure [31]. The antibody proved to be highly specific, detecting no more than a single band per lane.

RESULTS

Since the presence of detergent severely inhibits such P450-dependent reactions as androgen hydroxylations [2, 8], isolation and purification of the putative $P450_{15\alpha AD}$ were based upon absorbance at 417 nm, characteristic of flavo- and hemoproteins, P450 content, and reactivity to monoclonal antibodies raised against the major female-specific rat P450 2C12.

The elution profile of the solubilized microsomes from the Fractogel column (Fig. 1) had four heme absorbance peaks, of which only the Lubrol-eluted peak was assayable for P450 and reacted positively with anti-P450 2C12. This P450-Fractogel-isolated peak contained proteins that were either intensely reactive (fractions 115–120) toward anti-P450 2C12 or weakly reactive (fractions 122–135) to the antibody, the latter recently identified as a highly selective P450-dependent testosterone 2α-hydroxylase [8]. Fractions 117–120 were combined, dialyzed, concentrated, and applied to a DEAE-Sephacel column from which three substantial peaks containing P450 were eluted (Fig. 2). However, only the first peak eluted with a linear KCl gradient reacted with anti-P450 2C12. Next, fractions from this peak were pooled, dialyzed, concentrated, and applied to a hydroxylapatite column from which basically only a single P450-containing peak was eluted with a linear potassium phosphate gradient, reacting with anti-P450 2C12 and exhibiting a maximal dithionate-reduced CO-binding absorbance at 450 nm (Fig. 3).

The progressive purification of the putative $P450_{15\alpha AD}$ by column chromatography, shown in an SDS–polyacrylamide gel, demonstrates the isolation of an apparent single major protein with intense reactivity towards anti-P450 2C12 from the hydroxylapatite column (Fig. 4). With the use of molecular weight markers, the molecular weight of the purified protein was estimated from unstained markers to be 53,000, consistent with the molecular weights of known forms of P450 [2].

Regioselectivity and stereospecificity of steroid hydroxylations catalyzed by the putative $P450_{15\alpha AD}$ are shown from autoradiographic results of thin-layer chromatograms of androstenedione and testosterone metabolites (Fig. 5).

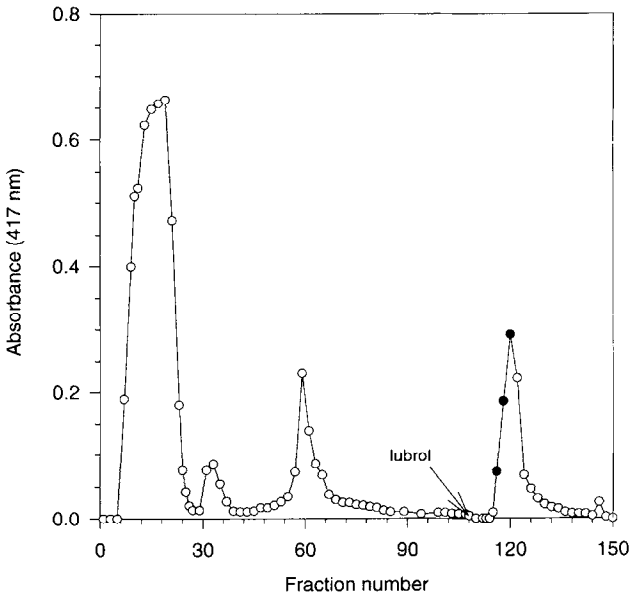

FIG. 1. Elution profile from the Fractogel EMD TMAE-650 (M) anion exchange column. Solubilized and dialyzed microsomes were loaded on the column according to the method described under Materials and Methods. Fractions 1–23 were collected during sample application, fractions 44–60 were collected during washing with the equilibrium buffer, and fractions 115–135 (cytochrome P450) were eluted with 0.1% Lubrol. Fractions represented by solid circles were the most intensely reactive to anti-P450 2C12 and were the source of material applied to the DEAE-Sephacel column (Fig. 2). Each fraction contained 10 mL of eluate.

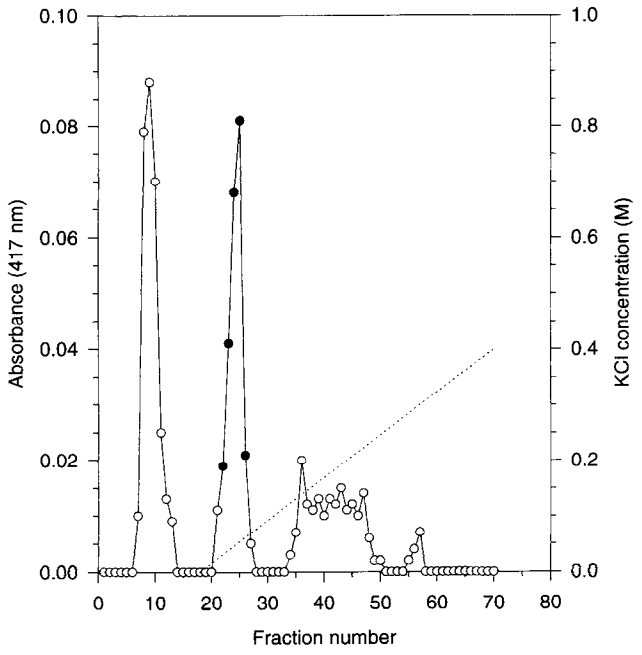

FIG. 2. Elution profile from the DEAE-Sephacel column. Fractions 115–120 eluted from the Fractogel column with Lubrol were pooled, dialyzed, and chromatographed on a DEAE-Sephacel column as described under Materials and Methods. Fractions 1–5 were collected during sample application, and fractions 6–17 were collected during washing with the equilibration buffer. A linear gradient of 0 to 0.3 M KCl was started at fraction 18 and ended at fraction 70. Each fraction contained 5 mL of eluate. All peaks were assayable for P450, but only the second peak (fractions 20–28) reacted to anti-P450 2C12. Fractions represented by solid circles were the source of material applied to the hydroxylapatite column (Fig. 3).

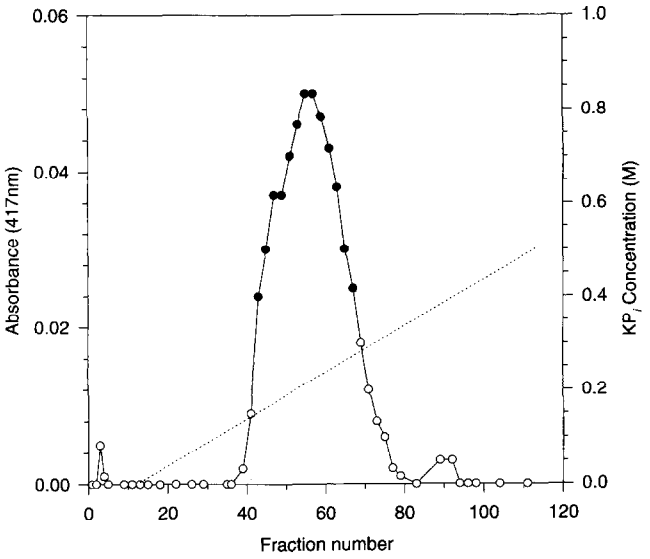

FIG. 3. Elution profile from hydroxylapatite chromatography. Pooled fractions 22–26 from the Sephacel column (Fig. 2) were dialyzed, concentrated, and adsorbed to the hydroxylapatite column as described under Materials and Methods. Fractions 1–10 were collected during sample application, and a linear gradient of 5–500 mM potassium phosphate containing 0.4% sodium cholate was started at fraction 14 and ended at fraction 115. One milliliter of eluate was collected in each fraction. Fractions 38–78 contained the putative $P450_{15\alpha AD}$, and fractions represented by solid circles were the source of material used to characterize the enzyme.

The catalytic activities toward androgen of the purified protein derived from the hydroxylapatite column was basically limited to the 7α- and 15α-hydroxylation of androstenedione and the 7α- and 11α-hydroxylation of testosterone*, the latter steroid being a considerably less effective substrate (Table 1). Curiously, whereas the purified protein exhibited testosterone 11α-hydroxylase activity, no such activity was detectable in the microsomes (Fig. 5); a similar phenomenon has been reported with several isoforms of rat P450 [32, 33]. As regards drug metabolism, the purified protein had an enhanced capacity for benzphetamine and cocaine demethylation, but had no measurable hexobarbital hydroxylase activity (Table 1).

We estimate by spectrophotometric analysis that the purified $P450_{15\alpha AD}$ eluted from the hydroxylapatite column had a specific content of 2.7 nmol of P450/mg protein, and according to western blot analysis was 3% of the total hepatic microsomal P450. The low specific content calculated for $P450_{15\alpha AD}$ may be explained by our observation, not unique to this isoform [34], of marked absorption of $P450_{15\alpha AD}$ at 420 nm, which increased with duration of the ferrous state at the expense of the 450 nm absorption during the spectral assay.

* The hydroxylated metabolites of testosterone and androstenedione were identified by their identical mobilities to known standards, run on HPLC, and one- and two-dimensional silica gel thin-layer chromatograms, using several different solvent systems.

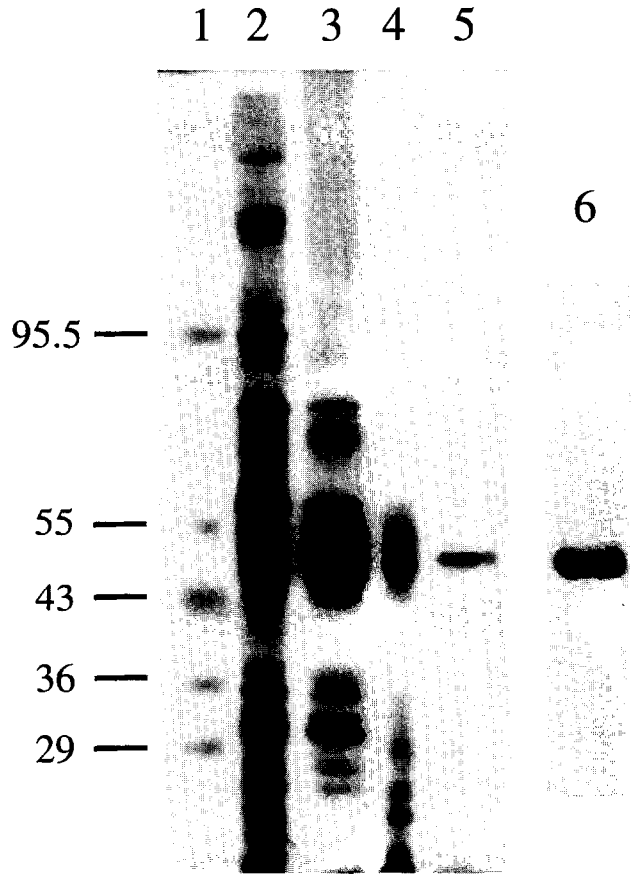

FIG. 4. SDS–PAGE of purified $P450_{15\alpha AD}$. Proteins were resolved on a 7.5% acrylamide gel, stained with Coomassie brilliant blue R-250, and destained with methanol:acetic acid. Lane 1 contains molecular weight standards, lane 2 contains 20 μg of microsomal protein, lane 3 contains 20 μg of protein eluate from the Fractogel column (fractions 115–120, Fig. 1), lane 4 contains 10 μg of protein from the DEAE-Sephacel column (fractions 22–26, Fig. 2), lane 5 contains 1 μg of purified $P450_{15\alpha AD}$ from the hydroxylapatite column (fractions 42–68, Fig. 3), and lane 6 is a western blot of 1 μg of purified $P450_{15\alpha AD}$ (same as applied to lane 5) probed with monoclonal antibody against rat P450 2C12. [Resolution of the purified protein from the hydroxylapatite column on 12% and 15% acrylamide gels did not alter the appearance of the single band (data not shown).]

The selective inhibition of steroid hydroxylations by polyclonal antibodies prepared against our purified protein was investigated by examining the effects of various concentrations of the antisera on microsomal (200 μg protein) metabolism of androstenedione (Fig. 6). The results indicate a selective and concentration-dependent inhibition of both androstenedione 7α- and 15α-hydroxylations, the former reaction being more sensitive to the inhibitory effect of the antisera. Whereas as little as 10 μg of antisera protein inhibited microsomal androstenedione 7α-hydroxylation by 90%, about 3 times this amount of antisera was required to block 90% of androstenedione 15α-hydroxylase activity, possibly suggesting the presence of a microsomal $P450_{15\alpha AD}$-immuno-related androstenedione 15α-hydroxylase. In contrast, the antisera had little inhibitory

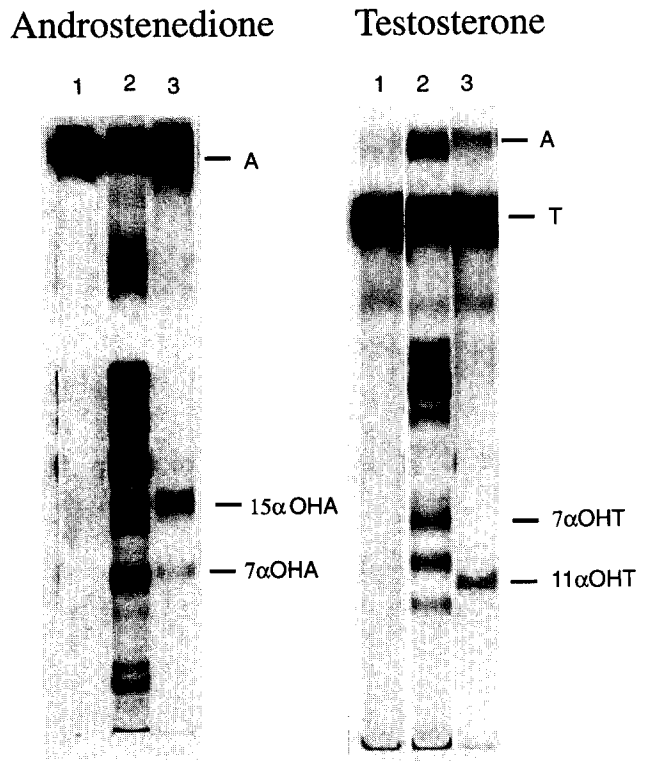

FIG. 5. Autoradiograms of one-dimensional thin-layer chromatography of androstenedione and testosterone metabolites formed by microsomes and putative $P450_{15\alpha AD}$. (See Materials and Methods for details.) The androgen metabolites were formed by a control system in which the microsomes were added after the reaction was terminated (lanes 1), an incubate containing microsomes (lanes 2), and a complete reconstituted system containing both the reductase and purified enzyme (lanes 3). Complete reconstitution systems excluding the purified $P450_{15\alpha AD}$ or the NADPH-cytochrome P450-reductase produced androgen metabolites that were indistinguishable from that in lanes 1 (not shown). Abbreviations: A, androstenedione; T, testosterone; 15αOHA, 15α-hydroxyandrostenedione; 11αOHT, 11α-hydroxytestosterone; 7αOHA, 7α-hydroxyandrostenedione; and 7αOHT, 7α-hydroxytestosterone.

effect on any other androstenedione hydroxylation; the effects on 6β- and 7β-hydroxylations are presented in Fig. 6. The antibody produced a maximal 60% inhibition of microsomal benzphetamine N-demethylation, suggesting that $P450_{15\alpha AD}$ may be responsible, in large measure, for the *in vivo* metabolism of the xenobiotic. Preimmune sera had no inhibitory effect on any of the reactions studied (data not shown). It may be concluded from the immuno-inhibition studies that $P450_{15\alpha AD}$ is the enzyme that appears to be almost exclusively responsible for androstenedione 7α- and 15α-hydroxylations.

Database-derived comparisons of the amino-terminal sequence of the purified murine $P450_{15\alpha AD}$ with various mammalian isoforms indicate that the murine enzyme shares the greatest sequence similarity with members of the P450 2C subfamily. Putative murine $P450_{15\alpha AD}$ had the greatest sequence similarity (i.e. 90%) with recently identified murine P450 2c-29 [14]. Although the next greatest

TABLE 1. Oxidative metabolism of androgens and xenobiotics catalyzed by purified murine $P450_{15\alpha AD}$

	Activity (nmol/min/nmol P450)
Androstenedione 7α-hydroxylation	0.07 ± 0.03
Androstenedione 15α-hydroxylation	1.02 ± 0.13
Testosterone 7α-hydroxylation	0.03 ± 0.01
Testosterone 11α-hydroxylation	0.19 ± 0.04
Benzphetamine N-demethylation	75.18 ± 11.39
Cocaine N-demethylation	47.42 ± 8.61
Hexobarbital 3-hydroxylation	UD*

The reconstitution system used to assay the monooxygenase activities is described under Materials and Methods. Values are means + SD, N = 3.
* Undetectable activity.

sequence similarity (i.e. 81%) was with rat P450 2C7, our protein was completely unreactive to antibodies raised against the rat isoform (data not presented). Other members of the P450 2C subfamily had a 60–70% sequence similarity with $P450_{15\alpha AD}$ (Table 2). Antibodies against rat P450 2C12, which were used in the present study to monitor the purification of the murine cytochrome, were intensely reactive to $P450_{15\alpha AD}$, whereas antibodies against rat P450 2C11 and human P450 2C9 were only moderately reactive to the murine isoform (data not presented).

Western blot analysis of hepatic $P450_{15\alpha AD}$ (Fig. 7) in seven commonly used inbred strains of mice and the widely utilized outbred CD-1 indicates that expression of the murine protein is either female-predominant (AKR/J, C57BL/6J, CD-1, BALB/cJ, and 129/J) or sex-independent (A/J, C3H/HeJ, and DBA/2J). Comparison of microsomal $P450_{15\alpha AD}$ in several tissues from CD-1 female mice showed the greatest concentration in liver, followed by highly substantial levels in lung, undetectable concentrations in kidney, and faint levels in total brain (Fig. 8).

DISCUSSION

Based upon SDS–PAGE profiles, reconstituted activity, selective inhibition by polyclonal antisera, and amino-terminal sequence analysis, we have identified an isoform of murine hepatic P450 having highly specific regioselectivity and stereospecificity for 15α-hydroxylation of androstenedione. In fact, in a reconstitution system containing our purified protein, referred to as $P450_{15\alpha AD}$, 7α- and 15α-hydroxyandrostenedione were the only detectable androstenedione metabolites, with the latter accounting for >95% of the total product. Although testosterone was a much less effective substrate for $P450_{15\alpha AD}$, at least 90% of the metabolites produced by the murine isoform were 7α- and 11α-hydroxytestosterone, with the latter androgen representing 75–80% of the total testosterone metabolites. In addition, $P450_{15\alpha AD}$ had no measurable hexobarbital hydroxylase activity, but did contribute significantly to multicytochrome P450-dependent [2, 7] benzphetamine and cocaine demethylation.

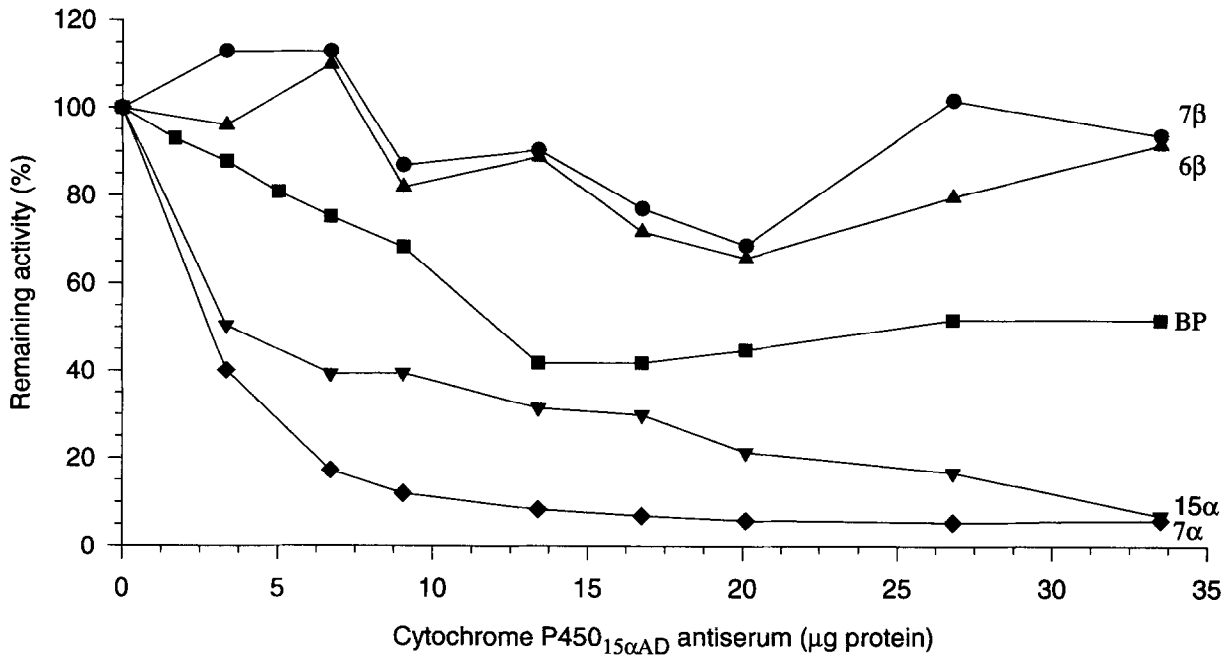

FIG. 6. Inhibition of androstenedione hydroxylations and benzphetamine demethylation in intact hepatic microsomes by polyclonal antisera prepared against purified $P450_{15\alpha AD}$. (See Materials and Methods for details.) Various amounts of antisera were preincubated with intact microsomes, and the reaction was initiated by the addition of the NADPH-generating system. Androstenedione metabolites were separated by one-dimensional thin-layer chromatography, scraped off the chromatograms, and quantified by scintillation counting. Each data point represents the mean of two determinations. Key: 7β, 7β-hydroxyandrostenedione (●); 6β, 6β-hydroxyandrostenedione (▲); BP, benzphetamine demethylase activity (■); 15α, 15α-hydroxyandrostenedione (▼); and 7α, 7α-hydroxyandrostenedione (◆). The syntheses of 2α- 15α-, 16α-, and 16β-hydroxyandrostenedione were unaffected by the presence of the antisera (data not shown). Preimmune sera had no inhibitory effect on any of the reactions (data not shown).

Benzphetamine N-demethylation is a rather ubiquitous activity expressed by many isoforms of P450 [2], some of which may or may not normally contribute to the metabolism of the drug *in vivo*. The fact that antibodies against murine P450 2d-9 inhibit 90% of the purified protein's benzphetamine demethylase activity but are ineffective in blocking the demethylase activity in microsomes [7] suggests that in the presence of the complete complement of microsomal P450s, isoforms other than P450 2d-9 are responsible for benzphetamine demethylation. Our finding that anti-$P450_{15\alpha AD}$ inhibited 60% of microsomal benzphetamine demethylation suggests that this murine isoform is normally a significant contributor to the metabolism of the drug*. Furthermore, comparing the very substantial

* Antibodies against murine P450 PBIII, a putative member of the 2c subfamily, sharing a high degree of *N*-terminal amino acid similarity with $P450_{15\alpha AD}$, also block 60% of constituent hepatic microsomal benzphetamine demethylase activity [35].

TABLE 2. *N*-terminal sequence analysis of murine $P450_{15\alpha AD}$ and comparison to other P450 2C and murine isoforms*

	Residue																				Mol. mass (kDa)	Similarity (%)
	1						7							14						21		
$P450_{15\alpha AD}$ - mouse	M	D	L	V	V	F	L	A	L	T	L	S	X	L	I	L	L	S	X	W	R	53	
P450 2c-29 - mouse	M	D	L	V	V	F	L	A	L	T	L	S	C	L	I	L	L	S	*L*	W	R	50	90
P4502C7 - rat	M	D	L	V	*T*	F	L	*V*	L	T	L	S	*S*	L	I	L	L	S	*L*	W	R	51	81
P450 2C6 - rat	M	D	L	V	M	*L*	L	*V*	L	T	L	*T*	C	L	I	L	L	S	*I*	W	R	51	71
P450 2C11 - rat	M	D	*P*	V	*L*	*V*	L	V	L	T	L	S	*S*	L	*L*	L	L	S	*L*	W	R	51	67
P450 2C9 - human	M	D	*S*	*L*	V	V	L	V	L	C	L	S	C	L	*L*	L	L	S	*L*	W	R	50	62
P450 2C12 - rat	M	D	*P*	*F*	V	V	L	V	L	*S*	L	S	*F*	L	*L*	L	L	*Y*	*L*	W	R	51	57
P450 2d-9 - mouse	M	*E*	L	*L*	*T*	*G*	*T*	*D*	L	*W*	*P*	*V*	*A*	*I*	*F*	*T*	*V*	*I*	*F*	*I*	*L*	49	14
P450 2e-1 - mouse	M	*A*	*V*	*L*	*G*	*I*	*T*	*V*	*A*	*L*	L	*V*	*W*	*I*	*A*	*T*	L	*L*	*L*	*V*	*S*	NA	*14*
P450 2a-4 - mouse	M	*L*	*T*	*S*	*G*	*L*	L	*L*	*V*	*A*	*A*	*V*	*A*	*F*	*L*	*S*	*V*	*L*	*V*	*L*	M	48	10

* Accession numbers: D17674 (P450 2c-29 - mouse), P05179 (P450 2C7 - rat), P05178 (P450 2C6 - rat), P08683 (P450 2C11 - rat), A41506 (P450 2C9 - human), P11510 (P450 2C12 - rat), A27384 (P450 2d-9 - mouse), X62595 (P450 2e-1 - mouse), and J03549 (P450 2a-4 - mouse). Residues that differ from those identified in murine $P450_{15\alpha AD}$ are presented in *italics*. X, unidentified amino acid; NA, not available. Listed molecular masses were determined from the purified protein and not deduced from the cDNA.

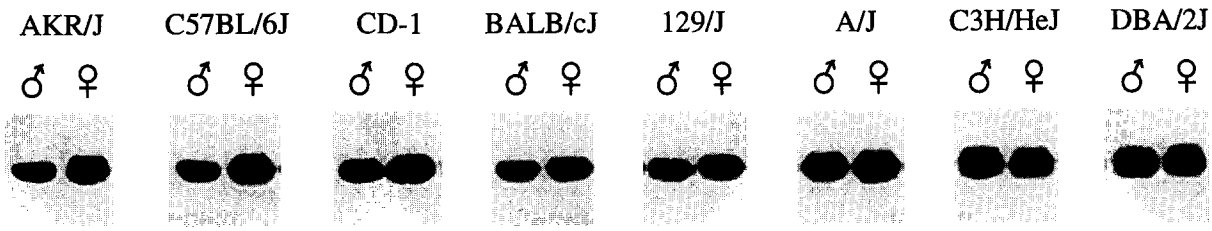

FIG. 7. Western blots probed with polyclonal antibody to $P450_{15\alpha AD}$ protein in liver microsomes isolated from male (♂) and female (♀) mice of eight different strains. (See Materials and Methods for details.) Western blot analysis of microsomes from 2–3 additional animals in each group was similar to that presented here.

benzphetamine and cocaine demethylase activities of $P450_{15\alpha AD}$ with its lower androgen hydroxylating capacity may suggest that steroids are not the endogenous substrates for the enzyme. In this regard, pulmonary tissue contains an unexpectedly high level of $P450_{15\alpha AD}$ (compared to the usual 10% of hepatic total P450 [36]), and mouse lung is enriched with isoforms of P450 that do not exhibit significant affinity for steroids [37].* Moreover, the *N*-terminal amino acid sequence of murine P450 2c-29 [14] may be the same as $P450_{15\alpha AD}$, and the former enzyme strongly catalyzes the oxygenation of tetrahydrocannabinol [39]. Thus, while androstenedione 15α-hydroxylation may be a specific indicator of $P450_{15\alpha AD}$ activity, it may not reflect the physiologic function of the enzyme.

In contrast to the rat where an individual form of hepatic P450 may comprise up to 50% of the total P450 [40, 41] and in humans where much of drug metabolism is dependent upon just a few forms of P450 [42], we have found that $P450_{15\alpha AD}$ accounts for about 3% of the total P450 in female CD-1 mouse liver microsomes, which is similar in magnitude to that reported for other constitutive forms of murine hepatic P450 [6–8, 35]. Unless there exists a unique subset of murine P450 whose stability and/or solubility precludes their being isolated by existing methodologies, it is difficult to understand how any major putative form of murine P450, as seen in rats, could have eluded detection. Perhaps, unlike in the rat and human, there are no predominant forms of murine hepatic P450 that account for more than 5–10% of the total constituent P450 pool.

Comparing the *N*-terminal sequence of our putative P450 with previously identified isoforms suggests that $P450_{15\alpha AD}$ is a member of the P450 2c subfamily.† If we disregard the two unidentified amino acid residues in the *N*-terminal sequence of murine $P450_{15\alpha AD}$, then we find that the isoform shares 100% sequence similarity with the recently cloned murine P450 2c-29 [14] (previously identified from the purified protein as P450 MUT-2 [39]). Furthermore, the identified residues in the *N*-terminal amino acid sequences of the purified murine proteins P450 PBIII, a potent benzphetamine demethylase [35], and $P450_{THC}$, like MUT-2 [39], a potent cannabinoid hydroxylase [44], also share 100% sequence similarity with $P450_{15\alpha AD}$. Although the protein-derived molecular masses of murine P450 MUT-2 (50 kDa [39]), P450 PBIII (51 kDa [35]), $P450_{THC}$ (47 kDa [44], and $P450_{15\alpha AD}$ (53 kDa) are dissimilar, their generally potent benzphetamine demethylase activities and, more importantly, their identical *N*-terminal amino acid sequences suggest that not only are these isoforms members of the murine 2c family, but may actually be the same protein, i.e. P450 2c-29.

Whereas rat P450 2C7 has the greatest sequence similarity (81%) (for a non-murine isoform) with putative $P450_{15\alpha AD}$, and exhibits the same female-predominance [45], antibodies against the rat isoform were completely unreactive to $P450_{15\alpha AD}$. In contrast, anti-rat P450 2C11 and anti-human P450 2C9 were moderately reactive to $P450_{15\alpha AD}$, and anti-rat P450 2C12, whose antigen shared only a 57% *N*-terminal sequence similarity, was intensely reactive to murine $P450_{15\alpha AD}$. (The disassociation between *N*-terminal amino acid sequence similarities and antigenicity can be explained by the fact that the immunogenic epitopes of a protein are likely to be located within its hydrophilic carboxy-terminal end.)

Rats and mice express opposite gender differences in drug metabolism [4]. Adult rats exhibit a sexual dimorphism in

* The absence of immuno-detectable $P450_{15\alpha AD}$ in kidney of CD-1 females is in agreement with previous studies reporting that renal total P450 levels in female mice are only 35% of male values, with some selective isoform activities being only 1–2% of that measured in males [38].

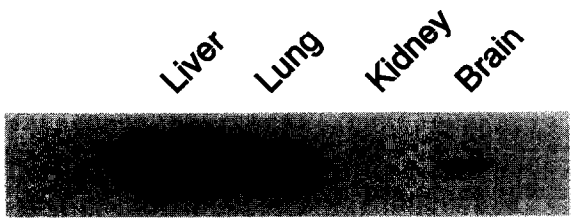

FIG. 8. Western blots probed with polyclonal antibody to $P450_{15\alpha AD}$ protein in liver, lung, kidney, and brain microsomes isolated from CD-1 female mice. (See Materials and Methods for details.) Western blot analysis of tissue microsomes from 2–3 additional animals was indistinguishable from that presented here.

† Although murine P450 2a-4 and $P450_{15\alpha AD}$ are both androgen 15α-hydroxylases, the former isoform can effectively 15α-hydroxylate androstenedione as well as testosterone [43], whereas $P450_{15\alpha AD}$ 15α-hydroxylase activity is restricted to androstenedione. Moreover, the two isoforms share virtually no similarity in their *N*-terminal amino acid sequences (Table 2).

P450-dependent monooxygenases in which the activities are generally 3- to 5-fold higher in the male [4, 46]. Mice, on the other hand, have a reversed sexual dimorphism in which the enzyme activities are 40–60% greater in females [16, 17]. The magnitude of this smaller, but consistent sexual dimorphism in murine monooxygenases is due to the occurrence of a less than 2-fold gender difference (F > M) in sex-dependent P450-catalyzed activities (compared with many 10- to 20-fold sex differences [M > F] in the rat [4, 46]), and by the large number of microsomal monooxygenases that are sex-independent [16]. In agreement with this paucity of male-predominant P450-dependent activities, we have observed a modest (<2-fold), but clear female-predominance of a putative murine P450 2c isoform (i.e. hepatic $P450_{15\alpha AD}$) in five of eight mice strains, with no strain expressing male-predominance. Although to our knowledge there are no previous studies examining the sex-dependence of murine P450 2c isoforms, rat P450 2C7 and 2C6 having the greatest *N*-terminal amino acid sequence similarity (for non-murine isoforms) to $P450_{15\alpha AD}$, both exhibit, like $P450_{15\alpha AD}$, a modest female-predominance in the rat [33, 45]. Since previous studies comparing gender differences in murine P450 have been limited to only a few isoforms using just a small number of strains, it is difficult to determine if our cross-strain $P450_{15\alpha AD}$ findings are consistent with sex differences of other murine isoforms.

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References

1. Gonzalez F, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40:** 243–288, 1989.
2. Ryan DE and Levin W, Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol Ther* **45:** 153–239, 1990.
3. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K and Nebert DW, The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trival names of enzymes, and nomenclature. *DNA Cell Biol* **12:** 1–51, 1993.
4. Shapiro BH, Agrawal AK and Pampori NA, Gender differences in drug metabolism regulated by growth hormone. *Int J Biochem Cell Biol* **27:** 9–20, 1995.
5. Schenkman JB, Steroid metabolism by constitutive cytochromes P450. *J Steroid Biochem Mol Biol* **43:** 1023–1030, 1992.
6. Harada N and Negishi M, Mouse liver testosterone 15α-hydroxylase (cytochrome $P\text{-}450_{15\alpha}$). Purification, regioselectivity, stereospecificity, and sex-dependent expression. *J Biol Chem* **259:** 1265–1271, 1984.
7. Harada N and Negishi M, Mouse liver testosterone 16α-hydroxylase (cytochrome $P\text{-}450_{16\alpha}$). Purification, regioselectivity, stereospecificity, and immunochemical characterization. *J Biol Chem* **259:** 12285–12290, 1984.
8. Sharma MC and Shapiro BH, Purification and characterization of constituent testosterone 2α-hydroxylase (cytochrome $P450_{2\alpha}$) from mouse liver. *Arch Biochem Biophys* **316:** 478–484, 1995.
9. Devore K, Harada N and Negishi M, Characterization of testosterone 16α-hydroxylase ($I\text{-}P\text{-}450_{16\alpha}$) induced by phenobarbital in mice. *Biochemistry* **24:** 5632–5637, 1985.
10. Noshiro M, Lakso M, Kawajiri K and Negishi M, Rip locus: Regulation of female-specific isozyme ($I\text{-}P\text{-}450_{16\alpha}$) of testosterone 16α-hydroxylase in mouse liver, chromosome localization, and cloning of P-450 cDNA. *Biochemistry* **27:** 6434–6443, 1988.
11. Lindberg R, Burkhart B, Ichikawa T and Negishi M, The structure and characterization of type I $P\text{-}450_{15\alpha}$ gene as major steroid 15α-hydroxylase and its comparison with type II $P\text{-}450_{15\alpha}$. *J Biol Chem* **264:** 6465–6471, 1989.
12. Negishi M, Lindberg R, Burkhart B, Ichikawa T, Honkakoski P and Lang M, Mouse steroid 15α-hydroxylase gene family: Identification of type II $P\text{-}450_{15\alpha}$ as coumarin 7-hydroxylase. *Biochemistry* **28:** 4169–4172, 1989.
13. Itoh S, Satoh M, Abe Y, Hashimoto H, Yanagimoto T and Kamataki T, A novel form of mouse cytochrome P450 3A (Cyp3a-16). Its cDNA cloning and expression in fetal liver. *Eur J Biochem* **226:** 877–882, 1994.
14. Matsunaga T, Watanabe K, Yamamoto I, Negishi M, Gonzalez FJ and Yoshimura H, cDNA cloning and sequence of CYP2C29 encoding P-450 MUT-2, a microsomal aldehyde oxygenase. *Biochim Biophys Acta* **1184:** 299–301, 1994.
15. Freeman JE, Stirling D, Russell AL and Wolf CR, cDNA sequence, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1. *Biochem J* **281:** 689–695, 1992.
16. Pampori NA and Shapiro BH, Effects of neonatally administered monosodium glutamate on the sexually dimorphic profiles of circulating growth hormone regulating murine hepatic monooxygenases. *Biochem Pharmacol* **47:** 1221–1229, 1994.
17. MacLeod JN, Sorensen MP and Shapiro BH, Strain independent elevation of hepatic monooxygenase enzymes in female mice. *Xenobiotica* **17:** 1095–1102, 1987.
18. Morgan ET, Rönnholm M and Gustafsson J-Å, Preparation and characterization of microsomal antibodies recognizing unique epitopes on sexually differentiated rat liver cytochrome P-450 isozymes. *Biochemistry* **26:** 4193–4200, 1987.
19. Sharma MC, Jeong S-J, Sharma MR and Shapiro BH, Simultaneous isolation of NADPH-cytochrome P-450 reductase and cytochrome P-450 using tentacle ion-exchange chromatography and interspecies comparison of the reductase activity. *Pharmacology* **51:** 331–340, 1995.
20. Shapiro BH and Szczotka SM, Androgenic repression of hexobarbitone metabolism and action in Crl:CD-1 (ICR)BR mice. *Br J Pharmacol* **81:** 49–54, 1984.
21. Raucy LJ and Lasker MJ, Isolation of P450 enzymes from human liver. *Methods Enzymol* **206:** 577–587, 1991.
22. Agrawal AK, Pampori NA and Shapiro BH, Thin-layer chromatographic separation of regioselective and stereospecific androgen metabolites. *Anal Biochem* **224:** 455–457, 1995.
23. Cochin J and Axelrod J, Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normophine. *J Pharmacol Exp Ther* **125:** 105–110, 1959.
24. Boelsterli UA, Lanzotti A, Goldlin C and Oertle M, Identification of cytochrome P-450IIB1 as a cocaine-bioactivating isoform in rat hepatic microsomes and in cultured rat hepatocytes. *Drug Metab Dispos* **20:** 96–101, 1992.

25. Omura T and Sato R, The carbon-monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239:** 2370–2378, 1964.
26. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
27. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
28. Matsudaira P, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262:** 10035–10038, 1987.
29. Reim D, Hembach P and Speicher DW, Evaluation of the blot cartridge for enhanced gas phase sequencing at maximum sensitivity. In: *Techniques in Protein Chemistry* (Ed. Angeletti RH), pp. 53–60. Academic Press, New York, 1992.
30. Waxman DJ, Rat hepatic P450IIA and P4501IC subfamily expression using catalytic, immunochemical, and molecular probes. *Methods Enzymol* **206:** 249–267, 1991.
31. Pampori NA, Pampori MK and Shapiro BH, Dilution of the chemiluminescence reagents reduces the background noise on western blots. *Biotechniques* **18:** 588–590, 1995.
32. Wood AW, Ryan DE, Thomas PE and Levin W, Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J Biol Chem* **258:** 8839–8847, 1983.
33. Waxman DJ, Dannan GA and Guengerich FP, Regulation of rat hepatic cytochrome P-450: Age-dependent expression, hormonal imprinting and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24:** 4409–4417, 1985.
34. Miranda CL, Wang J-L, Henderson MC and Buhler DR, Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. *Arch Biochem Biophys* **268:** 227–238, 1989.
35. Honkakoski P and Lang MA, Mouse liver phenobarbital-inducible P450 system: Purification, characterization, and differential inducibility of four cytochrome P450 isozymes from the D2 mouse. *Arch Biochem Biophys* **273:** 42–57, 1989.
36. Sonderfan AJ, Arlotto MP and Parkinson A, Identification of the cytochrome P-450 isozymes responsible for testosterone oxidation in rat lung, kidney and testis: Evidence that cytochrome P-450a (P450IIA1) is the physiologically important testosterone 7α-hydroxylase in rat testis. *Endocrinology* **125:** 857–866, 1989.
37. Villard PH, Seree E, Lacarelle B, Therene-Fenoglio MC, Barra Y, Attolini L, Bruguerole B, Durand A and Catalin J, Effect of cigarette smoke on hepatic and pulmonary cytochromes P450 in mouse: Evidence for CYP2E1 induction in lung. *Biochem Biophys Res Commun* **202:** 1731–1737, 1994.
38. Henderson CJ, Scott AR, Yang CS and Wolf CR, Testosterone-mediated regulation of mouse renal cytochrome P-450 isoenzymes. *Biochem J* **266:** 675–681, 1990.
39. Watanabe K, Narimatsu S, Yamamoto I and Yoshimura H, Oxygenation mechanism in conversion of aldehyde to carboxylic acid catalyzed by P-450 isozyme. *J Biol Chem* **266:** 2709–2711, 1991.
40. Morgan ET, MacGeoch C and Gustafsson J-Å, Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. *J Biol Chem* **260:** 11895–11898, 1985.
41. MacGeoch C, Morgan ET and Gustafsson J-Å, Hypothalamopituitary regulation of cytochrome $P\text{-}450_{15\beta}$ apoprotein levels in rat liver. *Endocrinology* **117:** 2085–2092, 1985.
42. Guengerich FP, Catalytic selectivity of human cytochrome P450 enzymes: Relevance to drug metabolism and toxicity. *Toxicol Lett* **70:** 133–138, 1994.
43. Harada N and Negishi M, Substrate specificities of cytochrome P-450, C-$P\text{-}450_{16\alpha}$ and $P\text{-}450_{15\alpha}$, and contribution to steroid hydroxylase activities in mouse liver microsomes. *Biochem Pharmacol* **37:** 4778–4780, 1988.
44. Bornheim LM and Correia MA, Purification and characterization of the major hepatic cannabinoid hydroxylase in the mouse: A possible member of the cytochrome P-450IIC subfamily. *Mol Pharmacol* **40:** 228–234, 1991.
45. Bandiera S and Dworschak C, Effects of testosterone and estrogen on hepatic levels of cytochromes P450 2C7 and P450 2C11 in the rat. *Arch Biochem Biophys* **296:** 286–295, 1992.
46. Waxman DJ, Regulation of liver-specific steroid metabolizing cytochromes P450: Cholesterol 7α-hydroxylase, bile acid, 6β-hydroxylase, and growth hormone-responsive steroid hormone hydroxylases. *J Steroid Biochem Mol Biol* **43:** 1055–1072, 1992.