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NADPH- and hydroperoxide-supported 17β-estradiol hydroxylation catalyzed by a variant form (432L, 453S) of human cytochrome P450 1B1

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Abstract

Human cytochrome P450 1B1 (CYP1B1) catalyzes the hydroxylation of 17β-estradiol (E_2) at C-4, with a lesser activity at C-2. The E_2 4-hydroxylase activity of human CYP1B1 was first observed in studies of MCF-7 breast cancer cells. Sequencing of polymerase chain reaction products revealed that CYP1B1 expressed in MCF-7 cells was not the previously characterized enzyme but a polymorphic form with leucine substituted for valine at position 432 and serine substituted for asparagine at position 453. To investigate the NADPH- and organic hydroperoxide-supported E_2 hydroxylase activities of the 432L, 453S form of human CYP1B1, the MCF-7 CYP1B1 cDNA was cloned and the enzyme was expressed in *Sf*9 insect cells. In microsomal assays supplemented with human NADPH:cytochrome P450 oxidoreductase, the expressed 432L, 453S form catalyzed NADPH-supported E_2 hydroxylation with a similar preference for 4-hydroxylation as the 432V, 453N form, with maximal rates of 1.97 and 0.37 nmol $(min)^{-1}$(nmol cytochrome P450)$^{-1}$ for 4- and 2-hydroxylation, respectively. Cumeme hydroperoxide efficiently supported E_2 hydroxylation by both the 432V, 453N and 432L, 453S forms at several-fold higher rates than the NADPH-supported activities and with a lesser preference for E_2 4- versus 2-hydroxylation (2:1). The hydroperoxide-supported activities of both forms were potently inhibited by the CYP1B1 inhibitor, 3,3′,4,4′,5,5′-hexachlorobiphenyl. These results indicate that the 432V, 453N and 432L, 453S forms of CYP1B1 have similar catalytic properties for E_2 hydroxylation, and that human CYP1B1 is very efficient in catalyzing the hydroperoxide-dependent formation of catecholestrogens. Published by Elsevier Science Ltd.

Keywords: Cytochrome P450; Genetic polymorphism; Estrogen metabolism; Catecholestrogens

1. Introduction

The catecholestrogens, 2- and 4-hydyroxyestradiol (2- and 4-OHE_2), are agonists of the estrogen receptor (ER) and are capable of activating ER-mediated gene transcription [1,2]. There is also considerable evidence that catecholestrogens mediate physiologic responses in the anterior pituitary, ovary and uterus, and by mechanisms that are distinct from those of the parent hormone, 17β-estradiol (E_2) [1,3–5]. In addition to its proposed physiologic role in implantation [4], 4-OHE_2 is a known carcinogen. The carcinogenicity of 4-OHE_2 was established in the Syrian hamster, in which treatment of male animals with 4-OHE_2 leads to a high incidence (80–100%) of renal tumors [6,7]. In the initiation of estrogen-induced carcinogenesis, it is thought that the catecholestrogens are formed and are further oxidized to semiquinone and quinone forms which form adducts with DNA [8,9] and are substrates for redox-cycling reactions in which free radicals are produced that ultimately cause damage to lipids, proteins and DNA [10–13].

Due to their high reactivity and rapid metabolic clearance [14], both the physiologic and carcinogenic

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effects of catecholestrogens in estrogen target tissues are likely to be mediated by local synthesis of these metabolites rather than from circulating metabolites. Catecholestrogen synthetic activity has been observed in a number of extrahepatic estrogen target tissues, and peroxidative as well as NADPH-supported mechanisms of catechol estrogen formation have been described [15–17]. While catecholestrogen synthetic activities have been identified in microsomes from various tissues, it is often not known what specific enzymes catalyzed the NADPH-dependent and peroxidative activities. In recent years a number of cytochromes P450 (P450) have been characterized at the molecular level, and their E_2 hydroxylase activities have been determined. To date, the most catalytically efficient catecholestrogen synthetic enzyme identified is human cytochrome P450 1B1 (CYP1B1), which catalyzes the NADPH-supported hydroxylation of 17β-estradiol (E_2) predominantly at the C-4 position with a lesser activity at C-2 [18–22]. CYP1B1 is expressed in a number of extrahepatic tissues including breast tissue and tumors [23–28].

The E_2 4-hydroxylase activity of human CYP1B1 was first identified in MCF-7 breast cancer cells as one of the activities induced by the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [18,29,30]. In recent studies, the reverse-transcriptase polymerase chain reaction (RT-PCR) was used to quantify CYP1B1 mRNA levels in MCF-7 cells [31]. Sequencing of these PCR products revealed that the CYP1B1 expressed in MCF-7 cells was not the previously characterized enzyme [19,32], but a polymorphic form with leucine substituted for valine at position 432 and serine substituted for asparagine at position 453. In recent years there has been considerable interest in the potential roles of genetic polymorphisms and in particular variant enzyme forms with altered activities toward endogenous and exogenous substrates as determinants of susceptibility to disease. To investigate the NADPH- and organic hydroperoxide-supported E_2 hydroxylase activities of the 432L, 453S form of human CYP1B1 and to compare them to those of the 432V, 453N form, the MCF-7 CYP1B1 cDNA was cloned and the enzyme was expressed in *Spodoptera frugiperda* (*Sf*9) insect cells.

2. Methods and materials

2.1. Isolation of RNA and DNA from MCF-7 cells

MCF-7 cells were cultured as described [29]. At confluence, cultures were treated with 10 nM TCDD for 24 h prior to isolation of total RNA by phenol guanidinium thiocyanate extraction [33]. Genomic DNA was isolated from untreated MCF-7 cultures as described [34].

2.2. Polymerase chain reaction

Reverse transcription of 2.5 μg of total RNA was primed with oligo-dT using Superscript II reverse transcriptase followed by RNaseH according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD). PCR of the exon 2/3 CYP1B1 cDNA fragment was performed as described [31]. PCR of the CYP1B1 sequence from MCF-7 genomic DNA was performed with primers designed from the human *CYP1B1* gene sequence [35]. The primers were: forward, 5′-TCTCTCTCCACATTAAACACC-3′ (gene sequence nucleotides 7854 to 7874 of intron 2; GenBank accession number U56438) and reverse, 5′-GCCCACT-GAAAAAATCATC-3′ (nucleotides 8235 to 8217 of exon 3). To obtain a full-length CYP1B1 cDNA for cloning and expression in *Sf*9 cells, the coding region of the CYP1B1 cDNA from the ATG start site (in bold in the primer sequence) to the TAA stop codon (in bold in the primer sequence) was amplified with the high-fidelity Vent DNA polymerase (New England Biolabs, Beverly, MA) with 4 mM $MgSO_4$ by using the following primers: forward, with added *Kpn*I site (underlined), 5′-TAAGGTACC**ATG**GGCACCAGCCTCA-GC-3′ (cDNA nucleotides 347 to 364; GenBank accession number U03688) and reverse, with added *Sal*I site (underlined), 5′-CGGTCGAC**TTA**TTGGCAAGTTT-CCTTGGC-3′ (nucleotides 1958 to 1978). After 1 min of denaturation at 97°C, cDNA was amplified for 30 cycles of 97°C for 15 s and 72°C for 4 min with a final elongation for 10 min at 72°C.

2.3. Cloning of the MCF-7 CYP1B1 cDNA

The PCR product representing the cDNA of the complete CYP1B1 coding sequence was purified by electrophoresis through 2% agarose, and the band corresponding to a size of 1649 bp was excised and extracted with QIAquick gel extraction kit (Qiagen, Santa Clarita, CA). This CYP1B1 cDNA was cloned into PCR-Blunt (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Recombinant plasmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen) and screened by *Kpn*I/*Sal*I restriction endonuclease digestion.

2.4. CYP1B1 expression in Sf 9 cells

The clone for CYP1B1 was expressed in *Sf*9 insect cells by subcloning it into the *Bam*HI/*Not*I sites of pVL1393 baculovirus transfer vector (PharMingen, San Diego, CA). After insertion into this vector, the nucleotide sequence of the entire CYP1B1 insert was verified by DyeDeoxy terminator cycle sequencing on an Applied Biosystems (Perkin Elmer, Foster City, CA)

model 377 sequencer by staff of the Wadsworth Center Molecular Genetics Core. The recombinant baculovirus was cotransfected with linearized BaculoGold DNA (PharMingen) and amplified in *Sf*9 cells as described [36]. The recombinant baculovirus was used to infect *Sf*9 cells plated at a density of 4.5×10^5 cells/cm^2 in TNM-FH medium with 10% fetal bovine serum (FBS) at a multiplicity of infection of 10 plaque-forming units per cell. After 24 h, the medium was replaced with fresh TNM-FH containing 20% FBS and 4 μg/ml hemin. To recover the microsomal fraction containing the expressed human CYP1B1, the cells were harvested 72 h after infection, washed twice with phosphate-buffered saline and resuspended in 10 mM Tris-acetate buffer, pH 7.4, containing 150 mM KCl. Cells were lysed by homogenization with a Polytron (Brinkmann,Westbury, NY) with three 10-s passes followed by three 30-s bursts of sonication using a probe sonicator. The nuclear fraction and cell debris were removed by centrifugation at $10\,000 \times g$ for 20 min; the supernatants were recovered and centrifuged at $100\,000 \times g$ to produce the microsomal pellets. Microsomes were resuspended at 10 mg protein per ml in the homogenization buffer with 20% (v/v) glycerol and stored at −80°C.

2.5. Characterization of cDNA-expressed CYP1B1

The P450 content of the *Sf*9 microsomes containing human CYP1B1 was determined by the CO difference spectral method [37]. Western immunoblots of sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) with 10% acrylamide were probed with anti-human CYP1B1 antibodies from the human CYP1B1 Western blotting kit (Gentest, Woburn, MA) according to the manufacturer's protocol, with the exceptions that the primary rabbit anti-human CYP1B1 antibody was used at 1:2000 dilution and the secondary horseradish peroxidase-conjugated goat anti-rabbit antibody was used at 1:5000 dilution. The immunoreactive proteins were visualized by use of enhanced chemiluminescence (Pierce, Rockford, IL).

2.6. Determination of E_2 hydroxylase activity

The E_2 hydroxylase activity was determined as described [29,30], with some modifications. Microsomal incubations (250 μl) contained 5 mM $MgCl_2$, 0.05 to 20 μM of E_2 as substrate and 20 pmol of cDNA-expressed CYP1B1 in 0.1 M phosphate buffer, pH 7.4, containing 2 mM ascorbic acid. Microsomes containing the 432V, 453N form of CYP1B1 co-expressed with human NADPH:cytochrome P450 oxidoreductase (OR) in *Sf*9 cells were obtained from Gentest. In determinations of the NADPH-supported E_2 hydroxylase activity of the 432L, 453S variant of CYP1B1, cDNA-expressed human OR (Panvara, Madison, WI) was added in a 9:1 ratio to P450. For evaluation of the cumene hydroperoxide-supported activities, 2 pmol of CYP1B1 was used per assay, microsomal protein content was maintained at 70 μg per assay by addition of control *Sf*9 microsomes (Gentest) and the NADPH cytochrome P450 oxidoreductase was omitted. In studies of the inhibitory effects of PCB 169 [38], varying concentrations (0.1 nM to 10 μM) were added to the reaction mixtures prior to preincubation. The incubations were pre-warmed at 37°C for 5 min, and enzymatic reactions were initiated with either NADPH (1.4 mM final concentration) or cumene hydroperoxide (500 μM final concentration). After 10 min, the reactions were terminated by the addition of two volumes of ice-cold 30 mM ascorbic acid, addition of internal standards and immediate extraction with ethyl acetate. The extracts were dried over anhydrous sodium sulfate, evaporated to dryness under N_2 and derivatives were prepared by reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce) containing 10% (v/v) pyridine. These derivatized samples were analyzed by GC/MS with selected-ion monitoring [29].

3. Results

3.1. Sequences of complementary and genomic DNA encoding CYP1B1

The sequence obtained for the MCF-7 CYP1B1 cDNA revealed three single-nucleotide polymorphisms within exon 3, each of which has been previously reported in a study of normal individuals [39]. These include a substitution of C for G at nucleotide 1640, which results in substitution of leucine for valine at amino-acid position 432, a substitution of C for T at nucleotide 1693, which would not affect the encoded amino-acid sequence, and substitution of G for A at nucleotide 1704, which results in the substitution of serine for asparagine at position 453. These same nucleotide substitutions were observed when genomic DNA was isolated from MCF-7 cells and the relevant regions amplified by PCR and sequenced directly (Fig. 1). The fact that double peaks were not observed in the sequencing electropherograms at these nucleotide positions indicates that MCF-7 cells are homozygous at each of these loci. After cloning into the pVL 1393 baculovirus transfer vector, sequencing of the entire CYP1B1 insert indicated that the substitutions of leucine for valine at position 432 and serine for asparagine at position 453 were the only amino acid changes encoded by this cDNA when compared with the previously reported sequence [32].

3.2. Characterization of cDNA-expressed 432L, 453S human CYP1B1

Analysis of microsomes from CYP1B1-transfected *Sf*9 cells by Western immunoblot showed a single band with an apparent molecular mass of 55 kDa, whereas analysis of an equal quantity of microsomal protein from control *Sf*9 cells failed to show the immunoreactive band (Fig. 2). Spectral analysis of the P450 content of several preparations of microsomes from CYP1B1-transfected *Sf*9 cells indicated expression levels of 20–76 pmol of P450 per mg of microsomal protein.

3.3. Catalytic properties of cDNA-expressed human CYP1B1

Microsomes prepared from *Sf*9 cells expressing the 432L, 453S form of human CYP1B1, when supplemented with cDNA-expressed human OR, catalyzed NADPH-dependent catechol estrogen formation predominantly at the C-4 position with a lesser activity at C-2 (Fig. 3). Rates of E_2 2- and 4-hydroxylation were maximal with a ratio of added OR to P450 of 9:1 (data not shown). At this maximal level of added OR, values of 1.98 ± 0.05 nmol $(\text{min})^{-1}$ $(\text{nmol P450})^{-1}$ and 3.34 ± 0.30 μM were determined for V_{max} and K_m, respectively, for E_2 4-hydroxylation, and 0.37 ± 0.03 nmol $(\text{min})^{-1}$ $(\text{nmol P450})^{-1}$ and 5.3 ± 1.3 μM for V_{max} and K_m, respectively, for E_2 2-hydroxylation. The E_2 hydroxylase activities of microsomes from *Sf*9 cells in which the 432V, 453N form was co-expressed with OR were likewise evaluated; values of 1.32 ± 0.07 nmol $(\text{min})^{-1}$ $(\text{nmol P450})^{-1}$ and 0.96 ± 0.24 μM for V_{max} and K_m for E_2 4-hydroxylation and values of 0.32 ± 0.03 nmol $(\text{min})^{-1}$ $(\text{nmol P450})^{-1}$ and 1.6 ± 0.5 μM for V_{max} and K_m for E_2 2-hydroxylation were obtained.

The catecholestrogen synthetic activities of the 432V, 453N and 432L, 453S forms in reactions supported by cumene hydroperoxide were also investigated. Cumene hydroperoxide (500 μM) effectively supported 4- and 2-hydroxylation of E_2 by both forms of CYP1B1. The E_2 dependence of the cumene hydroperoxide-supported activities of the 432L, 453S and 432V, 453N forms is shown in Fig. 4. The kinetic data are summarized in

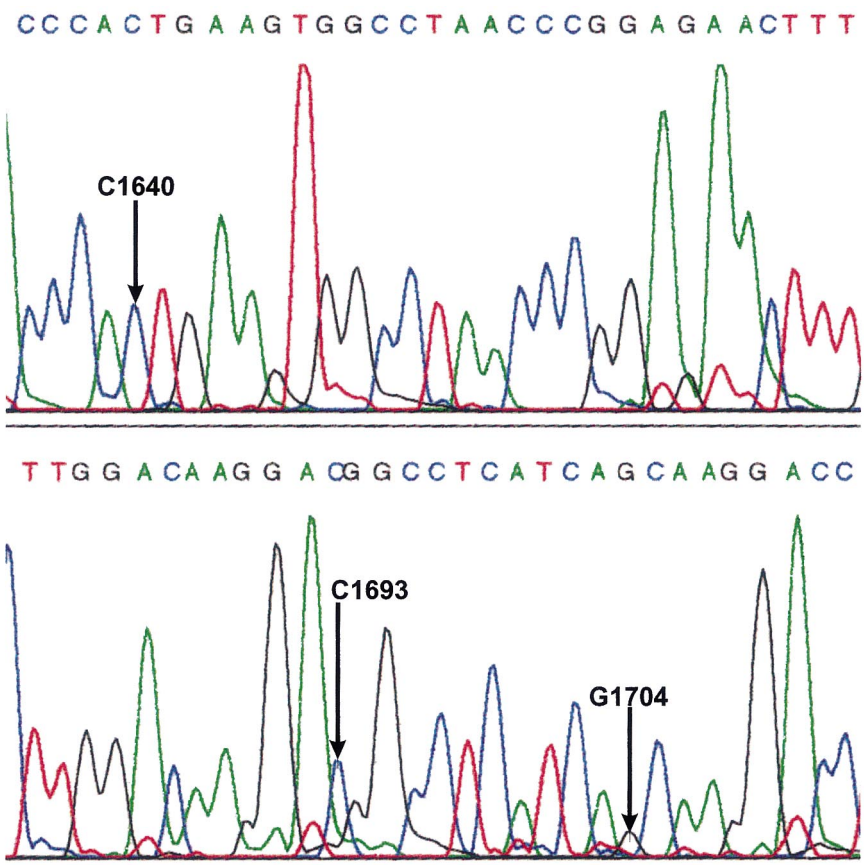

Fig. 1. Nucleotide sequence of PCR-amplified genomic DNA from MCF-7 cells. Genomic DNA was isolated from MCF-7 cells and amplified by 30 cycles of PCR with the forward primer in intron 2 and the reverse primer in exon 3 as described in Section 2. The product DNA was gel-purified and sequenced directly. Portions of a sequencing electropherogram are shown for the regions of the sequence with differences at cDNA nucleotide positions 1640 (codon 432), 1693 (codon 449), and 1704 (codon 453) indicated by the arrows.

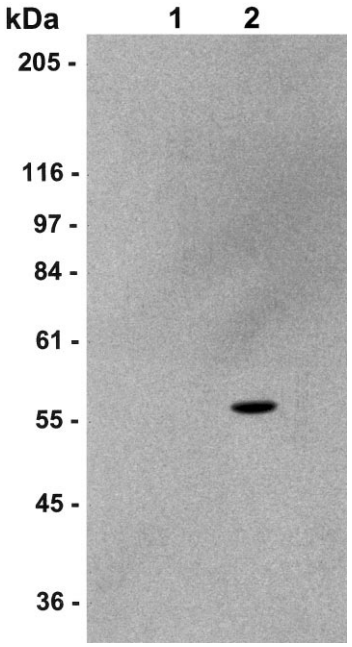

Fig. 2. Western immunoblot of the 432L, 453S variant of human CYP1B1 expressed in *Sf*9 cells. Samples of microsomal protein (32 μg) from control (lane 1) and CYP1B1-PVL1393-infected *Sf 9* cells (lane 2) were resolved by SDS-PAGE, electroblotted to nitrocellulose, and detected with anti-CYP1B1 antibodies and enhanced chemiluminescence. The immunoreactive band in lane 2 represents 1 pmol of cDNA-expressed CYP1B1.

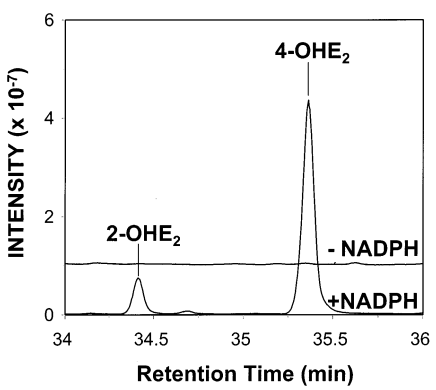

Fig. 3. NADPH-supported E_2 hydroxylation catalyzed by 432L, 453S variant of human CYP1B1. Microsomes from *Sf*9 cells expressing the 432L, 453S variant of human CYP1B1 were incubated with cDNA-expressed human OR and 10 μM E_2 for 10 min with or without addition of NADPH as described in Section 2. E_2 metabolites were then extracted and metabolite derivatives were prepared and analyzed by GC/MS. Shown are GC/MS ion chromatograms at m/z 504, the molecular ion of OHE_2 trimethylsilyl derivatives, for incubation without (upper trace) or with (lower trace) the addition of NADPH. The retention times of the 2- and 4-OHE_2 derivatives were as indicated.

Table 1. Cumene hydroperoxide-supported activities of both forms showed higher maximal velocities, higher values of apparent K_m for E_2 and a lower ratio of 4- to 2-hydroxylation (2:1) than were observed for the NADPH-supported activities.

PCB 169 was recently found to potently inhibit CYP1B1 activity in TCDD-treated MCF- 7 cells and the NADPH-supported activity of the 432V, 453N form expressed in *Saccharomyces cerevisiae* [38]. In this study we found that PCB 169 inhibited the cumene hydroperoxide- supported 4- and 2-hydroxylation of E_2 catalyzed by both the 432V, 453N and 432L, 453S forms of CYP1B1 with very similar efficacy (Table 1).

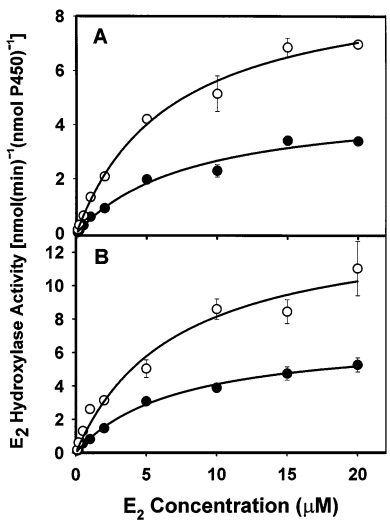

Fig. 4. Effect of varying E_2 concentration on the cumene hydroperoxide-supported activities of the 432V, 453N and 432L, 453S forms of human CYP1B1 expressed in *Sf* 9 cells. Shown are the rates of E_2 4-hydroxylation (○) and 2-hydroxylation (●) in the presence of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 15 or 20 μM E_2 and 500 μM cumene hydroperoxide catalyzed by the 432L, 453S (panel A) and 432 V, 453 N (panel B) forms of human CYP1B1. Data are the means ± S.E. of three independent experiments.

Table 1
Kinetic evaluation of the cumene hydroperoxide-supported E_2 hydroxylase activities of the 432V, 453N and 432L, 432S forms of human CYP1B1[a]

	432V, 453N CYP1B1	432L, 432S CYP1B1
E_2 4-hydroxylation		
K_m (μM)	7.05 ± 2.01	6.73 ± 0.98
V_{max} (nmol (min)$^{-1}$ (nmol)$^{-1}$)	13.9 ± 1.6	9.41 ± 0.53
IC_{50} PCB169 (nM)	50 ± 0.73	56 ± 18
E_2 2-hydroxylation		
K_m (μM)	7.11 ± 0.73	8.3 ± 2.0
V_{max} (nmol (min)$^{-1}$ (nmol)$^{-1}$)	7.02 ± 0.28	4.87 ± 0.51
IC_{50} PCB169 (nM)	45 ± 17	61 ± 17

[a] Data are the mean ± S.E.. Values of V_{max} and K_m were estimated by fitting the data presented in Fig. 4 to the Michaelis–Menton equation as described [18,30]. Values of EC_{50} for inhibition of E_2 hydroxylase activity by PCB 169 were estimated from concentration-response data as described [38].

4. Discussion

Results reported here indicate that the initial characterizations of the E_2 4-hydroxylase activity of human CYP1B1 in MCF-7 cells [18,29,30] were of the 432L, 453S polymorphic form of CYP1B1, as supported by nucleotide sequencing of both genomic and complementary DNA of CYP1B1. The first full-length human CYP1B1 cDNA obtained was cloned from a cDNA library prepared from the kerotinocyte cell line, SCC-12F, and encodes the 432V, 453N form [32]. This cDNA was used to express the 432V, 453N form and to demonstrate the E_2 4-hydroxylase activity of human CYP1B1 [19], confirming the earlier hypothesis that CYP1B1 was the TCDD-inducible E_2 4-hydroxylase in MCF-7 cells [18].

The V432L and N453S polymorphisms represent two of the six polymorphisms of human CYP1B1 that have been identified in population studies to date. Two others which result in amino-acid substitutions are glycine substituted for arginine at position 48 and serine substituted for alanine at position 119. A silent polymorphism at cDNA nucleotide 1693 (T→C) which does not change the aspartic acid at position 449 and a substitution of T for C at nucleotide 3793 of the gene sequence, which is 13 base pairs upstream of the ATG, were also identified [39]. Genetic analysis of a group of normal individuals, 50 British and 50 Turkish, showed that 48R, 119A, 432L, 1693C, and 3793C were more common in this population with allele frequencies of 0.71, 0.71, 0.715, 0.705 and 0.76, respectively [39]. The MCF-7 cDNA cloned and expressed in the current study encoded the more common alleles 48R, 119A, 432L, and 1693C; the only lower-frequency allele encoded in the cloned MCF-7 CYP1B1 cDNA was 453S.

Epidemiologic studies have been initiated to evaluate the potential roles of CYP1B1 polymorphisms in susceptibility to disease. Bailey et al. [40] investigated the V432L and N453S polymorphisms and reported that, while none of the genotypes showed a significant association with breast cancer, homozygosity for the 432V form of human CYP1B1 was strongly associated with the expression of estrogen and progesterone receptors in breast cancer cases. Fritsche et al. [41] investigated the V432L CYP1B1 polymorphism in a German population and reported that the CYP1B1*1/*2 (heterozygous at position 432) and CYP1B1*2/*2 (homozygous for 432V) genotypes were significantly more frequent in patients with colorectal carcinoma compared with a control group.

Since there are known instances when P450 polymorphisms result in a null phenotype or enzymes with altered activity, a key question regarding the potential roles of polymorphic forms of human CYP1B1 in disease susceptibility is whether the amino-acid changes of these polymorphisms eliminate or alter the catalytic activities of the enzyme. Recent genetic studies of primary congenital glaucoma (PCG) have unexpectedly been informative on this issue. As a result of a search for the gene defect that causes PGC, truncating and frame-shift mutations of the human *CYP1B1* gene were identified that were linked to the disorder, strongly suggesting that it is the locus on chromosome 2p21 that is associated with PGC [39]. Additionally, eight rare missense mutations in the *CYP1B1* gene were identified that segregated with PCG. Based on a molecular model of CYP1B1 prepared by alignment with the soluble bacterial P450s for which the structures have been solved, the PGC-associated missense mutations mapped to conserved core structures which, when altered by amino-acid substitutions, may be defective in heme incorporation and result in inactive enzyme. If functional CYP1B1 is necessary to prevent primary congenital glaucoma, then both alleles of each of the polymorphisms at amino-acid positions 48, 119, 432 and 453 should encode active forms of CYP1B1, as these rather common polymorphisms do not segregate with PCG and, based on the molecular model, map outside the conserved core structures of CYP1B1. Evidence to date is consistent with this hypothesis. Based on the expression of various CYP1B1 alleles in *E. coli* [22] and *S. cerevisiae* [19], it appears that either allele at positions 119 and 432 encodes an active E_2 hydroxylase, and the 453S form expressed in *Sf*9 cells in the current study is likewise active in catecholestrogen synthesis. While enzyme activity does not appear to be profoundly affected by the amino-acid substitutions at positions 119, 432 and 453, there is the possibility that more subtle changes in catalytic properties may result from these polymorphisms.

Our comparisons of the activities of the 432L, 453S and 432V, 453N variants suggest that there are, at most, only relatively minor differences in the catalytic properties of these forms of human CYP1B1. The 432L, 453S form catalyzed NADPH-supported E_2 4- and 2-hydroxylation in approximately a 5:1 ratio of the rates, which is comparable to what we observed for the 432V, 453N form [19]. The values of V_{max} and K_m for NADPH-supported E_2 4- and 2-hydroxylation reported here for the 432L, 453S form differed from those determined for the 432V, 453N form; however, the nature of the interaction of the P450 with OR may have been a confounding factor in these experiments. The fact that the human OR was not expressed in the *Sf*9 microsomal membrane along with the 432L, 453S form but was added just prior to the assay may have affected the kinetic parameters of NADPH-supported activities, as reconstituted versus co- expressed P450 enzyme systems often differ in kinetic parameters [42]. The value of K_m for 4-hydroxylation catalyzed by the 432L, 453S form expressed in *Sf*9 cells with added OR was 3.34 ± 0.30 μM, whereas for microsomes from TCDD-treated

MCF-7 cells, which our current results indicate was the activity of the 432L, 453S form of CYP1B1, the value was 0.81 ± 0.20 μM [30]. These results suggest that in the normal eukaryotic microsomal membrane with OR present, the value of K_m for E_2 4-hydroxylation catalyzed by the 432L, 453S form is lower than when OR is added to microsomal preparations. The values of K_m determined for E_2 4-hydroxylation catalyzed by the 432V, 453N form expressed in *S. cerevisiae* [19], which was supported by endogenous OR, and, in the current study, co-expressed with human OR in *Sf*9 cells were 0.78 ± 0.24 and 0.96 ± 0.24 μM, respectively.

Potential differences in the complex interactions between P450 and OR are not confounding factors when the organic hydroperoxide-supported metabolism is investigated, as this 'peroxide shunt' of the P450 catalytic mechanism does not require OR or electrons from NADPH [43–45]. Analysis of the hydroperoxide-supported hydroxylase activity provides for a more direct comparison of potential effects of the polymorphisms on portions of the catalytic mechanism involving binding of the steroid substrate and the active oxygen species. The comparison of the cumene hydroperoxide-supported activities showed similar values of apparent K_m for E_2 2- and 4-hydroxylation, although the values obtained for V_{max} for both 4- and 2-hydroxylation were about 30% higher for the 432V, 453N than the corresponding values for the 432L, 453S form. In addition, the two forms showed very similar sensitivity to inhibition of the cumene hydroperoxide-supported activities by PCB 169.

The observed ability of the two forms of CYP1B1 to efficiently catalyze cumene hydroperoxide-supported E_2 hydroxylation gives rise to the question of whether organic hydroperoxides produced within cells can support CYP1B1-catalyzed E_2 hydroxylation as well. Lipid hydroperoxides are formed in normal and cancerous tissue, and these compounds have been shown to serve as active oxygen donors in P450-catalyzed reactions [46,47]. CYP1A1 utilizes lipid peroxides in the conversion of diethylstilbestrol to diethylstilbestrol quinone and the conversion of 2- and 4-OHE_2 to the corresponding quinone metabolites, which has been proposed as a key step in estrogen-induced carcinogenesis [6,9–13]. While the possible role of human CYP1B1 in the oxidation of catechol estrogens to their respective quinones has not been investigated, the possibility that CYP1B1 may catalyze multiple steps in the initiation of estrogen carcinogenesis is an area for further research.

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