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Catalytic properties of polymorphic human cytochrome P450 1B1 variants

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Four polymorphic human cytochrome P450 (CYP) 1B1 allelic variants, namely Arg48,Ala119,Leu432,Asn453, Arg48,Ser119,Leu432,Asn453, Arg48,Ala119,Val432,Asn453 and Arg48,Ser119,Val432,Asn453, were expressed in *Escherichia coli* together with human NADPH-P450 reductase and the recombinant proteins (in bacterial membranes) were used to assess whether CYP1B1 polymorphisms affect catalytic activities towards a variety of P450 substrates, including diverse procarcinogens and steroid hormones. Activities for activation of 19 procarcinogens to DNA-damaging products by these four CYP1B1 variants in a *Salmonella typhimurium* NM2009 *umu* response system were found to be essentially similar, except that a Arg48, Ser119,Leu432,Asn453 variant was slightly more active (1.2- to 1.5-fold) than the other three CYP1B1 enzymes in catalyzing activation of (+)- and (–)-benzo[*a*]pyrene-7,8-diols, 7,12-dimethylbenz[*a*]anthracene-3,4-diol, benzo[*g*]chrysene-11,12-diol, benzo[*b*]fluoranthene-9,10-diol,2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline, 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-aminofluorene. Kinetic analysis of 17β-estradiol hydroxylation showed that V_{max} values for 4-hydroxylation ranged between 0.9 and 1.5 nmol/min/nmol P450 for 4-hydroxylation and 0.3 and 0.6 nmol/min/nmol P450 for 2-hydroxylation in these CYP1B1 variants, with K_m values ranging from 1 to 9 μM. Interestingly, the ratio of product formation of 4-hydroxyestradiol to 2-hydroxyestradiol was higher for the Val432 variants of CYP1B1 variants than the Leu432 variants of the enzyme. The same trend was noted in the ratio of estrone 4-hydroxylation to estrone 2-hydroxylation catalyzed by CYP1B1 variants. Mutation in the CYP1B1 genes also affected the K_m and V_{max} values in the 6β-hydroxylation of testosterone and 6β- and 16α-hydroxylation of progesterone. These results indicate that the polymorphisms in the human *CYP1B1* gene cause some alterations in catalytic function towards procarcinogens and steroid hormones and thus may make some contribution to susceptibilities of individuals towards mammary and lung cancers in humans.

Abbreviations: B[*g*]C, benzo[*g*]chrysene; B[*b*]F, benzo[*b*]fluoranthene; B[*a*]P, benzo[*a*]pyrene; B[*c*]P, benzo[*c*]phenanthrene; CYP, cytochrome P450; DB[*a*,*l*]P, dibenzo[*a*,*l*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; hNPR, human NADPH-P450 reductase; IQ, 2-amino-3-methylimidazo [4,5-*f*]quinoline; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; Trp-P-1, 3-amino-1,4-dimethyl5*H*-pyrido[4,3-*b*]indole; diol, used in the text to designate the prefix 'dihydroxydihydro' for individual polycyclic hydrocarbons.

Introduction

Kawajiri and co-workers have reported that *Msp*I and Ile–Val polymorphisms of the *CYP1A1* gene appear to be related to the susceptibility towards lung cancer, but not breast cancer, in a Japanese population (1,2). The mechanisms by which the cytochrome P450 (CYP) 1A1 polymorphisms determine the susceptibilities of individuals towards lung cancer are not known at present, however, it has been suggested that some differences in the catalytic activities of these CYP1A1 variants towards activation of lung carcinogens such as benzo[*a*]pyrene (B[*a*]P) to active metabolites that initiate cellular transformations in this organ might be involved (3–5). Very recently, we have reported evidence suggesting that CYP1B1, as well as CYP1A1, is also very active in catalyzing the activation of lung carcinogens such as (+)- and (–)-B[*a*]P-7,8-diol, dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P)-11,12-diol, benzo[*g*]chrysene (B[*g*]C)-11,12-diol, benzo[*c*]phenanthrene (B[*c*]P)-3,4-diol, 7,12-dimethylbenz[*a*]anthracene (DMBA)-3,4-diol, 5-methylchrysene-1,2-diol and 5,6-dimethylchrysene-1,2-diol (6). Some of these chemicals are also known to be mammary carcinogens in experimental animals and recent studies have established that CYP1B1 is expressed at substantial levels in human mammary epithelial cells (7–9).

Another interesting finding is the observation that CYP1B1 catalyzes the oxidation of 17β-estradiol at a higher rate at the 4 position than at the 2 position; the former metabolite has been suggested to be involved in development of breast cancer in humans (10–12). CYP1A1 and CYP1A2 oxidize 17β-estradiol preferentially at the 2 position (12,13).

It has recently been reported that there are at least six polymorphisms of the *CYP1B1* gene in humans and that polymorphisms are found leading to amino acid replacements of Arg by Gly, Ala by Ser, Leu by Val and Asn by Ser at codons 48, 119, 432 and 453 (14–16). Thus, it is of interest to know whether the mutated CYP1B1 proteins change in catalytic functions towards environmental procarcinogens and estrogens.

In this paper we examine the effects on catalytic properties of the two types of allelic variants, with amino acid replacements of Ala by Ser at codon 119 and of Leu by Val at codon 432. We expressed CYP1B1 variants in *Escherichia coli* in which plasmids pCW'/1B1^{RALN}/hNPR, pCW'/1B1^{RSLN}/hNPR, pCW'/1B1^{RAVN}/hNPR and pCW'/1B1^{RSVN}/hNPR were introduced; the resulting bacterial membranes containing CYP1B1 variants Arg48,Ala119,Leu432,Asn453, Arg48,Ser119,Leu432,Asn453, Arg48,Ala119,Val432,Asn453 and Arg48,Ser119, Val432,Asn453, respectively, together with human NADPH-

P450 (hNPR) reductase (17) were used for measurement of catalytic activities. Catalytic properties of CYP1B1 variants examined in this study were determined using 19 procarcinogens, 17β-estradiol, estrone, testosterone and progesterone.

Materials and methods

Chemicals

B[*a*]P, (+)- and (–)-B[*a*]P-7,8-diols were purchased from the National Cancer Institute Chemical Carcinogen Repository Midwest Research Institute (Kansas City, MO). DB[*a*,*l*]P-11,12-diol, DMBA-3,4-diol, B[*g*]C-11,12-diol, B[*c*]P-3,4-diol and benzo[*b*]fluoranthene (B[*b*]F)-9,10-diol were kindly provided by Dr Stephen S.Hecht (University of Minnesota, Minneapolis, MN). Other chemicals and reagents used in this study were obtained from sources described previously or were of the highest quality commercially available (17–19).

Construction of different genotype CYP1B1 cDNAs expression plasmids

The cDNA fragments were amplified from Human Fetal-Kidney Quick Clone cDNA (Clontech, Palo Alto, CA) with LATaq DNA polymerase, using the following primers: 5′-primer, 5′-TATCGGATCCAAGGTCCCAGTTCCTT-CTCG-3′; 3′-primer, 5′-AACTGGATCCTGAAGAACCGCTGGGTATGG-3′. PCR products contained the full-length coding region of the *CYP1B1* gene. Variant sequences were introduced into the monocistronic *CYP1B1* expression plasmid pCW'/1B1 (construct 3) described previously (17), which encodes Arg at position 48, Ala at position 119, Val at position 432 and Asn at position 453. The CYP1B1 Arg48,Ser119,Val432,Asn453 variant was prepared by replacing the *Pfl*MI–*Eco*RI fragment of the original expression plasmid (containing codon 119) with the cognate fragment of the amplified cDNA encoding Arg at position 48, Ser at 119, Leu at 432 and Asn at 453. The CYP1B1 Arg48,Ser119,Leu432,Asn453 variant was prepared by cloning the *Pfl*MI–*Ppu*MI fragment (containing codons 119, 432 and 453) from the amplified cDNA encoding Ser at 119, Leu at 432 and Asn at 453 into the expression vector in place of the original (Ala119,Val432,Asn453) sequence. Bicistronic constructs of CYP1B1 Arg48,Ser119,Val432,Asn453 and Arg48, Ser119,Leu432,Asn453 variants were then prepared by subcloning the respective P450 cDNAs from the monocistronic expression plasmids as *Nde*I–*Xba*I fragments into the original bicistronic expression plasmid, pCW'/1B1/hNPR (17). Finally, the CYP1B1 Arg48,Ser119,Leu432,Asn453 variant was prepared in bicistronic format by replacing the *Nde*I–*Eco*RI fragment (containing codon 119) of the bicistronic vector encoding CYP1B1 Arg48,Ser119,Leu432,Asn453 with the cognate fragment from the bicistronic vector for the original variant. In each case, replacement of cDNA cassettes was confirmed by diagnostic gain or loss of restriction sites. The presence of specific mutations at codons 119 and 432 was further confirmed by single-stranded confirmation polymorphism analysis. In all cases the N-terminal codon modifications found necessary to achieve expression of CYP1B1 in *E.coli* and present in the original expression vector (17) were retained. All expression constructs used in this study encoded Arg at position 48 and Asn at position 453.

Bacterial 'bicistronic' CYP1A1, CYP1A2, CYP2E1 and CYP3A4 systems were prepared as described (17,20).

Expression

Recombinant CYP1A1 and CYP1B1 variants were co-expressed in *E.coli* with hNPR and bacterial membranes were harvested and characterized as described previously (17,20). Briefly, ~0.1 μg of plasmid DNA was introduced into DH5α competent cells (40 μl of stock bacterial suspension) by a heat shock procedure. A single colony was picked and the bacteria were grown in LB medium containing 100 μg ampicillin/ml and 0.1% glucose (w/v) at 37°C for 24 h. Following dilution of the bacteria with 100 vol of TB medium containing 1.0 mM thiamine, 0.25 ml of a trace element solution/l, 100 μg ampicillin/ml, 1.0 mM IPTG and 0.5 mM 5-(δ)-aminolevulinic acid (S-ALA), the cells were grown for 24 h at 30°C with shaking at 150 r.p.m. (using triple baffle flasks) in a Bio-Shaker (type BR-300LF, Taitec Co., Tokyo).

Steroid hydroxylation activities

17β-Estradiol and estrone hydroxylation activities were determined in a standard incubation mixture (final volume 0.25 ml) containing variants of recombinant P450s (0.2 μM), 50 mM potassium phosphate buffer (pH 7.4), an NADPH generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate and 0.5 U of glucose 6-phosphate dehydrogenase/ml, 1.5 mM ascorbic acid and estradiol or estrone (100 μM). Product formation was determined as described previously (13).

Testosterone and progesterone hydroxylation activities were determined by the methods described previously (21).

Genotoxicity assay

P450-dependent activation of procarcinogens to reactive products that cause induction of *umu* gene expression in tester strain *Salmonella typhimurium* NM2009 was determined in systems containing *E.coli* membranes (in which CYP1A1/hNPR or CYP1B1/hNPR had been expressed using bicistronic vectors) as described previously (6,22). Standard incubation mixtures included P450 (10 pmol) and 2.5 μM procarcinogen in a final volume of 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH generating system and 0.75 ml of bacterial suspension. Incubation conditions for *E.coli* membrane and reconstitution systems were as described above. Induction of *umu* gene expression is presented as units of β-galactosidase activity/min/nmol P450 (23).

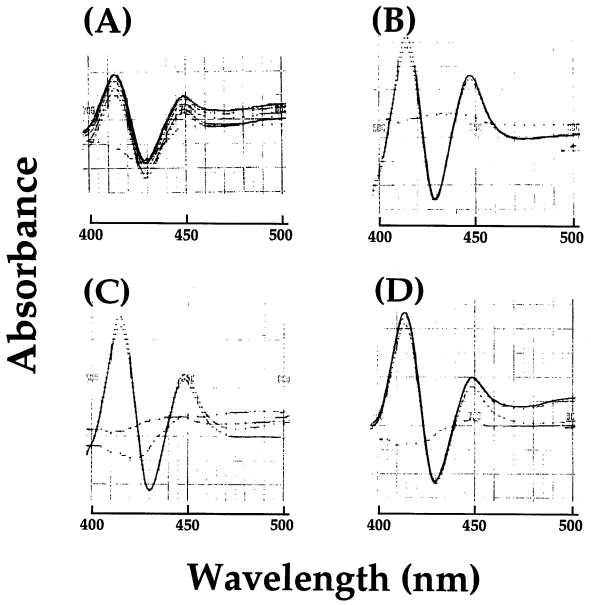

Fig. 1. Carbon monoxide difference spectra of membranes in *E.coli* expressing CYP1B1 variants Arg48,Ala119,Leu432,Asn453 (**A**), Arg48,Ser119,Leu432,Asn453 (**B**), Arg48,Ala119,Val432,Asn453 (**C**) and Arg48,Ser119,Val432,Asn453 (**D**) expressed in *E.coli* membranes in combination with hNPR.

Other assays

P450 was estimated spectrally by the method of Omura and Sato (24). Protein concentrations were estimated by the method of Lowry *et al.* (25).

Statistical analysis

Kinetic parameters for the hydroxylation of 17β-estradiol, testosterone and progesterone by recombinant human P450 enzymes were estimated using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for non-linear regression analysis. Statistical analysis was performed by Student's *t*-test.

Results

Expression of CYP1B1 variants in E.coli

The plasmids pCW'/1B1^{RALN}/hNPR, pCW'/1B1^{RSLN}/hNPR, pCW'/1B1^{RAVN}/hNPR and pCW'/1B1^{RSVN}/hNPR were introduced into *E.coli* and the bacterial membranes were isolated. CO difference spectra showed that all of the constructs produced active CYP1B1 proteins of Arg48,Ala119,Leu432, Asn453, Arg48,Ser119,Leu432,Asn453, Arg48,Ala119,Val432, Asn453 and Arg48,Ser119,Val432,Asn453, respectively, in the membranes (Figure 1). Yields of P450, as determined by the method of Omura and Sato (24), were ~75, ~170, ~170 and ~116 nmol/l medium, respectively, and the spectra in all of the CYP1B1 preparations showed the wavelength maximum at 446 nm in the reduced CO complex.

Procarcinogen activation by CYP1B1 variants

Procarcinogens, including two polycyclic aromatic hydrocarbons, 10 dihydrodiols, five heterocyclic aryl amines, 2-aminofluorene and 3-methoxy-4-aminoazobenzene, were used to determine and compare the catalytic activities of four

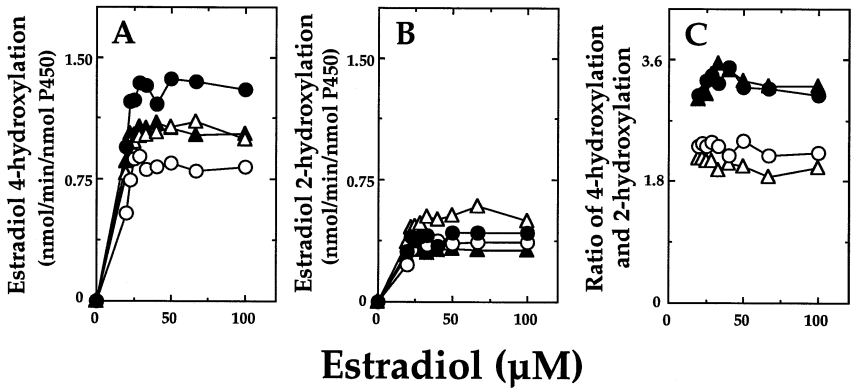

Fig. 2. Concentration dependence of 17β-estradiol 4-hydroxylation (**A**) and 2-hydroxylation (**B**) by CYP1B1 variants Arg48,Ala119,Leu432,Asn453 (○), Arg48,Ser119,Leu432,Asn453 (△), Arg48,Ala119,Val432,Asn453 (●) and Arg48,Ser119,Val432,Asn453 (▲) expressed in *E.coli* membranes in combination with hNPR.

Table I. Activation of procarcinogens by human CYP1B1 variants and CYP1A1 co-expressed with hNPR in *E.coli* membranes

Procarcinogen	Procarcinogen activation (*umu* units/min/nmol P450)				
	CYP1B1 variant				CYP1A1
	Ala119,Leu432	Ser119,Leu432	Ala119,Val432	Ser119,Val432	
B[*a*]P	330 ± 30	330 ± 20	290 ± 40	300 ± 30	320 ± 30
(+)B[*a*]P-7,8-diol	730 ± 10	920 ± 40	730 ± 80	760 ± 90	830 ± 90
(-)B[*a*]P-7,8-diol	580 ± 90	720 ± 10	590 ± 70	660 ± 70	630 ± 30
DB[*a,l*]P-11,12-diol	780 ± 80	880 ± 70	800 ± 70	830 ± 30	850 ± 80
Chrysene-1,2-diol	510 ± 80	616 ± 70	490 ± 30	540 ± 80	750 ± 110
5-Methylchrysene-1,2-diol	1000 ± 140	1080 ± 130	950 ± 90	990 ± 170	1060 ± 60
5,6-Dimethylchrysene-1,2-diol	390 ± 40	440 ± 30	400 ± 50	370 ± 80	460 ± 40
DMBA	80 ± 20	80 ± 20	70 ± 40	80 ± 10	260 ± 50
DMBA-3,4-diol	930 ± 40	1130 ± 20	880 ± 80	850 ± 50	940 ± 60
B[*g*]C-11,12-diol	430 ± 60	530 ± 20	370 ± 40	340 ± 50	380 ± 30
B[*c*]P-3,4-diol	240 ± 10	280 ± 40	190 ± 30	170 ± 30	80 ± 10
B[*b*]F-9,10-diol	670 ± 60	820 ± 80	700 ± 40	590 ± 10	510 ± 40
Trp-P-1	870 ± 90	830 ±100	800 ± 60	890 ± 60	610 ± 60
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	260 ± 40	240 ± 30	200 ± 30	220 ± 30	240 ± 30
MeIQ	560 ± 120	780 ± 90	560 ± 110	610 ± 40	920 ± 90
MeIQx	580 ± 50	570 ± 40	450 ± 140	470 ± 60	720 ± 140
IQ	700 ± 80	910 ± 50	680 ± 80	700 ± 30	720 ± 100
2-Aminofluorene	770 ± 80	830 ± 110	820 ± 10	810 ± 90	740 ± 40
3-Methoxy-4-aminoazobenzene	390 ± 70	350 ± 70	410 ± 20	360 ± 70	950 ± 50

Data are means ± range of duplicate determinations.
All CYP1B1 variants used in this work contained Arg48 and Asn453.

CYP1B1 variants in the membranes of *E.coli* to form reactive metabolites in the tester strain *S.typhimurium* NM2009 (Table I). Human recombinant CYP1A1 co-expressed with hNPR in membranes of *E.coli* was also used for comparison. Activities for activation of 19 procarcinogens to DNA-damaging products by these four CYP1B1 variants in *S.typhimurium* NM2009 were found to be essentially similar, except that the Ser119,Leu432 variant activity was slightly higher (1.2- to 1.5-fold) that those of the other three CYP1B1 enzymes in catalyzing activation of (+)- and (–)-B[*a*]P-7,8-diols, DMBA-3,4-diol, B[*g*]C-11,12-diol, B[*b*]F-9,10-diol,2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-aminofluorene.

Recombinant CYP1A1 gave relatively similar activities to CYP1B1 for procarcinogen activation, except that the former enzyme had higher activities than the latter enzyme for chrysene-1,2-diol, DMBA, MeIQ, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 3-methoxy-4-aminoazobenzene and had lower activities for B[*c*]P-3,4-diol and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1).

17β-Estradiol and estrone hydroxylation by CYP1B1 variants

The dependence of the formation of 4-hydroxy- and 2-hydroxyestradiol on the concentration of 17β-estradiol was examined for four variants of CYP1B1 (Figure 2). 4-Hydroxylation activity was higher than 2-hydroxylation activity in all of the four CYP1B1 enzymes examined. 4-Hydroxylation of 17β-estradiol was highest with the Ala119,Val432 variant and lowest with the Ala119,Leu432 variant. Interestingly, the ratio of 17β-estradiol 4-hydroxylation to 2-hydroxylation was higher for the two Val432 than for the two Leu432 variants for all of the substrate concentrations used; the ratio in the former two cases was 3–3.6 while the latter two showed ratios of ~2. The V_{max}/K_m ratio for 17β-estradiol 4- and 2-hydroxylation by these four CYP1B1 variants was essentially similar, except

Table II. Kinetic analysis of the hydroxylation of 17β-estradiol by recombinant human CYP1B1 variants expressed in *E.coli* which co-express hNPR

CYP1B1 variant	17β-Estradiol hydroxylation						V_{max} ratio
	4-Hydroxylation			2-Hydroxylation			
	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m	K_m (μM) (nmol/min/nmol P450)	V_{max}	V_{max}/K_m	4-/2-
Ala119,Leu432	5.1 ± 4.2	0.91 ± 0.10	0.18	6.9 ± 4.3	0.42 ± 0.04	0.06	2.2
Ser119,Leu432	5.2 ± 2.3	1.15 ± 0.07	0.22	8.4 ± 3.2	0.61 ± 0.05	0.07	1.9
Ala119,Val432	5.3 ± 2.8	1.45 ± 0.10	0.27	5.7 ± 2.8	0.46 ± 0.03	0.08	3.2
Ser119,Val432	2.5 ± 1.9	1.10 ± 0.06	0.44	0.9 ± 1.3	0.33 ± 0.01	0.37	3.3

Data are means ± SE.
All CYP1B1 variants used in this work contained Arg48 and Asn453.

Table III. Estrone hydroxylations by CYP1B1 variants and other human P450 enzymes co-expressed in *E.coli* with hNPR

P450	Estrone hydroxylation (nmol product/min/nmol P450)		Ratio
	4-Hydroxylation	2-Hydroxylation	4-/2-
CYP1B1 variants			
Ala119,Leu432	0.24 ± 0.03	0.17 ± 0.02	1.4
Ser119,Leu432	0.31 ± 0.03[a]	0.22 ± 0.02[a]	1.5
Ala119,Val432	0.50 ± 0.05[a]	0.28 ± 0.03[a]	1.8
Ser119,Val432	0.42 ± 0.04[a]	0.25 ± 0.02[a]	1.7
CYP1A1	0.03 ± 0.01	0.55 ± 0.07	0.06
CYP1A2	0.07 ± 0.01	0.75 ± 0.06	0.09
CYP2E1	<0.01	<0.01	
CYP3A4	0.04 ± 0.01	0.22 ± 0.02	0.26

[a]$P < 0.05$ as compared with activities by the Ala119,Leu432 variant.
Data are means ± range of triplicate determinations.
All CYP1B1 variants used in this work contained Arg48 and Asn453.

that the ratio was highest in the Ser119,Val432 variant, with K_m values being low (Table II). Again, it was shown that the ratio of V_{max} values for 4-hydroxylation to 2-hydroxylation was higher for the two Val432 variants.

The ratio of 17β-estradiol 4-hydroxylation versus 2-hydroxylation was determined to be 0.03, 0.03 and 0.09 for human (bicistronic) CYP1A1, CYP1A2 and CYP3A4, respectively (co-expressed with hNPR in bacterial membranes), at a substrate concentration of 100 μM (detailed results not shown).

Estrone 4-hydroxylation was higher than 2-hydroxylation for all four CYP1B1 variants and the ratio of 4-hydroxylation to 2-hydroxylation was determined to be 1.4–1.5 for Leu432 variants and 1.7–1.8 for the Val432 variants (Table III). The ratio of 4- to 2-hydroxylation of estrone was very low in human bicistronic CYP1A1, 1A2 and 3A4. CYP2E1 catalyzed the 2- and 4-hydroxylation of 17β-estradiol (data not shown) and of estrone (Table III) at very low levels.

Testosterone and progesterone hydroxylation by CYP1B1 variants

Testosterone 6β-hydroxylation activities were higher for the Leu432 than for the Val432 variants, with the V_{max}/K_m ratio being several fold higher for the former two enzymes than the latter enzymes (Table IV). V_{max} values of progesterone 6β- and 16α-hydroxylation activities were lower in the Leu432 than the Val432 variants. However, the ratio of V_{max} to K_m values were essentailly similar for four CYP1B1 variants examined.

Discussion

Numerous studies have shown that polymorphisms in P450 genes cause defects in the expression of the proteins or changes in the catalytic function of the resultant mutated enzymes (26,27). Individuals who have mutations in their *CYP2D6* and *CYP2C19* genes have been reported to have very low catalytic activity towards typical substrates such as debrisoquine and *S*-mephenytoin, respectively, and appear to show severe pharmacological and toxic effects when administered the usual dosages of certain drugs (28–30). It has also been reported that polymorphisms in the *CYP1A1*, *2E1* and *2D6* genes relate to susceptibilities in the incidence of lung and breast cancers in humans (3,31). Among the studies reported so far, Kawajiri and co-workers have shown that *Msp*I and Ile–Val polymorphisms of the *CYP1A1* gene are associated with a high susceptibility to squamous cell carcinoma of the lung; genotype C in the *Msp*I polymorphism and genotype *Val*/*Val* in the Ile–Val polymorphism among the patients are more than twice as frequent as they are among controls in the Japanese population (1,2). It is interesting to note that a point mutation in the Ile–Val polymorphism causes an amino acid replacement of Ile for Val at residue 462 in the heme-binding region and studies suggest that the Val variant of CYP1A1 shows slightly higher catalytic activity for oxidation of B[*a*]P (4,32).

Genetic polymorphism in the human *CYP1B1* gene has recently been reported by Stoilov *et al.* (14), Bejjani *et al.* (16) and Bailey *et al.* (15) and the results have suggested that there are at least six genetic polymorphisms in the human *CYP1B1* gene. Of the mutations in the *CYP1B1* gene examined, amino acid replacements occur at codons 48, 119, 432 and 453 leading to the replacement of Arg by Gly, Ala by Ser, Leu by Val and Asn by Ser, respectively (14,15).

In this study we determined whether two changes at codons 119 and 432 of the *CYP1B1* gene cause alterations in catalytic properties towards a variety of substrates, including procarcinogens and gonadal steroid hormones. Four recombinant CYP1B1 variants (Arg48,Ala119,Leu432,Asn453, Arg48,Ser119,Leu432,Asn453, Arg48,Ala119,Val432,Asn453 and Arg48,Ser119,Val432,Asn453) were co-expressed in *E.coli* together with hNPR and the activities of the recombinant proteins were characterized. The results obtained here can be summarized as follows. Activities for activation of 19 procarcinogens to DNA-damaging products by these four CYP1B1 variants in

Table IV. Kinetic analysis of testosterone 6β-hydroxylation and progesterone 6β- and 16α-hydroxylation by recombinant human CYP1B1 variants expressed in *E.coli*

CYP1B1 variant	Testosterone 6β-hydroxylation			Progesterone hydroxylation					
				6β-Hydroxylation			16α-Hydroxylation		
	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m
Ala119,Leu432	17 ± 7	2.2 ± 0.3	0.13	25 ± 11	0.6 ± 0.1	0.02	23 ± 8	2.3 ± 0.3	0.10
Ser119,Leu432	26 ± 16	2.5 ± 0.6	0.10	74 ± 32	1.4 ± 0.4	0.02	46 ± 17	4.1 ± 0.8	0.09
Ala119,Val432	48 ± 23	1.1 ± 0.3	0.02	167 ± 69	3.4 ± 1.0	0.02	154 ± 69	8.3 ± 2.7	0.05
Ser119,Val432	54 ± 23	1.5 ± 0.3	0.03	109 ± 42	2.4 ± 0.6	0.02	75 ± 24	5.4 ± 1.0	0.07

Testosterone 6β-hydroxylation and progesterone 6β- and 16α-hydroxylation activities were determined at substrate concentrations between 20 and 100 μM, an incubation time of 10 min and a P450 content of 25 pmol in a final incubation mixture of 0.25 ml.
All CYP1B1 variants used in this work contained Arg48 and Asn453.
Data are means ± SE.

S.typhimurium NM2009 were found to be essentially similar, except that the Ser119,Leu432 variant was found to be slightly more active (1.2- to 1.5-fold) than the other three CYP1B1 enzymes in catalyzing activation of some of the dihydrodiols of polycyclic aromatic hydrocarbons. The kinetic analysis of 17β-estradiol hydroxylation showed that the ratio of product formation of 4-hydroxyestradiol versus 2-hydroxyestradiol was higher in the Val432 than the Leu432 CYP1B1 variants (in terms of V_{max} but not V_{max}/K_m). The same trend was also noted in estrone 4-hydroxylation, for which both the Arg48,Ala119,Val432,Asn453 and Arg48,Ser119,Val432,-Asn453 CYP1B1 variants gave slightly higher rates than did the Arg48,Ala119,Leu432,Asn453 and Arg48,Ser119,Leu432,-Asn453 variants of CYP1B1. Finally, the two CYP1B1 Leu432 variants were found to have higher V_{max}/K_m ratios than the Val432 variants for 6β-hydroxylation of testosterone. These results support the view that mutations at codons 119 and 432 of the CYP1B1 enzyme cause some alterations in substrate specificity and catalytic activity. The results of changes in 4-hydroxylation of estrogens seen in the Val432 variants of CYP1B1 are of interest, since it has been suggested that 4-hydroxylation of 17β-estradiol and estrone is one of the causes of breast cancer in humans (12,33).

We found in this study that the ratio of 4-hydroxylation to 2-hydroxylation of estrogens by recombinant CYP1A1, CYP1A2 and CYP3A4 was 0.03, 0.03 and 0.09, respectively, when 17β-estradiol (at 100 μM) was used as substrate and 0.06, 0.09 and 0.26, respectively, when estrone (at 100 μM) was used as substrate. These rates were extremely low as compared with those of CYP1B1 variants, where the ratio of 4-hydroxylation versus 2-hydroxylation of 17β-estradiol and estrone was determined to be between 1.9 and 3.3 and between 1.4 and 1.8, respectively, suggesting again the importance of the CYP1B1 enzymes in the formation of 4-hydroxyestrogens in humans.

Recombinant CYP1A1 and CYP1B1 enzymes in *E.coli* membranes (in which the reductase was co-expressed) were examined for their abilities to activate 19 procarcinogens to genotoxic metabolites in *S.typhimurium* NM2009 (Table I). The results suggested that the activities of CYP1A1 and CYP1B1 enzymes were essentially similar for procarcinogen activation, except for the activities when B[*c*]P-3,4-diol, chrysene-1,2-diol, DMBA, MeIQ, MeIQx, 3-methoxy-4-aminoazobenzene and Trp-P-1 were used as substrates. The results obtained in this study were somewhat different from those of our previous work using recombinant CYP1B1 in yeast microsomes and purified CYP1A1 isolated from membranes of *E.coli*: in both systems only the *CYP1A1* or *CYP1B1* cDNA, respectively, using monocistronic vectors, had been introduced and the activities were measured after adding rabbit liver NADPH-P450 reductase to the reaction mixtures (6). Especially, procarcinogen activation activities by CYP1A1 in the reconstituted system in the previous work were very much lower than those obtained in this work using *E.coli* membranes co-expressing CYP1A1 and hNPR. It should, however, be mentioned that numerous recent studies have suggested the usefulness of recombinant P450 enzymes co-expressing NADPH-P450 reductase in studies of roles of P450 enzymes in the biotransformation of drugs, toxic chemicals and procarcinogens (13,17,20,34–36).

In a preliminary account, Watanabe *et al*. recently analyzed two types (Ala119Ser and Leu432Val) of CYP1B1 polymorphisms in the Japanese population and have reported

that frequency distributions of each allele with combination genotypes of (Arg48)Ala119,Leu432, (Gly48)Ser119,Leu432, (Arg48)Ala119,Val432 and (Gly48)Ser119,Val432 are 0.746, 0.100, 0.136 and 0.018, respectively; the two polymorphisms Arg–Gly at codon 48 and Ala–Ser at codon 119 have been shown to be genetically associated, namely Arg is linked to Ala and Gly to Ser, respectively; in contrast the polymorphisms of Ala–Ser and Leu–Val appear to be genetically independent of each other (J.Watanabe, T.Shimada, E.M.J.Gillam, T.Ikuta, K.Suematsu, Y.Higashi and K.Kawajiri, unpublished observations). This study was designed to examine the effect of different amino acids at positions 119 and 432 of CYP1B1 on alterations in the catalytic properties of CYP1B1 using recombinant CYP1B1 forms engineered to contain each of the variant amino acids at these positions. Thus, two of the variants assessed in this work, Arg48,Ala119,Leu432,Asn453 and Arg48,Ala119,Val432,Asn453, represent alleles that occur at reasonable frequency in nature (14,15). It is unclear as yet whether the other variants are found at significant frequencies in the wider population. The influence of the changes at codons 48 and 453 were not assessed in this study, but this work is underway in our laboratories.

In conclusion, the present study examined the catalytic functions of the CYP1B1 proteins representing major allelic variants towards a variety of substrates, including procarcinogens and gonadal steroid hormones, that have been considered to be activated by CYP1B1 enzymes to active metabolites which cause initiation of cellular transformation in mammary glands and lung (6,33,37,38). Inter-individual differences in activation of procarcinogens or metabolism of procarcinogens and estrogens originating from genetic polymorphisms of the human *CYP1B1* gene may contribute to human susceptibility to cancers and will be the subject of further investigation.

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