Table 1 Primers used for site-directed mutagenesis

Mutation	Primer name	Sequence	Location
227G>A	CYP2E1*2-FP	5′-GTGGGCTCGCAGC**A**CATGGTGGTGATG-3′	214–240
	CYP2E1*2-RP	5′-CATCACCACCATG**T**GCTGCGAGCCCAC-3′	
1165G>A	CYP2E1*3-FP	5′-CCAAGGGCACAGTC**A**TAGTGCCAACTCTGG-3′	1,151–1,180
	CYP2E1*3-RP	5′-CCAGAGTTGGCACTA**T**GACTGTGCCCTTGG-3′	
535G>A	CYP2E1*4-FP	5′-CGCGCCCTGCAAC**A**TCATAGCCGACATC-3′	522–549
	CYP2E1*4-RP	5′-GATGTCGGCTATGA**T**GTTGCAGGGCGCG-3′	

Bold and underlined letters indicate the mutation sites introduced by PCR-based mutagenesis

were spectrophotometrically measured as reduced carbon monoxide (CO) spectra according to the method of Omura and Sato (1964) using 91 $mM^{-1}cm^{-1}$ as an absorption coefficient for the 450–490 wavelength couple. Total CYP2E1 protein levels of holo- and apoforms in yeast cell microsomes were determined by Western blot analysis. Microsomal proteins (5.0 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli 1970) and electrotransferred to a polyvinylidene fluoride sheet as described by Towbin et al. (1979). The sheet was incubated with rabbit anti-human CYP2E1 antibody (diluted at 1:10,000) as the primary antibody and then with peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted at 1:5,000) as the secondary antibody. Immunoreactive proteins were visualized with chemifluorescence (enhanced chemiluminescence-plus reagents), and the band densities were relatively determined with ImageJ v1.34 (National Institutes of Health, MD, USA).

Assay for benzene hydroxylation and toluene methylhydroxylation

The activities of benzene hydroxylation and toluene methylhydroxylation in yeast cell microsomes expressing wild-type and variant CYP2E1s were determined by measuring the formation of phenol and benzyl alcohol, respectively, according to the methods reported previously by (Nakajima et al. 1991, 1993 and Hanioka et al. 1995) with some modifications. The incubation mixture contained benzene (0.5–50 mM) or toluene (0.2–20 mM) as a substrate, yeast cell microsomes expressing CYP2E1s (500 μg protein/mL) and an NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 2 U/mL glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$) in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 500 μL. Both substrates were dissolved in methanol. The final concentration of methanol in the incubation mixture was 1% (v/v). The reaction was initiated by the addition of the NADPH-generating system after preincubation at 37°C for 1 min. After incubation at 37°C for 10 min, the reaction was terminated by adding 100 μL of 15% zinc sulfate, followed by 100 μL of saturated barium hydroxide solution. The mixtures were spiked with 2.0 nmol of *p*-methylbenzyl alcohol as an internal standard and gently vortexed for 10 s. The supernatant was filtered with a polytetrafluoroethylene membrane filter (0.45 μm pore size, Millipore, Billerica, MA, USA), and a 50 μL portion of the filtrate was subjected to high-performance liquid chromatography (HPLC) with an Inertsil ODS-80A column (4.6 mm i.d. × 150 mm; GL Sciences, Tokyo, Japan). The column was maintained at 40°C. The products (phenol for benzene hydroxylation and benzyl alcohol for toluene methylhydroxylation) were eluted isocratically with water–acetonitrile (75:25, v/v) at a flow rate of 1.0 mL/min. UV detection was performed at 200 nm. Under these conditions, the retention times of phenol, benzyl alcohol and *p*-methylbenzyl alcohol were 6.4, 5.1 and 9.3 min, respectively. The limits of detection for phenol and benzyl alcohol were 50 and 100 pmol/mL with a signal-to-noise ratio of 3, respectively. Intra-day ($n = 5$) and inter-day ($n = 5$) precision did not exceed 10% in any of the assays.

Other methods

Protein concentrations of microsomes from yeast cells expressing wild-type and variant CYP2E1s were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. NADPH-cytochrome P450 reductase (OR) activities were measured as described previously, with a substrate concentration of 50 μM (Phillips and Langdon 1962). Chlorzoxazone 6-hydroxylation activities were determined by HPLC as described previously by (Hanioka et al. 2007). The substrate and protein concentrations of yeast cell microsomes were 50–5,000 μM and 500 μg protein/mL, respectively.

Data analysis

Kinetic parameters such as K_m and V_{max} for benzene hydroxylation, toluene methylhydroxylation and chlorzoxazone 6-hydroxylation were estimated by analyzing Michaelis–Menten plots using Prism v5.01 software

(GraphPad Software, San Diego, CA). Intrinsic clearance (CL_{int}) values were determined as the ratio of V_{max}/K_m. All values are expressed as the mean ± SD of three donors/animals or three separate experiments derived from independent preparations. Statistical comparisons were performed by one-way ANOVA with Dunnett's *post hoc* test using Prism v5.01 software. Differences were considered statistically significant when the p value was <0.05.

Results

Expression of wild-type and variant CYP2E1s in yeast cells

The expression levels of CYP2E1 proteins in microsomal fractions obtained from yeast cells transfected with wild-type and variant CYP2E1 cDNAs were examined by reduced CO difference spectral and Western blot analyses. The reduced CO difference spectra of yeast cell microsomes expressing CYP2E1.1, CYP2E1.2, CYP2E1.3 and CYP2E1.4 proteins showed a Soret peak at around 450 nm (Fig. 1). The expressed CYP level of CYP2E1.1 was 61.7 pmol/mg of microsomal protein. The levels of CYP2E1.3 and CYP2E1.4 were comparable to that of CYP2E1.1, whereas the level of CYP2E1.2 was 42.9% that of CYP2E1.1 (Table 2). The expression levels of wild-type and variant CYP2E1 proteins in yeast cell microsomes were also assessed by Western blot analysis which recognized both holo- and apoforms. All constructs, except the negative control (mock), yielded immunodetectable CYP2E1 protein (Fig. 2). The staining band intensities of variant CYP2E1s were 65.8–87.4% that of CYP2E1.1, and this difference was not significant (Table 2). OR activities in yeast cell microsomes expressing wild-type and variant CYP2E1s were further determined. OR activity of CYP2E1.1 was 0.17 μmol/min/mg protein, and there were no significant differences in the OR activities of wild-type and variant CYP2E1s.

Table 2 CYP contents and OR activities in microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	CYP		
	Reduced CO[a] (pmol/mg protein)	WB[b] (% of CYP2E1.1)	OR (μmol/min/mg protein)
CYP2E1.1	61.7 ± 21.1	100 ± 23	0.17 ± 0.04
CYP2E1.2	26.5 ± 5.75	65.8 ± 12.9	0.14 ± 0.02
CYP2E1.3	61.4 ± 17.4	85.3 ± 10.8	0.16 ± 0.03
CYP2E1.4	83.7 ± 10.9	87.4 ± 17.6	0.16 ± 0.02

Each value represents the mean ± SD of three separate experiments derived from independent preparations

[a] Reduced CO difference spectral analysis

[b] Western blot analysis

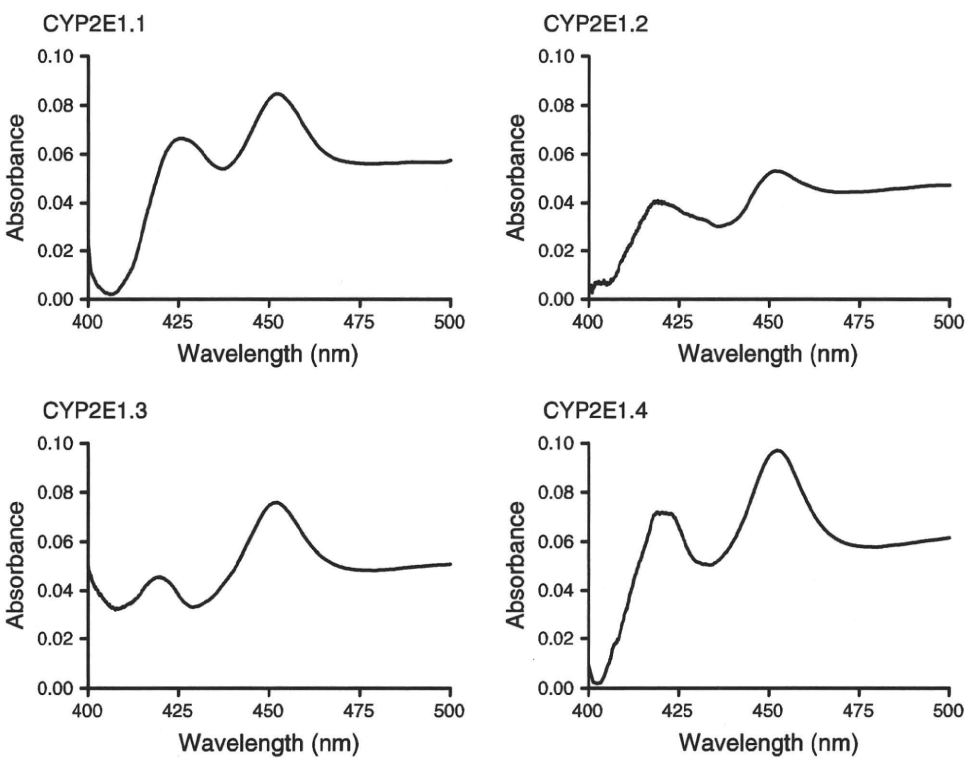

Fig. 1 Reduced CO difference spectra of yeast cell microsomes expressing wild-type and variant CYP2E1s. Pooled microsomes from three independent preparations are expressed. The microsomal protein concentrations used were 5.0 mg/mL

Benzene hydroxylation and toluene methylhydroxylation activities of wild-type and variant CYP2E1s expressed in yeast cells

Benzene hydroxylation and toluene methylhydroxylation activities in yeast cell microsomes expressing wild-type and variant CYP2E1s were determined, and kinetic analyses were then performed. No activity in yeast cell microsomes of the negative control was detected with any substrate (data not shown). The [*S*]-[*V*] plots and kinetic parameters are shown in Fig. 3 and Table 3 for benzene hydroxylation, and Fig. 4 and Table 4 for toluene methylhydroxylation, respectively. Benzene hydroxylation and toluene methylhydroxylation in yeast cell microsomes expressing wild-type and variant CYP2E1s showed single-site Michaelis–Menten kinetics. The K_m, V_{max} and CL_{int} values of CYP2E1.1 were 10.1 mM, 9.38 pmol/min/pmol CYP and 0.99 nL/min/pmol CYP for benzene hydroxylation, and 3.97 mM, 19.9 pmol/min/pmol CYP and 5.26 nL/min/pmol CYP for toluene methylhydroxylation, respectively. There were no significant differences in the K_m, V_{max} and CL_{int} values between wild-type CYP2E1 and each variant of CYP2E1 in any oxidative metabolism.

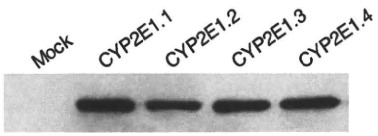

Fig. 2 Immunoblotting of yeast cell microsomes expressing wild-type and variant CYP2E1s. Pooled microsomes from three independent preparations are expressed. The microsomal protein levels applied were 5.0 μg/lane

Chlorzoxazone 6-hydroxylation activities of wild-type and variant CYP2E1s expressed in yeast cells

Chlorzoxazone 6-hydroxylation activities as a typical CYP2E1 probe in yeast cell microsomes expressing wild-type and variant CYP2E1s were also determined. The [*S*]-[*V*] plots and the kinetic parameters are shown in Fig. 5 and Table 5, respectively. Chlorzoxazone 6-hydroxylation

Table 3 Kinetic parameters for benzene hydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	10.1 ± 2.1	9.38 ± 2.15	0.99 ± 0.47
CYP2E1.2	10.2 ± 2.2	9.13 ± 2.47	0.95 ± 0.31
CYP2E1.3	13.6 ± 2.6	6.97 ± 2.80	0.56 ± 0.34
CYP2E1.4	13.6 ± 2.5	5.37 ± 1.70	0.40 ± 0.09

Each value represents the mean ± SD of three separate experiments derived from independent preparations

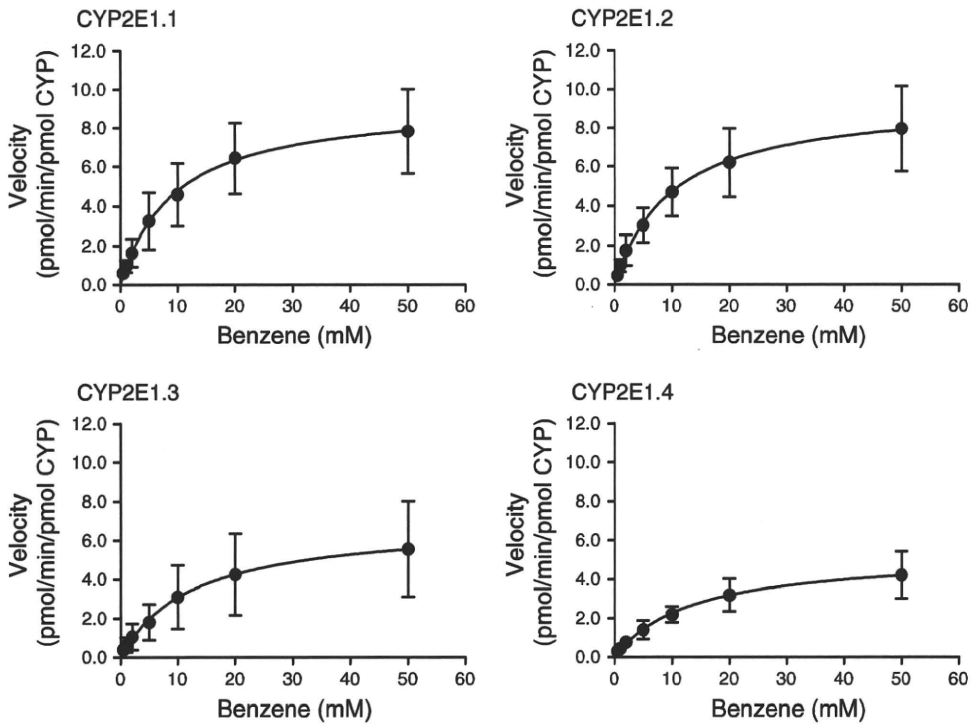

Fig. 3 [*S*]-[*V*] plots for benzene hydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.5–50 mM

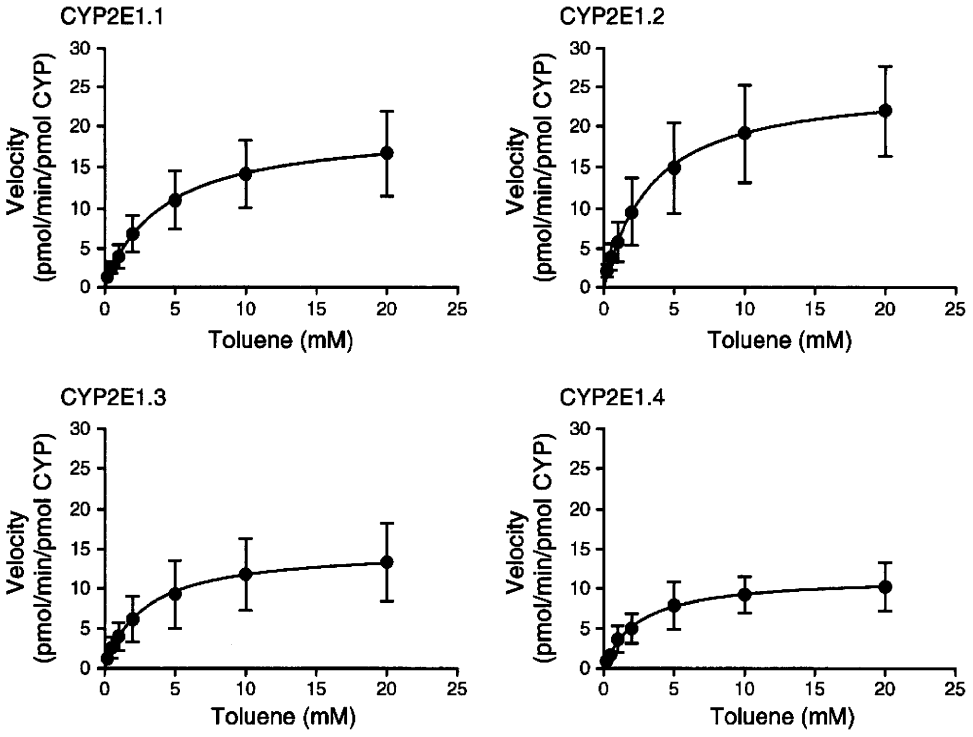

Fig. 4 [*S*]-[*V*] plots of toluene methylhydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.2–20 mM

Table 4 Kinetic parameters for toluene methylhydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	3.97 ± 1.02	19.9 ± 6.2	5.26 ± 2.12
CYP2E1.2	3.81 ± 1.41	25.9 ± 6.0	7.92 ± 4.42
CYP2E1.3	3.26 ± 1.39	15.3 ± 5.2	5.39 ± 2.92
CYP2E1.4	2.54 ± 0.43	11.6 ± 3.1	4.77 ± 2.06

Each value represents the mean ± SD of three separate experiments derived from independent preparations

by yeast cell microsomes expressing wild-type and variant CYP2E1s showed single-site Michaelis–Menten kinetics. The K_m, V_{max} and CL_{int} values of CYP2E1.1 were 1.57 mM, 9.78 pmol/min/pmol CYP and 6.12 nL/min/pmol, respectively. The K_m, V_{max} and CL_{int} values of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were not significantly different from those of wild-type CYP2E1.

Discussion

Benzene and toluene are common organic solvents currently in worldwide industrial usage, and a large number of workers are exposed to these chemicals (World Health Organization 1996). Previous reports have shown that CYP2E1 is a key enzyme involved in the toxicity of benzene and toluene (Guengerich et al. 1991; Tassaneeyakul et al. 1996; Kim et al. 1997; Nakajima et al. 1997). The metabolic activity of CYP2E1 toward xenobiotics both in vivo and in vitro has been reported to be extensive, and genetic polymorphism in *CYP2E1* has been considered to be at least partly responsible (Kim and O'Shea 1995; Lucas et al. 1995; Song 1996). Since *CYP2E1* polymorphism has been suggested to be closely associated with the incidence of several cancers, it may be an important risk factor for susceptibility to environmental pollutants (Song 1996; Tanaka et al. 2000). In this study, benzene hydroxylation and toluene methylhydroxylation were investigated in yeast cell microsomes expressing wild-type and variant CYP2E1s.

The expression of wild-type and variant CYP2E1 protein in yeast cells was confirmed by reduced CO difference spectral and Western blot analyses. Although there were no statistically significant differences in CYP levels between CYP2E1.1 and each variant CYP2E1 in any analysis, the decreasing tendency of holo- and apoproteins CYP levels of CYP2E1.2 was observed. Hu et al. (Hu et al. 1997) have reported that CYP2E1*2 cDNA reduces the expression level of protein to about 40% of CYP2E1*1 cDNA but not mRNA in the expression system using COS-1 cells. We have also previously found that the CYP level of CYP2E1.2 expressed in COS-1 cells was about 30% of CYP2E1.1, and that the transcription and translation rates for each expression plasmid of variant CYP2E1 were very similar to those for wild-type CYP2E1 (Hanioka et al.

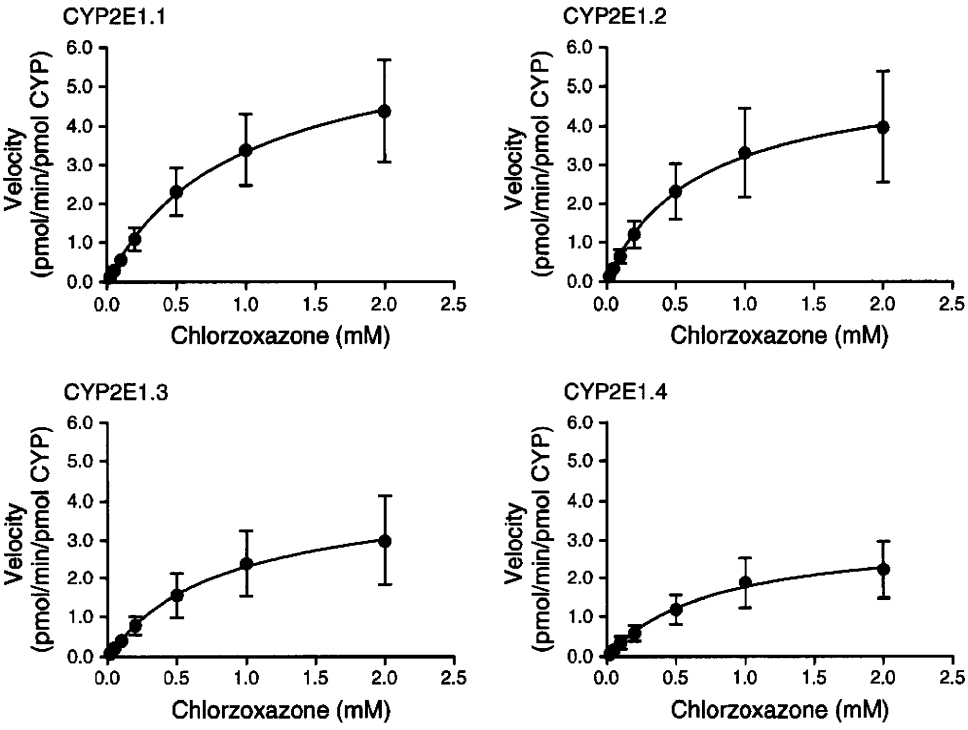

Fig. 5 [*S*]-[*V*] plots for chlorzoxazone 6-hydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.05–5.0 mM

Table 5 Kinetic parameters for chlorzoxazone 6-hydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	0.92 ± 0.11	6.51 ± 2.04	6.92 ± 1.67
CYP2E1.2	0.66 ± 0.10	5.41 ± 2.09	7.99 ± 2.10
CYP2E1.3	0.89 ± 0.16	4.41 ± 1.80	4.87 ± 1.55
CYP2E1.4	0.84 ± 0.13	3.24 ± 1.11	3.88 ± 1.35

Each value represents the mean ± SD of three separate experiments derived from independent preparations

2003). Thus, the expression profile of CYP2E1 obtained in this study using the yeast cell expression system agreed with those of previous reports, and it is possible that the reduction in the CYP expression level of CYP2E1.2 is due to the altered stability or folding efficiency of the protein.

Nedelcheva et al. (1999) and Tassaneeyakul et al. (1996) have reported that the activities of benzene hydroxylation and toluene methylhydroxylation were highly correlated with CYP2E1-specific activities and the immunoreactive CYP2E1 level in human liver microsomes, suggesting the importance of CYP2E1 in benzene and toluene metabolism; therefore, we examined the effect of *CYP2E1*2*, *CYP2E1*3* and *CYP2E1*4* on benzene hydroxylation and toluene methylhydroxylation using the recombinant CYP2E1 enzymes expressed in yeast cells. Additionally, chlorzoxazone 6-hydroxylation activities were also determined to characterize the enzymatic function of variant CYP2E1 enzymes toward a typical CYP2E1 substrate. All variant CYP2E1s with amino acid substitutions were capable of catalyzing benzene and toluene oxidation as well as wild-type CYP2E1 at the substrate concentrations examined, and the kinetics of all CYP2E1 enzymes fitted the typical Michaelis–Menten model. Although the in vitro metabolism of benzene and toluene in humans has been studied using liver microsomes and recombinant CYP enzymes (Tassaneeyakul et al. 1996; Kim et al. 1997; Nakajima et al. 1997; Nedelcheva et al. 1999), the kinetic parameters for benzene hydroxylation and toluene methylhydroxylation by recombinant CYP2E1 enzymes were estimated for the first time in this study. The K_m values for benzene hydroxylation and toluene methylhydroxylation of CYP2E1.1 were millimolar levels, and the values were generally higher than those of other typical CYP2E1-dependent oxidation by recombinant CYP2E1 and liver microsomes (Peter et al. 1990; Tassaneeyakul et al. 1993; Fairbrother et al. 1998; Shimada et al. 1999; Hanioka et al. 2003, 2007). The K_m, V_{max} and CL_{int} values for benzene hydroxylation and toluene methylhydroxylation of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1, and the kinetic parameters of chlorzoxazone 6-hydroxylation as a typical probe for CYP2E1 were not also affected by the corresponding amino acid substitutions of variant CYP2E1s. These findings suggest that Arg76His, Val389Ile and Val179Ile substitutions in the CYP2E1 enzyme hardly affect benzene and toluene metabolism.

X-ray crystal structures of several mammalian CYP enzymes (rabbit CYP2B4 and CYP2C5, and human CYP2C8, CYP2C9, CYP2D6 and CYP3A4) have been reported to date (Williams et al. 2000, 2003, 2004; Schoch et al. 2004; Scott et al. 2004; Rowland et al. 2006). Homology modeling of human CYP2E1 has been generated using CYP2C5 crystal structures as a template, and six substrate recognition sites (SRSs) and a putative active site for CYP2E1 have been identified (Lewis 2002, 2003; Lewis et al. 2003). Phe205 and Phe298 of CYP2E1 have been suggested to be able to form π–π stacking with the benzoxazole ring of chlorzoxazone, whereas Asp202 and The303 of CYP2E1 enter into hydrogen bonding with the chlorzoxazone hydroxyl and chloro groups, respectively. These amino acid residues are in SRS-2 and SRS-4 and have been regarded to assist in positioning the substrate cooperatively such that the 6-hydrogen is directly over the haem iron at the distance of 5.7 Å. The amino acids of interest in this study, Arg76, Val389 and val179, are not found in any SRSs; therefore, we consider that the profile of the metabolism of benzene and toluene by wild-type and variant CYP2E1s is similar.

In conclusion, we expressed three variant CYP2E1s as well as wild-type CYP2E1 in yeast cells, and the kinetics of benzene hydroxylation and toluene methylhydroxylation were determined. The K_m, V_{max} and CL_{int} values of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1 in any oxidative metabolism, and the kinetic parameters of chlorzoxazone 6-hydroxylation as a typical probe for CYP2E1 were not also affected by the corresponding amino acid substitutions of variant CYP2E1s. These findings may mean that the polymorphic alleles of *CYP2E1* causing amino acid substitutions are not directly associated with the metabolic activation of benzene and toluene. The information gained in this study should help to identify the variations in the toxicity of environmental pollutants.

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